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Phylogenetic systematics of subtribe Spiranthinae (Orchidaceae: Orchidoideae: Cranichideae) based on nuclear and plastid DNA sequences of a nearly complete generic sample

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Subtribe Spiranthinae is the most species-rich lineage of terrestrial Neotropical orchids, encompassing > 500 species and 40 genera. We conducted maximum parsimony and maximum likelihood phylogenetic analyses of DNA sequence data of plastid *matK-trnK* and *trnL-trnF* and nuclear ribosomal ITS sequences for 36 genera and 182 species of Spiranthinae plus appropriate outgroups. The results strongly support monophyly of Spiranthinae (minus *Discyphus*, Discyphinae and *Galeottiella*, Galeottiellinae) and five major lineages, namely monospecific *Cotylolabium* (sister to the remaining Spiranthinae) and the *Eurystyles*, *Pelexia*, *Spiranthes* and *Stenorrhynchos* clades. Eighteen of the 27 genera of Spiranthinae for which more than one species was included in our analyses are monophyletic. Paraphyly of large genera, such as *Cyclopogon* and *Sarcoglottis*, resulted from segregation of particular species or groups of species exhibiting minor modifications of structures directly involved in pollination (e.g. nectary, rostellum and viscidium). Conversely, polyphyly has resulted from convergent evolution of floral attributes in distantly related species (e.g. *Mesadenus*). Some of the morphological characters used traditionally for generic delimitation and in non-molecular cladistic analyses of Spiranthinae are discussed against the evolutionary framework set by our molecular trees, emphasizing putative synapomorphies and problems derived from inappropriate character coding or incorrect homology assessments. Our ancestral area analysis indicates that Spiranthinae originated in eastern South America, with subsequent migrations and secondary radiations in Mesoamerica and North America, plus a derived migration from the latter region to the Old World (*Spiranthes*).

ADDITIONAL KEYWORDS: Ancestral area – floral morphology – homology – homoplasy – molecular phylogenetics – pollination syndrome – taxonomy.

> ‘Modern orchidologists tend to take the view that any modifications in the reproductive organs of orchids, no matter how obscure, are probably of evolutionary importance because of the close correlation in the whole family between flower structure and pollinators. It is tempting to think that any character of evolutionary importance is *ipso facto* a character that will define a distinct genus, but obviously this is not true.’
>
> Rogers McVaugh, Orchidaceae,
> In *Flora Novo-Galiciana* Vol. 16 (1985).

INTRODUCTION

Subtribe Spiranthinae is the most species-rich clade of terrestrial orchids in the Neotropics, where most of the *c*. 40 genera and 520 species are found (Garay, 1982; Salazar, 2003b; Chase *et al.*, 2015). Spiranthinae were first recognized formally as ‘division’ Spiranthidae of tribe Neottieae in the early orchid classification of Lindley (1840), but 20th century systematists largely followed the circumscription of Spiranthinae outlined in the posthumously published synoptic classification of Orchidaceae by Schlechter (1926). In that work, Spiranthinae were distinguished by their more or less erect anther, fasciculate roots, basal leaves (Fig. 1) and margins of the labellum adherent (‘adnate’) to the sides of the column. Schlechter (1920) also carried out the first modern revision of the generic classification of Spiranthinae, recognizing 24 genera, including many proposed there for the first time, based on floral features such as presence of a spur, relative length and thickness of the column, presence of a column foot, lobulation of the stigma, and details of the rostellum and viscidium (Fig. 2). Using characters of the rostellum and viscidium, Schlechter grouped the genera into four unnamed alliances or Gattungsreihen, to which he gave informal names in a later work (Schlechter, 1926).

The generic classification of Spiranthinae proposed by Schlechter (1920) was criticized by the influential Harvard orchidologist Ames (e.g. Ames, 1922) for relying on ‘recondite’ column characters, and such disagreement among leading orchid specialists resulted in a long-standing lack of consensus in the approach to the generic classification of Spiranthinae. For instance, Harvard botanists who prepared orchid floras of various New World countries, strongly influenced by Ames’ views, placed nearly all species of Spiranthinae in an exceedingly broad genus *Spiranthes* Rich. *s.l.* (e.g. Correll, 1950; Williams, 1951; Ames & Correll, 1952; Schweinfurth, 1958), whereas other botanists followed Schlechter (Hoehne, 1945; Correa, 1955; Brieger, 1974–75; Garay, 1978).

Two further generic revisions of Spiranthinae were published, nearly simultaneously, in the early 1980s (Balogh, 1982; Garay, 1982). The most salient feature of these two treatments was their disagreement in the number of genera recognized and in the species composition of the genera (McVaugh, 1985: 295–296). Garay (1982), whose publication gained priority by 2 months, admitted that he had previously supported the ‘lumping’ approach of his earlier Harvard colleagues but radically changed his views when he had a chance to study the whole complex on his own, increasing the number of genera recognized to 44. Garay (1982) distinguished the genera based on the structure of the rostellum, but he also considered important the degree of fusion of the lateral sepals (forming a floral

tube) and position of the stigma, i.e. 'terminal' vs. 'anterior'. In contrast, Balogh (1982) recognized only 16 genera based on characteristics of the rostellum, pollinarium and viscidium, position of the entrance of the stylar channel and position of the lateral sepals, grouping most of the genera in four alliances similar in composition to the Gattungsreihen of Schlechter (1920, 1926). Balogh pioneered the application of cladistic methods to orchid classification, applying Hennigian argumentation (after Hennig, 1966) and manual optimization (i.e. without using specific algorithms) of the characters on a cladogram to assess relationships in the *Pelexia* Poit. ex Lindl. alliance (Burns-Balogh & Robinson, 1983) and her version of *Deiregyne* Schltr. (*sensu* Burns-Balogh, 1988). However, she did not attempt to carry out a phylogenetic assessment of the whole subtribe.

Dressler (1993) reviewed the classification of the orchid family, stressing the different generic treatments of Spiranthinae of Balogh (1982; also as Burns-Balogh, 1986b) and Garay (1982) and the scant discussion supporting either. Dressler (1993) adopted the generic scheme of Garay (1982), but stated that, at that point, one could not evaluate either of those classifications without redoing much of the work. Soon after, Szlachetko (1995a) proposed a new classification of Orchidaceae in which he divided Spiranthinae into three less-inclusive subtribes, namely Spiranthinae, Cyclopogoninae and Stenorrhynchidinae, based on differences in the structure of the rostellum and viscidium. He referred to these three groups as 'subclades' but did not provide any clear indication of which synapomorphies diagnose them, leaving aside the contradiction arising from his explicit rejection of cladistic methods in favour of the search for polythetic, 'homogeneous' groups in an evolutionary taxonomic context (Szlachetko, 1995a: 6). Szlachetko and co-workers subsequently proposed several new genera of Spiranthinae (e.g. Szlachetko, 1991a, b, 1993a, 1994a, b; González & Szlachetko, 1995; Szlachetko & González, 1996a, b, c; Szlachetko, González & Rutkowski, 2000, 2001), often splitting genera that they considered 'highly heterogeneous and difficult to define' on morphological grounds (e.g. Szlachetko *et al.*, 2001: 3).

Salazar *et al.* (2003) carried out the first molecular phylogenetic analysis of Spiranthinae, conducting maximum parsimony (MP) and Bayesian inference analyses of DNA sequences and insertion/deletion (indel) data from four plastid (*rbcL*, *matK–trnK*, *trnL* intron and *trnL–trnF* spacer) and one nuclear region (the ITS region of nuclear ribosomal DNA, hereafter nrITS) for 50 taxa, of which 24 species/21 genera were previously included in Spiranthinae. Their results showed that, with the exclusion of *Galeottiella* Schltr. (removed to a monogeneric subtribe by Salazar, Chase & Soto, 2002), Spiranthinae are monophyletic and strongly supported, whereas Schlechter's (1920) Gattungsreihen, Balogh's (1982) generic alliances and the narrowly defined subtribes of Szlachetko (1995a) are not monophyletic. Salazar *et al.* (2003) suggested that conflicts between strongly supported clades recovered in their analysis and the limits of taxa based solely on floral structures directly involved in pollination, such as the rostellum and viscidium, might reflect homoplasy in floral characters resulting from pressures from similar pollinators in distantly related groups. However, because they only analysed a fraction of the known diversity of Spiranthinae, the ensuing synoptical treatments of the genera of Galeottiellinae and Spiranthinae by Salazar (2003a, b, respectively) followed the generic concepts of Garay (1982) to minimize arbitrary changes lacking phylogenetic support, except for some changes resulting from the phylogenetic analysis of Salazar *et al.* (2003).

Szlachetko, Rutkowski & Mytnik (2005) criticized the analysis of Salazar *et al.* (2003) for having included only 24 (*c.* 6%) of the species of Spiranthinae. However, Szlachetko and co-workers approached the issue by conducting a molecular phylogenetic analysis of only 19 species of Spiranthinae *s.l.* and a single genomic region (nrITS), without offering a rationale for excluding taxa for which sequences of this and other DNA regions were already available (Górniak *et al.*, 2006). In any event, the analysis of Górniak *et al.* (2006) corroborated the results of Salazar *et al.* (2003) regarding both the non-monophyly of Szlachetko's narrowly delimited subtribes and the discovery of some 'unexpected' groupings in the molecular tree relative to morphological classifications.

Rutkowski, Szlachetko & Górniak (2008) published a book on the phylogeny and taxonomy of Spiranthinae, 'Stenorrhynchidinae' and 'Cyclopogoninae' in Central and South America (but also including Mexico, located in North America), in which they conducted phenetic and cladistic analyses of vegetative and floral morphological characters and molecular characters (DNA sequences of plastid *matK* and nrITS regions). All such analyses used the genera as terminal taxa, and therefore those authors did not attempt to evaluate generic monophyly. Moreover, their analyses were hampered by methodological inconsistencies, such as arbitrarily excluding *Spiranthes* from the morphological analyses, confounding the results of distance analyses with cladograms, using different sets of ingroup and outgroup taxa in their separate and combined DNA analyses without any justification, and failing to explain clearly how they conducted the analyses. Rutkowski *et al.* (2008) did not provide an articulate discussion summarizing the results of their various morphological analyses, which produced different groupings depending on whether floral,

vegetative or both classes of characters were included. They conceded that their DNA analyses 'do not confirm the monophyletic character of Spiranthinae and Stenorrhynchidinae *sensu* Szlachetko' (Rutkowski *et al.*, 2008: 95), but failed to cite their own previous molecular analysis (Górniak *et al.*, 2006), in which they arrived at similar conclusions. Most surprisingly, the taxonomic synopsis of Rutkowski *et al.* (2008) ignored their own molecular phylogenetic results and continued using artificial, non-monophyletic 'subtribes'.

Several recent molecular phylogenetic studies, although not focused specifically on Spiranthinae, have contributed to clarification of subtribal limits and relationships in Cranichideae. Figueroa *et al.* (2008) analysed DNA sequence data and anatomical root characters to assess relationships and morphological evolution among representatives of various genera of Cranichidinae, Prescottiinae and Spiranthinae. In turn, Álvarez-Molina & Cameron (2009) and Salazar *et al.* (2009) independently assessed the limits and relationships of Cranichidinae and Prescottiinae, but also included several representatives of Spiranthinae. Those studies supported the results and conclusions of Salazar *et al.* (2003) regarding subtribal relationships and the limits and monophyly of Spiranthinae to the exclusion of *Galeottiella* (removed to Galeottiellinae). Likewise, Salazar (2009) and Salazar *et al.* (2009, 2011b) clarified the systematic position of *Galeoglossum* A.Rich. & Galeotti, including its synonym, *Pseudocranichis* Garay, showing that it belongs in Cranichidinae and not in Spiranthinae as believed by Garay (1982) in the case of *G. thysanochilum* (Rob. & Greenm.) Salazar.

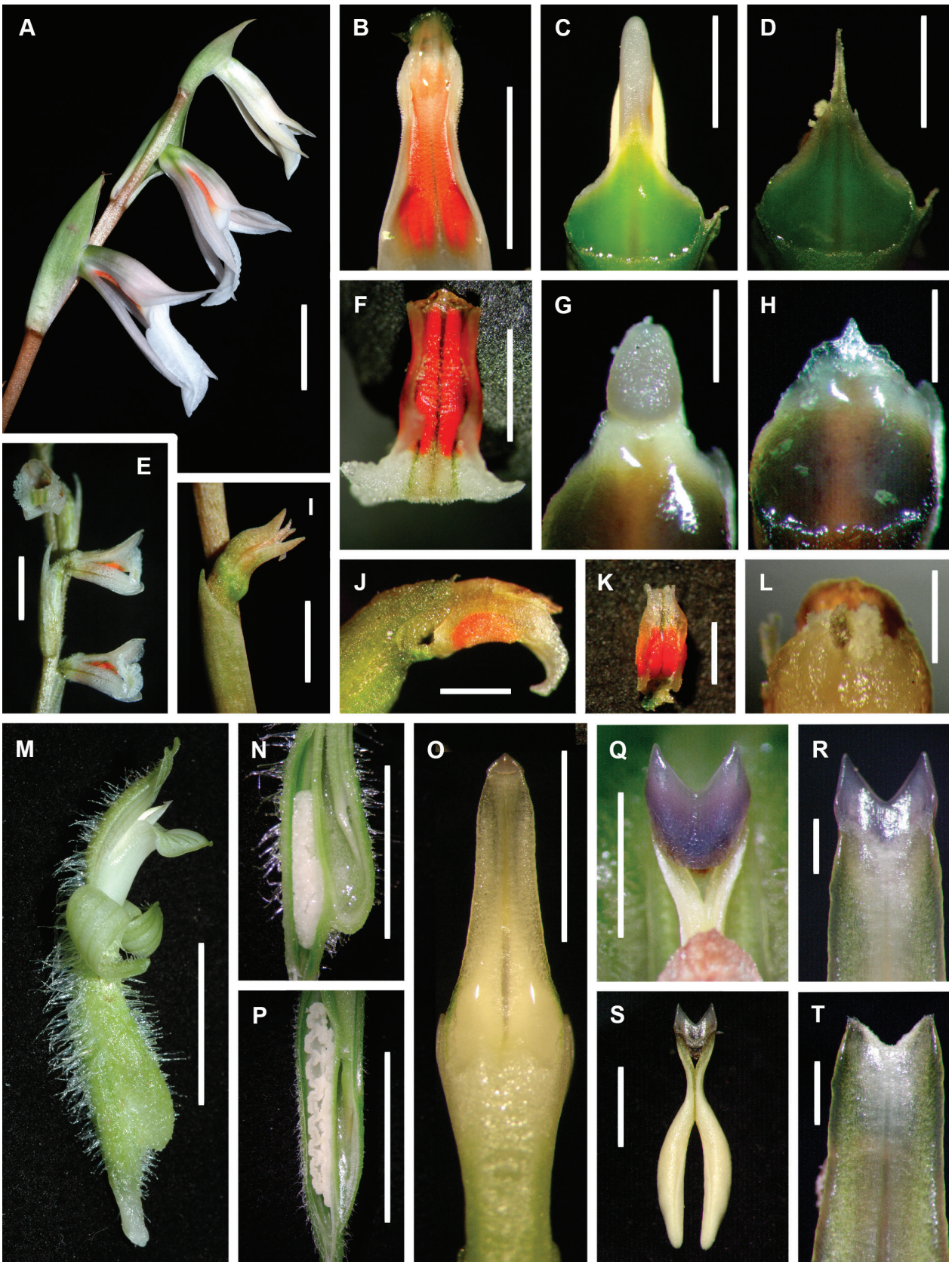
Recently, several papers have focused on molecular phylogenetics and floral evolution of particular species and clades of Spiranthinae, elucidating the systematic position of some taxa of uncertain affinity (Salazar & Ballesteros-Barrera, 2010; Batista *et al.*, 2011; Salazar & Dressler, 2011; Salazar & Jost, 2012; Borba *et al.*, 2014; Salazar, Cabrera & Figueroa, 2011a; Salazar, van den Berg & Popovkin, 2014; Salazar *et al.*, 2016). For instance, Batista *et al.* (2011) showed that *Nothostele* Garay is a member of Spiranthinae, not of Cranichidinae as suggested by Dressler (1993), and the analysis of Salazar *et al.* (2014) revealed that *Discyphus* Schltr., a morphologically distinctive monospecific genus previously included in Spiranthinae, does not fit there. They proposed a new subtribe for its single species, Discyphinae. The contribution by Borba *et al.* (2014) was significant in that it identified the monospecific, south-eastern Brazilian endemic *Cotylolabium* Garay as sister to all other Spiranthinae, which has important implications for inferring morphological character evolution.

Two recurring conclusions have arisen from the above-mentioned molecular phylogenetic and morphological studies. The first is that floral evolution in Spiranthinae is much more complex than cursory comparisons suggest. For instance, Salazar *et al.* (2011a) showed that the hummingbird-pollination syndrome, which includes odourless flowers, tubular, showily coloured perianth and bracts in tones of red, pink, orange or yellow, and a long, narrow rostellum with stiff, bristle-like rostellum remnant, evolved independently in distantly related clades such as *Dichromanthus* Garay, *Stenorrhynchos* Rich. ex Spreng. and other genera belonging to different major clades of Spiranthinae. Taxonomists focused only on floral characters have traditionally grouped these distantly related taxa into polyphyletic genera such as the different versions of *Stenorrhynchos s.l.* held, for instance, by Schlechter (1920), Balogh (1982), Garay (1982), Szlachetko (1995a) and Szlachetko *et al.* (2005).

The second recurring conclusion is that molecular studies have consistently recovered five major clades in Spiranthinae, namely: (1) the monospecific, eastern Brazilian endemic *Cotylolabium* as the strongly supported sister of the remainder of the subtribe; (2) the *Stenorrhynchos* clade; (3) the *Pelexia* clade; (4) the *Eurystyles* Wawra clade; and (5) the *Spiranthes* clade (clade names, for example, after Salazar *et al.*, 2003, 2011a, 2014, 2016; Batista *et al.*, 2011; Borba *et al.*, 2014). However, as pointed out by Borba *et al.* (2014), not all genera of Spiranthinae have been included in molecular phylogenetic analyses and several groups distributed in relatively inaccessible areas, mainly in South America, have not yet been analysed. Moreover, previous molecular phylogenetic studies of Spiranthinae have focused mostly on subtribal and generic relationships or on particular species or groups, and monophyly has not been assessed for most genera.

In this work, we build upon our previous studies to investigate phylogenetic relationships in Spiranthinae, analysing a nearly complete generic sample (*sensu*

Figure 1. Habit and vegetative features of selected Spiranthinae. A. *Sacoila hassleri* growing in a sandy savanna (Brazil, *Batista et al. 3137*). B. *Aulosepalum hemichreum* shortly before shedding the leaves growing on limestone (Mexico, *Salazar 6044*). C. *Cyclopogon calophyllus* in leaf litter (Brazil, *Salazar et al. 7793*). D. *Coccineorchis cernua* in deep leaf mould in an Andean cloud forest (Peru, *Edquen s.n.*). E. *Dichromanthus cinnabarinus* in a periodically mowed lawn on a traffic island (Mexico, *Salazar & Cabrera 6879*). F. Epiphytic *Lankesterella ceracifolia* (above, Argentina, *Salazar 7535*) and *Eurystyles auriculata* (below, El Salvador, *Salazar & Linares 7646*). G. *Sarcoglottis sceptrodes*, plant removed from soil to show the dense fascicle of fleshy roots (Mexico, *Salazar et al. 6584*). H. *Greenwoodiella wercklei*, roots produced at intervals on the rhizome (Dominican Republic, *Fragoso et al. 518*). Photographers: João A. N. Batista (A), Gerardo A. Salazar (B, C, E–H), José D. Edquen (D).



Chase *et al.*, 2015; Salazar *et al.*, 2016) and over one-third of the species currently accepted in the subtribe. We include DNA sequence data from one nuclear and three plastid DNA regions. The nuclear region consists of the internal transcribed spacers 1 and 2 and the intervening gene 5.8S of the nuclear ribosomal multigene family (nrITS; Baldwin *et al.*, 1995). The plastid regions include the *matK* gene plus partial 3′ *trnK* intron downstream *matK* (Hilu & Liang, 1997; Barthet *et al.*, 2015), the *trnL* group I intron and the *trnL–trnF* spacer (subsequently together referred to as the *trnL-F* region; Taberlet *et al.*, 1991). All these regions have been used previously, individually or in various combinations, for phylogenetic inference in Spiranthinae (Salazar *et al.*, 2003, 2011a, 2014, 2016; Górniak *et al.*, 2006; Rutkowski *et al.*, 2008; Salazar & Ballesteros-Barrera, 2010; Batista *et al.*, 2011; Salazar & Dressler, 2011; Salazar & Jost, 2012; Borba *et al.*, 2014) and other Cranichideae (Figueroa *et al.*, 2008; Álvarez-Molina & Cameron, 2009; Salazar *et al.*, 2009, 2011b; Cisternas *et al.*, 2012). We made an effort to achieve the best representation, as availability of material permitted, of the structural and ecological diversity of the subtribe over its geographical distribution worldwide, which required years of coordinated collecting effort by several collaborators. Our main aim is to assess generic monophyly and relationships as a foundation for subsequent systematic and evolutionary studies. We also discuss the value as phylogenetic and taxonomic markers of some floral morphological features in the light of our results and conduct an exploratory analysis of the ancestral distribution areas of the subtribe, major clades and genera.

MATERIAL AND METHODS

Taxon sampling

In total, 230 terminals were included in the phylogenetic analyses. These represent 182 species and 36 genera of Spiranthinae and 21 species/18 genera belonging to other subtribes of Cranichideae, namely Cranichidinae, Discyphinae, Galeottiellinae, Goodyerinae and Manniellinae (Salazar *et al.*, 2003; Álvarez-Molina & Cameron, 2009; Salazar *et al.* 2009; Batista *et al.*, 2011; Chase *et al.*, 2015; Supporting Information, Table S1). We are missing only monospecific *Aracamunia* Carnevali & I.Ramírez, monospecific *Cybebus* Garay, monospecific *Degranvillea* Determann and *Helonoma* Garay, which includes four species.

Molecular methods

Genomic DNA was extracted from fresh- or silica gel-dried plant tissue or from small leaf fragments, flower buds or pollinia taken from herbarium specimens. Extraction, amplification (PCR) and Sanger sequencing of DNA were carried out using standard protocols and the primers of Salazar *et al.* (2003).

Sequence editing and alignment

The bidirectional sequence reads were assembled and edited with Sequencher version 4 or 5 (GeneCodes Corp., Ann Arbor, MI, USA). Each DNA region (*matK–trnK*, *trnL–trnF* and ITS) was aligned separately using the L-INS-i algorithm implemented in the online interface of the software package MAFFT version 7 (Katoh & Standley, 2013; http://mafft.cbrc.jp/alignment/server/), with minor manual adjustment with Mesquite version 3.11 (Maddison & Maddison, 2016). In several instances, only partial sequences of one or more of the regions analysed were obtained, or one or two of the regions could not be sequenced for particular samples. The unavailable sequences or sequence portions were scored as missing data. The aligned matrix in Nexus format was deposited in the Dryad repository (doi:10.5061/dryad.9b9c1).

Phylogenetic analyses

We conducted MP and maximum likelihood (ML) analyses with the aim of comparing the patterns

Figure 2. Floral features of selected Spiranthinae. A–D. *Funkiella hyemalis* (A, Mexico, *Salazar 7633*; B–D, Mexico, *Salazar 6904*). A. Inflorescence. B. Proximal half of labellum showing the basal nectary (above) and thickened orange–red areas. C. Ventral view of column apex prior to removal of the pollinarium. D. Ventral view of column apex after removal of the pollinarium. E–H. *Funkiella parasitica* (Mexico, *Soto & Soto 10902*). E. Inflorescence. F. Labellum showing thickened orange–red areas. G. Ventral view of column apex prior to removal of the pollinarium. H. Ventral view of column apex after removal of the pollinarium. I–L. *Funkiella minutiflora* (Mexico, *Salazar et al. 9918*). I. Flower. J. Flower with the sepals and petals excised showing the labellum partially enfolding the column. K. Labellum showing thickened orange–red areas. L. Ventral view of column apex prior to removal of the pollinarium. M–O. *Sarcoglottis scintillans* (Mexico, *Salazar et al. 7436*). M. Flower. N. Longitudinal section of ovary and nectary. O. Ventral view of column prior to the removal of the pollinarium. P–T. *Sarcoglottis sceptrodes* (P: Mexico, *Figueroa 85*; Q–T, Mexico, *Martínez s.n.*). P. Longitudinal section of ovary and nectary. Q. Dorsal view of column apex showing the viscidium among the divergent pollinium apices. R. Ventral view of column apex showing the viscidium. S. Ventral view of pollinarium. T. Ventral view of column apex after removal of the pollinarium. Photographer: Gerardo A. Salazar. Scale bars: 10 mm (A, B, M, N, P); 5 mm (E); 3 mm (C, D, F, I, O); 1 mm (J, K, R, S, T); 0.5 mm (G, H, L, K).

of relationship and clade support generated by a method that does not require explicit models of nucleotide substitution (MP) with another that does (ML). Separate and combined MP analyses of plastid (*matK–trnK*/*trnL–trnF* regions) and nuclear DNA data (nrITS) were conducted with the software PAUP* version 4.0a150 for 32-bit Microsoft Windows (Swofford, 2016). Each analysis consisted of a heuristic search with 1000 replicates of random taxon order for the starting trees and TBR branch-swapping, saving in memory up to 20 most-parsimonious trees (MPTs) from each replicate to limit the time spent swapping in large islands of trees (Maddison, 1991). All characters were treated as unordered and equally weighted, and the individual positions of indel events postulated to account for length differences among sequences were treated as missing data. Clade support was evaluated with 1000 bootstrap replicates (Felsenstein, 1985), each consisting of 20 heuristic searches with random taxon order for the starting trees and TBR branch-swapping, saving up to 20 shortest trees per search.

ML analyses were conducted for separate and combined plastid and nuclear data with the program RAxML-HPC2 on XSEDE version 8.2.9 (Stamatakis, 2014) implemented in the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal 2.0 (Miller, Pfeiffer & Schwartz, 2010). One thousand rapid bootstrap replicates (Stamatakis, Hoover & Rougemont, 2008) were followed by a thorough ML search with the default value of 25 rate categories and the GTRGAMMA model for nucleotides, allowing separate estimation of all free model parameters for the *matK* gene, *trnK* intron excluding *matK*, *trnL* intron, *trnL–trnF* intergenic spacer and the nrITS region.

The phylogenetic trees were edited with FigTree version 1.4.0 (Rambaut, 2012) and Photoshop CC (Adobe Systems Inc., San Jose, CA, USA). Bootstrap percentages of 51–74, 75–89 and 90–100 were arbitrarily considered as weak, moderate and strong support, respectively.

Ancestral area reconstruction

We conducted an ancestral area analysis using the Bayesian binary Markov chain Monte Carlo method (BMM) for ancestral states implemented in the RASP software package (reconstruct ancestral state in phylogenies; Yu *et al.*, 2015). The tree obtained in our ML analysis of combined plastid and nuclear sequences was loaded into RASP and ten Markov chains were run for 5 000 000 generations, sampling every 1000 generations and discarding 20% of the trees sampled as burn-in. State frequencies were estimated using the F81 model and the among-site rate variation model was set to gamma. The maximum number of reconstructed ancestral areas for a clade was set to five. Eleven distributions were considered that represent major areas of endemism for the species included in the phylogenetic analyses: (A) temperate North America; (B) Mesoamerica (including Mexico and Central America south to the Panama/Colombia border); (C) Andean South America; (D) eastern South America; (E) Amazonia; (F) tropical Asia; (G) west tropical Africa; (H) New Caledonia; (I) Malagasy region; (J) Caribbean; and (K) temperate Eurasia and northern Africa. Distribution data were recorded from specimens housed in the herbaria studied (AMES, AMO, ANDES, ARIZ, ASU, BHCB, BM, CAS, CHAPA, COL, CORU, ENCB, F, FCME, GH, IBUG, IEB, JBSD, K, LL, MEXU, MG, MHES, MO, NY, PMA, QCA, QCNE, R, RB, SEL, SERO, TEX, UAMIZ, US, USJ, UVAL, VEN, W and XAL; acronyms according to Thiers, 2017), complemented with records from public databases. The latter include *Portal de datos abiertos UNAM* (https://datosabiertos.unam.mx/), *SEINET* (http://swbiodiversity.org/seinet/), *Tropicos* (http://www.tropicos.org/Home.aspx), *REFLORA* (http://floradobrasil.jbrj.gov.br/reflora/PrincipalUC/PrincipalUC.do) and the *World Checklist of Selected Plant Families* (http://apps.kew.org/wcsp/prepareChecklist.do;jsessionid=DC970EDFBCF052B4F2BFA4BCE576E9A2?checklist=selected_families%40%40002020120150657121).

RESULTS

Phylogenetic analyses

The plastid dataset consisted of 224 terminals and 4171 characters, of which 1237 (30%) were potentially parsimony-informative. The plastid MP analysis found 4160 MPTs with a length of 5397 steps, consistency index (CI) excluding uninformative characters of 0.40 and retention index (RI) of 0.80. The strict consensus included 4160 trees with clade support from the bootstrap analysis (Supporting Information, Fig. S1). The ML tree of the plastid dataset was topologically similar to that from the MP analysis, but support was usually slightly higher in the ML analysis (Supporting Information, Fig. S2). The major difference between the MP and ML plastid trees was the position of *Coccineorchis* Schltr., placed by MP as sister to the *Eurystyles* clade and by ML as sister to the *Pelexia* clade, but neither position obtained a bootstrap percentage (BP) > 50.

The nrITS dataset included 221 terminals and 760 characters, of which 382 (50%) were potentially parsimony-informative. The nrITS MP analysis found 11 500 MPTs with a length of 2691 steps, CI of 0.30 and RI of 0.79. The consensus tree (Supporting Information,

Fig. S3) is topologically similar to the tree obtained in the ML nrITS analysis, but, as in the plastid dataset, resolution and clade support were overall higher in the ML analysis (Supporting Information, Fig. S4). The major groups recovered by the MP and ML nrITS analyses are for the most part the same as those found in the plastid trees, except for the *Eurystyles* clade being nested in the *Spiranthes* clade in the nrITS analyses. The sister-group relationship between the *Eurystyles* clade and the clade that includes species of *Hapalorchis* Schltr. [and in the nrITS analyses *Pseudoeurystyles lorenzii* (Cogn.) Hoehne] was weakly (BP 73) and strongly supported (BP 90) by the MP and ML nrITS analyses, respectively (Supporting Information, Figs S3, S4).

The combined matrix consisted of 230 terminals and 4931 characters, of which 1619 (33%) were potentially parsimony-informative. The combined MP analysis resulted in 6400 MPTs with a length of 8158 steps, CI of 0.36 and RI of 0.79 (Supporting Information, Fig. S5). As in the separate analyses, resolution increased and more clades obtained strong support (BP ≥ 90) in the ML analysis (Figs 3–6) relative to the MP analysis, with some exceptions. These exceptions include the *Stenorrhynchos* clade, which in the MP combined analysis was strongly supported (BP 100), whereas in the ML analysis received weak support (BP 68). Likewise, a subclade of the *Pelexia* clade containing, among others, the species of *Cyclopogon* C.Presl *s.l.* was strongly supported by MP (BP 100), but weakly so by ML (BP 68). Given the similarity in the groups recovered by all analyses, and the greater resolution and overall bootstrap support of the tree resulting from the ML analysis of combined plastid and nuclear data, in the following we will use the latter for describing the phylogenetic results. For ease of visualization, Figures 3–6 show only the portion of the tree corresponding to Spiranthinae, divided into their major clades. The full combined ML tree is displayed in Supporting Information, Figure S6.

Cotylolabium lutzii (Pabst) Garay is sister of the remaining Spiranthinae, which consist of four major clades corresponding to the *Eurystyles*, *Spiranthes*, *Stenorrhynchos* and *Pelexia* clades identified in previous molecular phylogenetic analyses. The *Eurystyles* clade includes monospecific *Quechua* Salazar & Jost as the sister (BP 87) of a strongly supported clade (BP 100) including monophyletic *Lankesterella* Ames and *Eurystyles*. The *Eurystyles* clade is the strongly supported sister (BP 93) of the *Spiranthes* clade (BP 100), and the latter includes four main subclades, marked with numbered circles 1–4 in Figure 3. The first of these subclades (BP 100) includes *Pseudoeurystyles lorenzii* and *Hapalorchis*. The second subclade consists of *Funkiella* Schltr. as sister of a group encompassing *Sotoa* Salazar plus *Svenkoeltzia* Burns-Bal. in turn sister to *Beloglottis* Schltr. plus *Aulosepalum* Garay; all genera in this subclade for which more than one species was analysed are monophyletic and strongly supported. The third main subclade is *Spiranthes* (BP 100), and the fourth one encompasses a group (BP < 50) consisting of weakly supported *Physogyne* Garay plus *Pseudogoodyera* Schltr. (BP 77) and *Mesadenus* Schltr. plus *Greenwoodiella* Salazar, Hern.-López & J.Sharma (BP 79), and another clade that includes *Kionophyton* Garay, *Schiedeella* Schltr., *Dichromanthus* Garay and *Deiregyne* Schltr. (*sensu* Garay, 1982; Salazar, 2003b), all with BP 100.

The *Stenorrhynchos* clade (Fig. 4; BP 68) includes *Mesadenus glaziovii* (Cogn.) Schltr. (which renders *Mesadenus* polyphyletic), monophyletic *Stenorrhynchos* (clade 5) and monospecific *Thelyschista* Garay plus *Buchtienia ecuadorensis* Garay as successive sisters of a major group consisting of two subclades. The first subclade (6) is strongly supported (BP 99) and encompasses *Nothostele acianthiformis* (Rchb.f. & Warm.) Garay sister to *Eltroplectris* Raf. (BP 100). The second subclade (7) consists, on the one hand, of a weakly supported group (BP 63) with various species of *Pteroglossa* Schltr. and *Mesadenella* Pabst & Garay and another group (BP 65) that in turn consists of two clades: *Lyroglossa grisebachii* (Cogn.) Schltr. sister to *Pteroglossa macrantha* (Rchb.f.) Schltr. plus *Sacoila hassleri* (Cogn.) Garay (BP 66) and *Skeptrostachys* Garay (BP 95) sister to *Sacoila lanceolata* (Aubl.) Garay (BP 67). Hence, neither *Pteroglossa* nor *Sacoila* Raf. is monophyletic.

In the *Pelexia* clade, *Coccineorchis* is weakly associated with the rest (Fig. 5; BP 61) and *Sauroglossum elatum* Lindl. diverges next (BP 68). *Sarcoglottis* C.Presl (clade 8) is strongly supported (BP 100) and includes a group of Mexican/Central American species, *S. corymbosa* Garay to *S. cerina* (Lindl.) P.N.Don nested among mostly South American species. The sister of *Sarcoglottis* is a strongly supported clade (Fig. 5, clade 9; BP 100) encompassing polyphyletic *Pelexia* and some members of *Odontorrhynchus* M.N.Correa, *Brachystele* Schltr. and Andean *Sauroglossum corymbosum* (Lindl.) Garay. *Pelexia weberbaueriana* (Kraenzl. ex Schltr.) Schltr., *Sauroglossum corymbosum* and *Odontorrhynchus chlorops* (Rchb.f.) Garay form a strongly supported group (clade 10; BP 100) that is sister to a 'core' *Pelexia* clade, which includes three strongly supported groups: *Pelexia* section *Pachygenium* Schltr. (clade 11), *Brachystele* (with *Odontorrhynchus variabilis* Garay nested; clade 12) and *Pelexia* section *Pelexia* (clade 13). Relationships among these are not clearly resolved (e.g. the sister-group relationship between clades 12 and 13 attained a BP < 50). The remaining members of the *Pelexia* clade (Fig. 6) consist mostly of species of *Cyclopogon s.l.*, with *Veyretia* Szlach. and *Brachystele guayanensis* (Lindl.) Schltr. embedded in a derived position; hence, both *Brachystele* and *Cyclopogon* are polyphyletic.

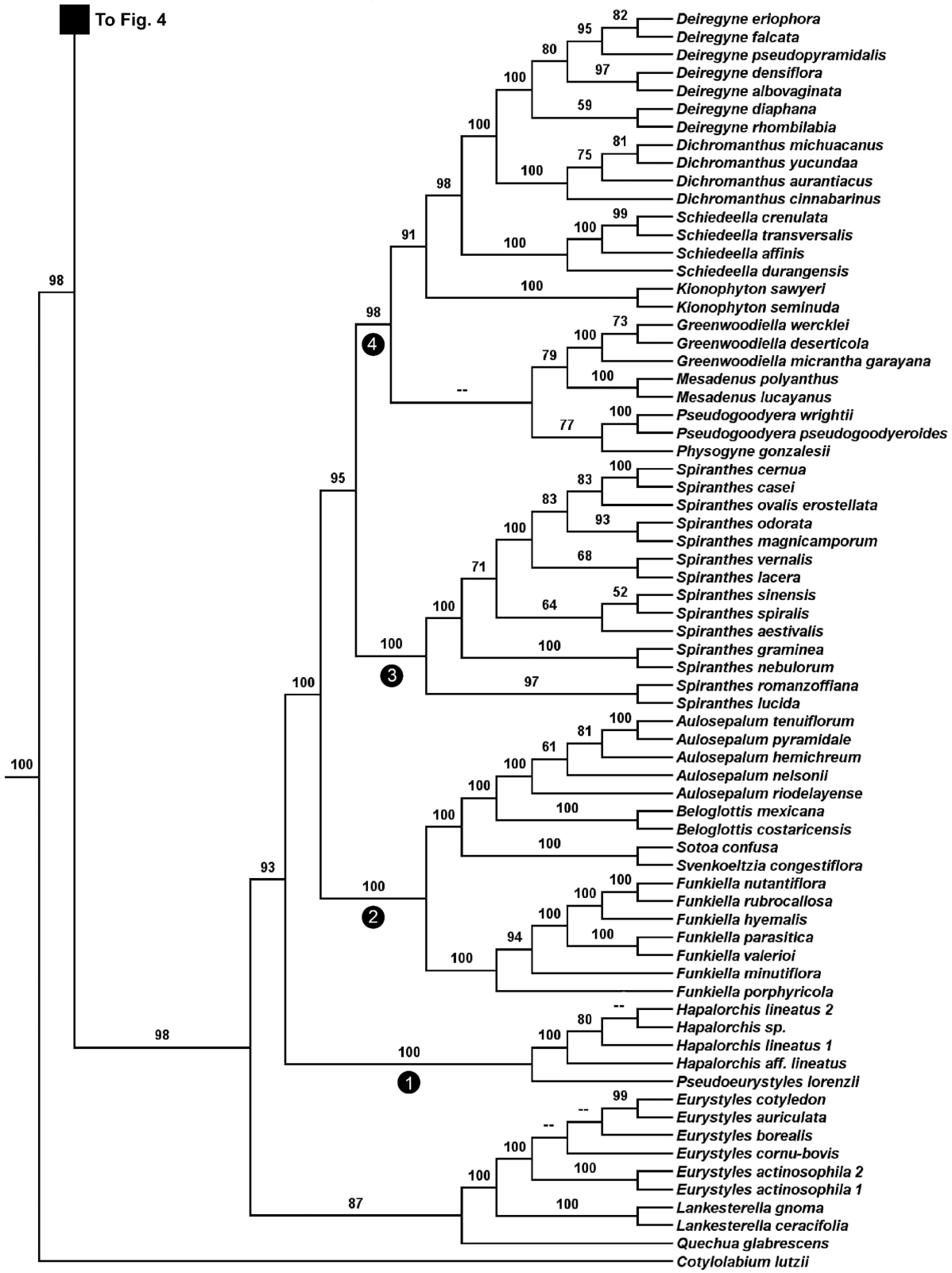


Figure 3. Maximum likelihood tree from the ML analysis of combined plastid and nuclear DNA sequences. Numbers above branches indicate bootstrap percentages > 50. For simplicity, outgroups were excluded. Numbered black circles mark clades discussed in the text.

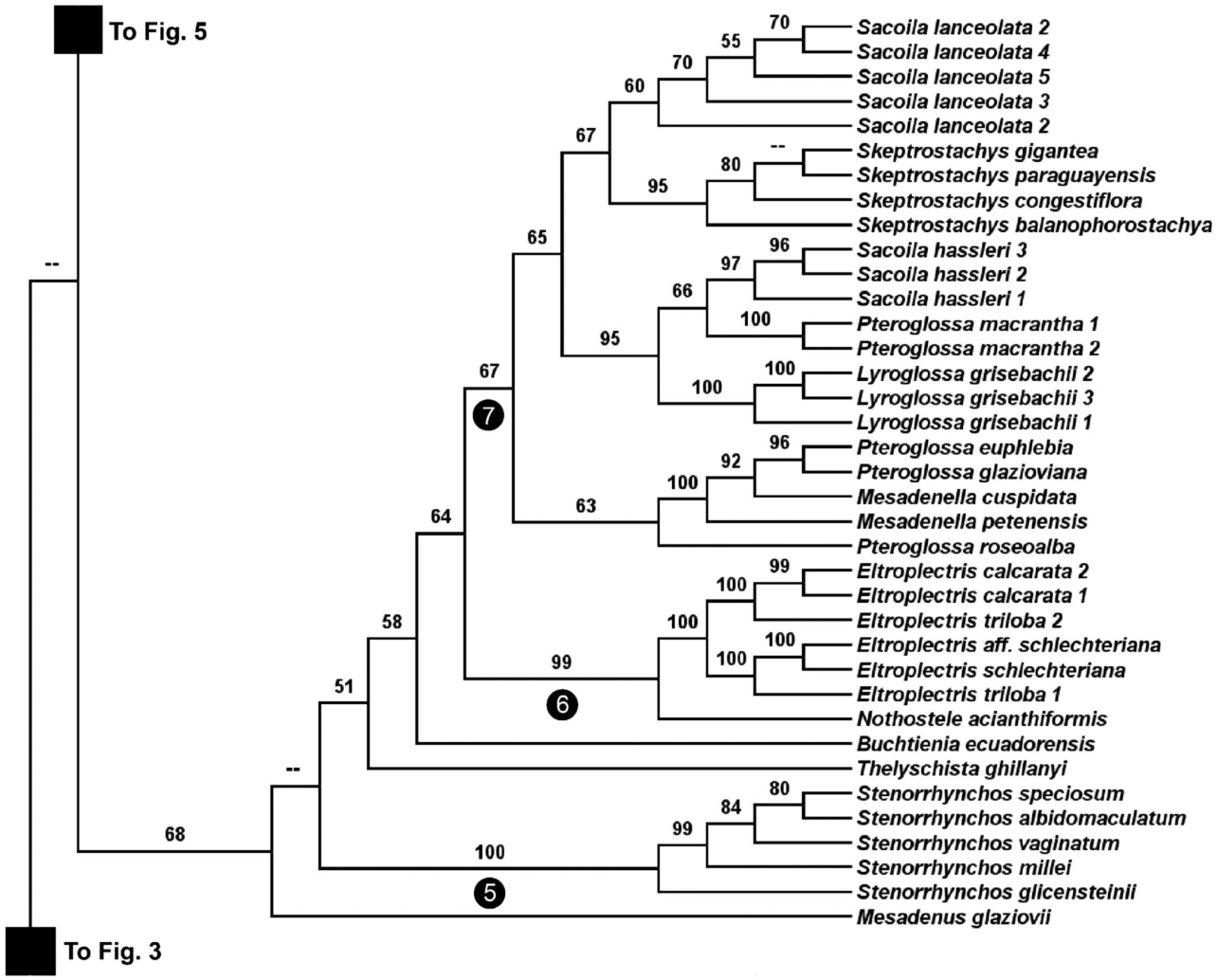


Figure 4. Maximum likelihood tree from the ML analysis of combined plastid and nuclear DNA sequences (continuation of Fig. 3). Numbers above branches indicate bootstrap percentages > 50. Numbered black circles mark clades discussed in the text.

Ancestral area analysis

The analysis identified eastern South America as the ancestral area for Spiranthinae, with a major dispersal to Mesoamerica in the last common ancestor (LCA) of the *Spiranthes* clade (Fig. 10; Supporting Information, Fig. S8). Two separate dispersals from Mesoamerica to North America [one for the LCA of the clade consisting of *S. lucida* (H.H.Eaton) Ames and *S. romanzoffiana* Cham. and the other for the LCA of the remainder of North American *Spiranthes*] and one to the Old World are inferred from our data. Additional dispersals to Mesoamerica include one of the two main subclades of *Sarcoglottis*, *Pelexia* section *Pelexia* (or *Pelexia s.s.*; see Discussion) and particular species or groups in *Eurystyles*, *Stenorrhynchos*, *Mesadenella* and *Cyclopogon*.

DISCUSSION

Overall phylogenetic relationships in Spiranthinae

The data sets analysed, separate and combined, irrespective of the method of analysis (MP or ML) recovered the same five main clades of Spiranthinae, with some topological differences among analyses that did not obtain strong bootstrap support. Such major lineages, namely *Cotylolabium* and the *Stenorrhynchos*, *Eurystyles*, *Spiranthes* and *Pelexia* clades (Figs 3–6) fully agree with those groups found in previous analyses of the same DNA regions but which included a much smaller taxonomic sample (e.g. Salazar *et al.*, 2003, 2011a, 2016; Batista *et al.*, 2011; Borba *et al.*, 2014). As in those works, the major clades received varying degrees of support, but the relationships among

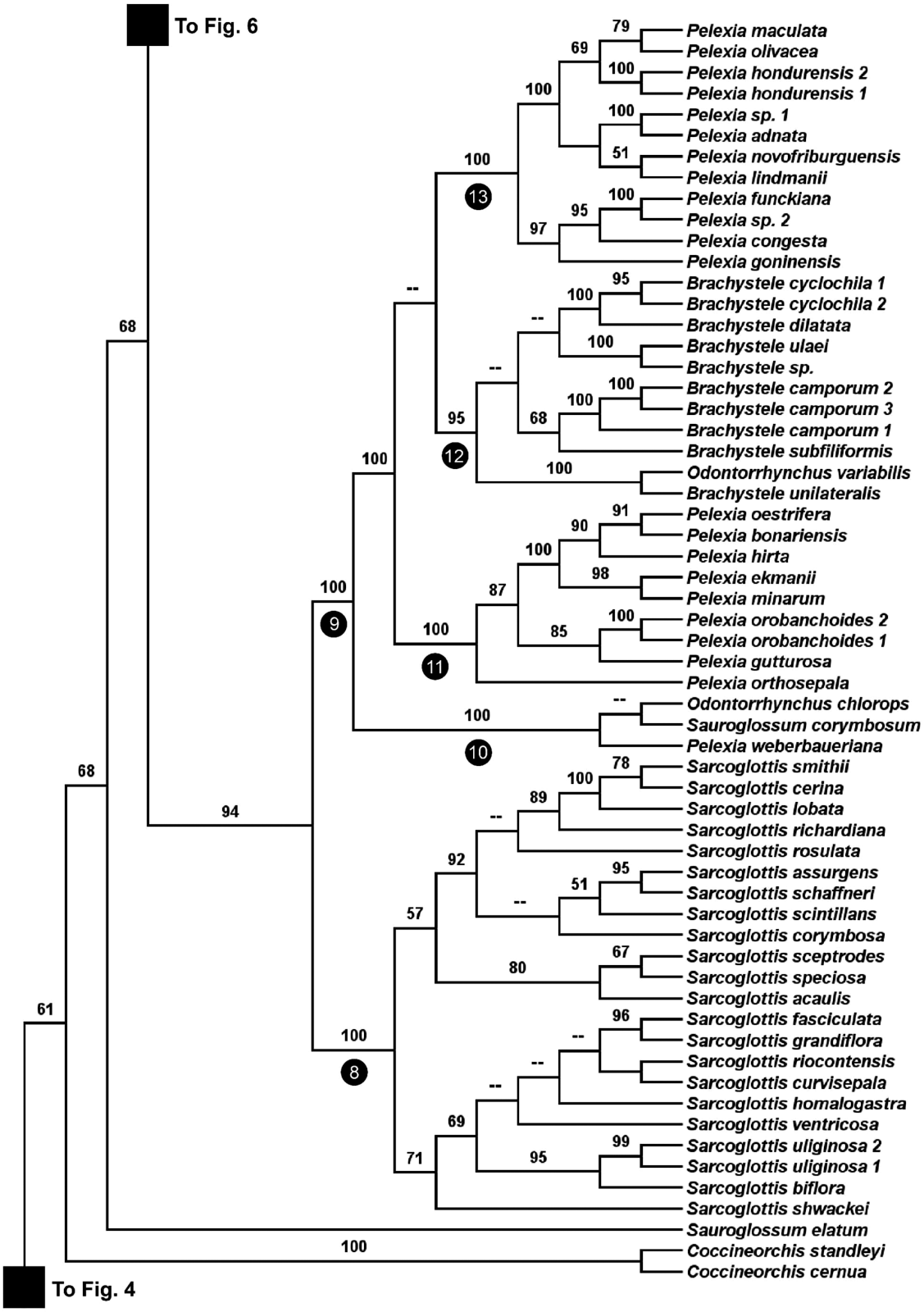


Figure 5. Maximum likelihood tree from the ML analysis of combined plastid and nuclear DNA sequences (continuation of Fig. 4). Numbers above branches indicate bootstrap percentages > 50. Numbered black circles mark clades discussed in the text.

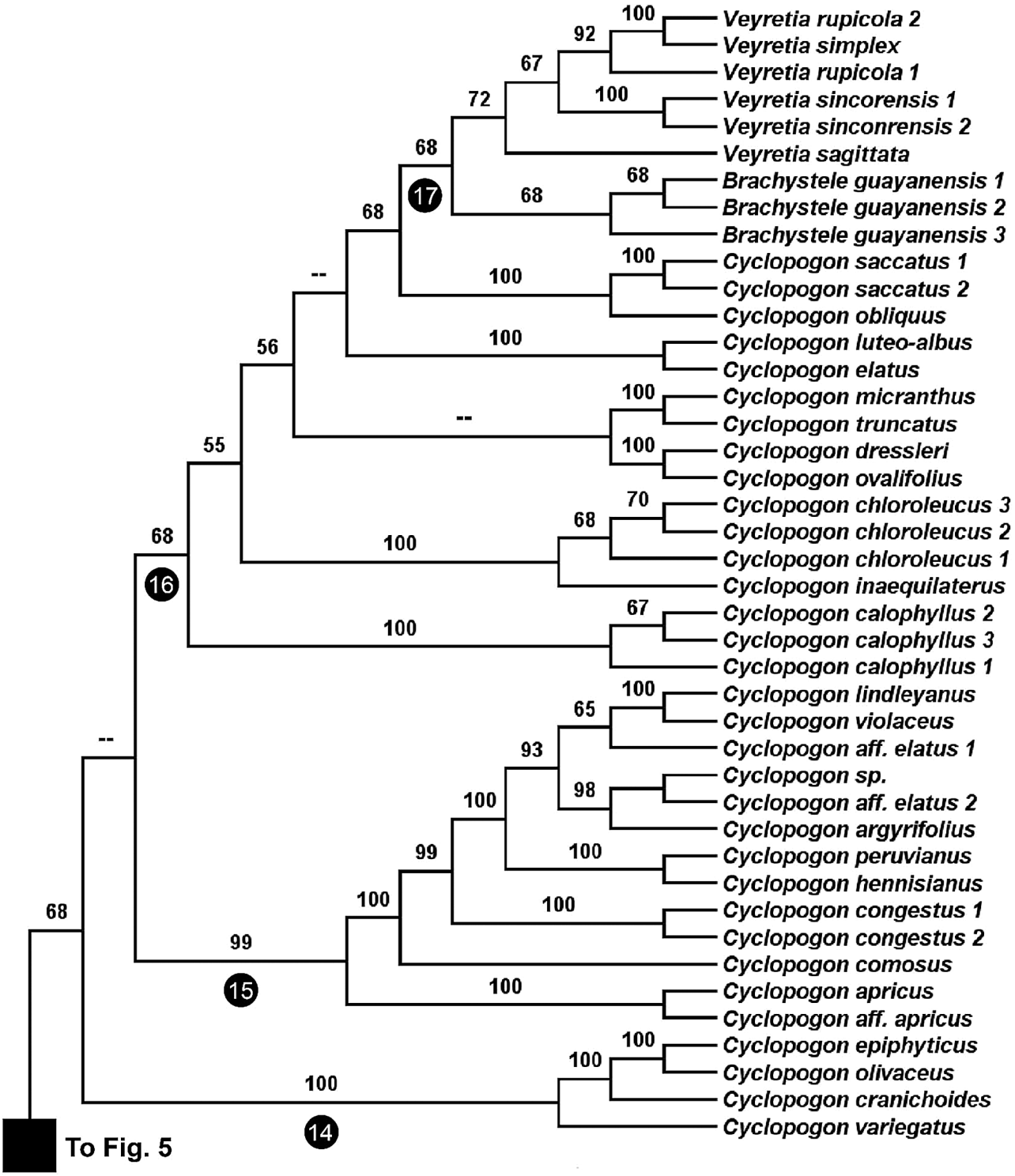


Figure 6. Maximum likelihood tree from the ML analysis of combined plastid and nuclear DNA sequences (continuation of Fig. 5). Numbers above branches indicate bootstrap percentages > 50. Numbered black circles mark clades discussed in the text.

them are not clearly resolved with the exception, in the present analysis, of a strongly supported sister-group relationship between the *Eurystyles* and *Spiranthes* clades (BP 91 and 98 in the combined MP and ML analyses, respectively). Lack of supported resolution for the relationships among the *Pelexia* and

Stenorrhynchos clades with respect to one another and to the group formed by the *Eurystyles*/*Spiranthes* clades might be a reflection of the short branches subtending those portions of the tree (Supporting Information, Fig. S7). The small number of molecular changes contrasts with the noticeable structural, ecological and distributional differences among the major clades of Spiranthinae (see later; Salazar *et al.*, 2003), a combination suggestive of a succession of cladogenetic events in a geologically short time interval during which little genetic change accumulated.

Generic monophyly and relationships

Of the 27 genera of Spiranthinae for which more than one species was included in our analyses, 18 were recovered as monophyletic. In the following, the genera are commented upon, in the context of the major clade to which they belong, in ascending branching order according to the tree depicted in Figures 3–6.

Cotylolabium

The single species in this genus was described originally as a species of *Stenorrhynchos*, but our results, like those from previous analysis that have included it, clearly place it as the sister of the rest of Spiranthinae (Borba *et al.*, 2014; Salazar *et al.*, 2016). Borba *et al.* (2014) provided detailed illustrations and discussion of the distinctive vegetative and floral attributes of *C. lutzii*, hypothesizing that the scented, bright yellow flowers with partially spreading lateral sepals and distally broadened labellum (Fig. 7A) correspond to a mellitophilous pollination syndrome.

Eurystyles *clade*

The monospecific, central Andean *Quechua* (Fig. 7B) was proposed as a distinct genus only recently based on MP and Bayesian inference analyses of the same DNA regions used here and detailed morphological comparisons (Salazar & Jost, 2012). Previously, *Quechua glabrescens* (T.Hashim.) Salazar & Jost had been included in *Spiranthes* or *Cyclopogon*. Overall, floral structure of *Q. glabrescens* is reminiscent of that of some species of *Hapalorchis* (Fig. 7E), and the early-diverging position of both *Quechua* and *Hapalorchis* in their respective major clades suggests that such floral structure might represent plesiomorphic (shared ancestral) traits in the group formed by the *Eurystyles* and *Spiranthes* clades. Vegetatively, *Q. glabrescens* sharply differs from *Hapalorchis* in having a rosette or sessile, linear-oblanceolate, fleshy leaves, in contrast to the petiolate, ovate, membranaceous leaves of *Hapalorchis*. The leaves of *Quechua* appear to persist over more than 1 year, a feature shared with its closest relatives (*Lankesterella* and *Eurystyles*).

Eurystyles and *Lankesterella* (Fig. 7C, D) form a strongly supported group, corroborating the results of previous molecular phylogenetic studies (e.g. Górniak *et al.*, 2006; Salazar & Dressler, 2011). Their unusual (in Spiranthinae) epiphytic habit and small rosettes of persistent, usually ciliate leaves (Fig. 1F) led Dressler (1981), Soto (1993) and Salazar (2005b) to argue for a close relationship between these two genera, despite differences in inflorescence and flower morphology. Burns-Balogh, Robinson & Foster (1985) stressed the unique features of the leaves, inflorescence and column of *Eurystyles*, *Synanthes* Burns-Bal., H.Rob. & M.S.Foster (here considered a synonym of *Eurystyles*; see later) and *Pseudoeurystyles* Hoehne and treated them as a distinct alliance in Spiranthinae. However, they did not compare them with *Lankesterella*, which Balogh (1982) had previously sunk as a section of *Stenorrhynchos*. Concerning the relationships of the *Eurystyles* alliance to other Spiranthinae, Burns-Balogh *et al.* (1985) considered the presence in some of its members of a supposedly plesiomorphic type of rostellum (i.e. one with an excised rostellar remnant similar to that in *Spiranthes*) as evidence of its early divergence from the 'basal stock' of the subtribe. Such a claim, however, is inconsistent with the derived position of *Spiranthes* in a different major clade (see below). Szlachetko (1992) noticed similarities in labellum morphology between some species of *Eurystyles* and *Lankesterella*, but in his classification (Szlachetko, 1995a; Szlachetko *et al.*, 2005; Rutkowski *et al.*, 2008), which emphasized characters of the column, placed the former in his version of Spiranthinae and the latter in 'Stenorrhynchidinae'. Such segregation is untenable on phylogenetic grounds given the sister-group relationship between these genera, strongly supported by vegetative and genetic evidence. Other than the recurrence of autogamy, nothing is known of natural pollination of *Eurystyles* and *Lankesterella*; Salazar & Dressler (2011) proposed that the differences in reproductive structure between these sister genera could be a reflection of different pollination mechanisms.

Mesoamerican *Eurystyles borealis* A.H.Heller has been associated, on morphological grounds, with Paraguayan *E. bertonii* (Burns-Bal., H.Rob. & M.S.Foster) Szlach. in *Synanthes* Burns-Bal., H.Rob. & M.S.Foster (Burns-Balogh *et al.*, 1985). However, these two species, each distributed at one extreme of the Neotropics, are auto-pollinating and the character that distinguishes them from other *Eurystyles* spp. (absence of a rostellum) probably evolved convergently. Previous studies have shown that absence of a rostellum is recurrent in auto-pollinating variants of various species of Spiranthinae (e.g.

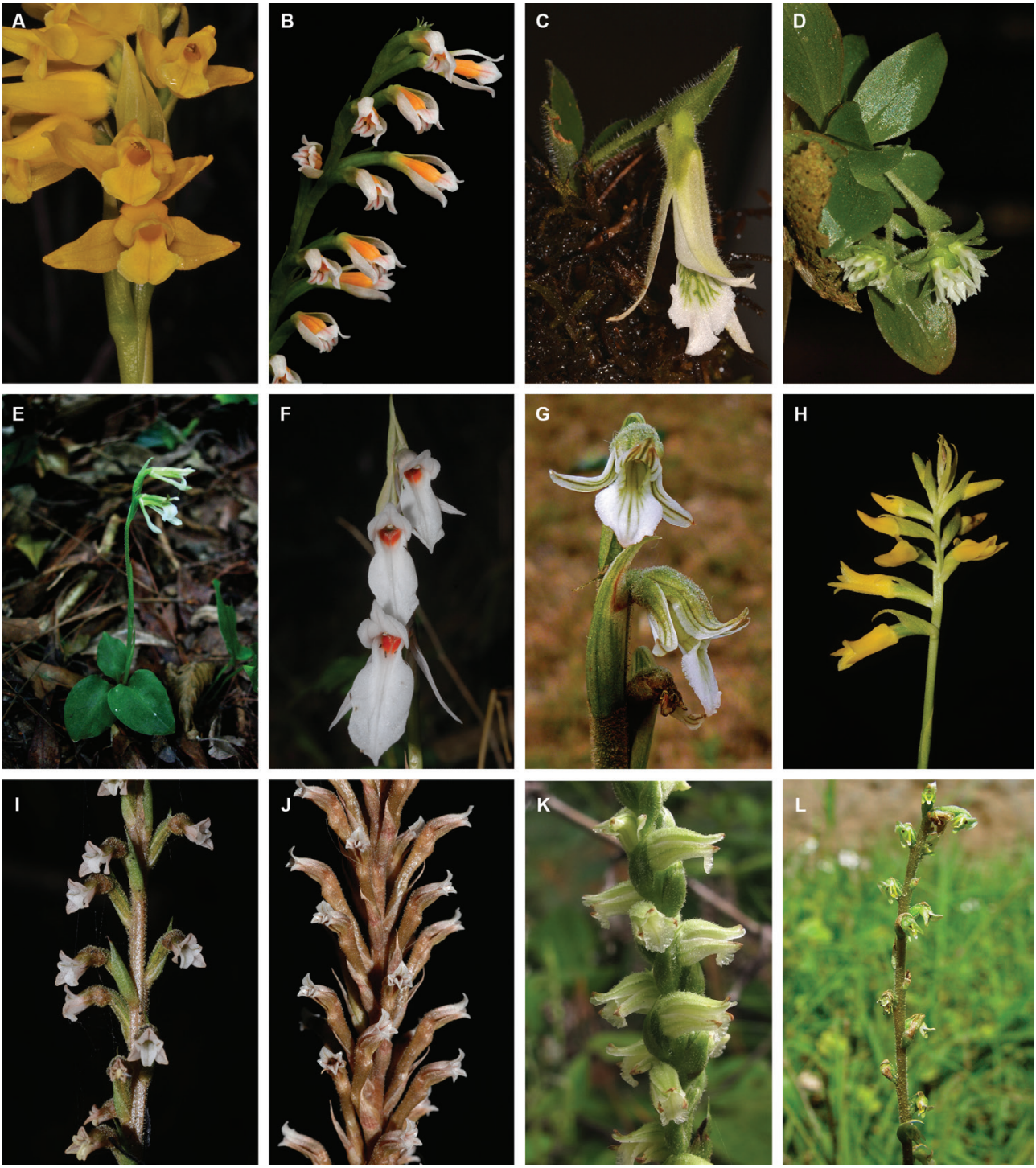


Figure 7. Inflorescences of selected members of Spiranthinae. A. *Cotylolabium lutzii* (Brazil, *Martins da Costa 326*). B. *Quechua glabrescens* (Ecuador, *Jost 7916*). C. *Lankesterella gnoma* (Brazil, *Batista s.n.*). D. *Eurystyles actinosophila* (Brazil, *Batista s.n.*). E. *Hapalorchis* aff. *lineatus* (Guatemala, *Salazar et al. 7699*). F. *Funkiella hyemalis* (Mexico, *Salazar et al. 9177*). G. *Sotoa confusa* (Mexico, *Hernández-López & Treviño-Carreón 85*). H. *Svenkoeltzia congestiflora* (Mexico, *Salazar 9507*). I. *Beloglottis mexicana* (Mexico, *Salazar et al. 9349*). J. *Aulosepalum tenuiflorum* (Mexico, *Salazar et al. 7427*). K. *Spiranthes nebulorum* (Mexico, *Beutelspacher s.n.*). L. *Physogyne gonzalesii* (Mexico, *Jiménez-Machorrro s.n.*). Photographers: Eduardo L. Borba (A), Lou Jost (B), João A. N. Batista (C, D), Gerardo A. Salazar (E, F, H–J), Tania Hernández-López (G), Carlos R. Beutelspacher (K), Rolando Jiménez-Machorro (L).

Catling, 1990; Szlachetko, 1992; Salazar *et al.*, 2016). *Eurystyles cornu-bovis* Szlach. is unique in the genus in having entire (i.e. not ciliate) leaf margins and pink (vs. greenish or whitish) flowers, among other minor details of its floral morphology (Szlachetko, 1992), but these features probably represent derived autapomorphies.

Spiranthes *clade*

This strongly supported group includes four main clades (1–4 in Fig. 3). In the first of these, our accession of *Pseudoeurystyles lorenzii* is sister, with strong support (BP 100), to *Hapalorchis*. Vegetative and floral similarities of *P. lorenzii* to the *Eurystyles* clade, and especially to *Lankesterella* (Szlachetko, 1992), might represent symplesiomorphies inherited from the most recent common ancestor shared by these clades, as suggested earlier for *Quechua*. However, because we were not able to include sequences of the plastid regions, this result should be considered with caution and will be explored further in another contribution (A. A. Bernal & E. C. Smidt, unpubl. data).

In the second major subclade of the *Spiranthes* clade (Fig. 3, clade 2), *Funkiella s.l.* (including *Microthelys* Garay, *Ecuadoria* Dodson & Dressler and, probably, *Stalkya* Garay; Salazar, 2003b; Solano-Gómez, Salazar & Jiménez-Machorro, 2011) is sister to a clade with *Sotoa* plus *Svenkoeltzia* and *Beloglottis* as the successive sisters of *Aulosepalum*. The monophyly of *Funkiella*, *Beloglottis* and *Aulosepalum* is strongly supported, as are the relationships among all these genera. *Funkiella* includes high-elevation species distributed on most major cordilleras from southern North America, Central America and the Greater Antilles south to Ecuador, which display a noticeable variation in flower size and rostellum remnant/pollinarium morphology (Fig. 2A–L). For instance, flowers of *F. minutiflora* (A.Rich. & Galeotti) Salazar & Soto Arenas are *c*. 3 mm long or less (Fig. 2I–K), have a shortly apiculate rostellum remnant and the viscidium is located centrally on the ventral surface of the pollinarium (Fig. 2L). This last feature, a centrally placed viscidium, is shared also by species of the polyphyletic and distantly related *Mesadenus* Schltr. (see later), and, outside Spiranthinae, by *Galeottiella* (Salazar *et al.*, 2002, 2003; Salazar, 2003a). In contrast, the sepals of *F. hyemalis* (A.Rich. & Galeotti) Schltr. (Figs 2A, 7F), the largest-flowered species of the genus, can exceed 25 mm in length, and the rostellum remnant is distinctly elongate and basally tridentate, with the viscidium attached to the distal part of the pollinarium (Fig. 2C, D). However, all *Funkiella* spp. share a putative morphological synapomorphy, i.e. possession of a red or orange thickened area on the labellum (Fig. 2B, F, K), and differences in flower size and column/pollinarium morphology probably reflect different pollination syndromes.

Monotypic *Sotoa* (Fig. 7G) is a geophyte restricted to the outskirts of the Chihuahuan Desert and other semi-arid regions of southern North America (southern USA south to the Mexican state of Oaxaca; Salazar & Ballesteros-Barrera, 2010). Its flowers are fragrant during daytime hours, predominantly white, sometimes with rosy suffusion, and the sepals and petals bear contrasting green or brownish veining, all of which suggests mellitophily. *Svenkoeltzia* encompasses four tenuously defined species, plants grow epiphytically or lithophytically in moist oak–coniferous forests in southern Mexico, and, in contrast to *Sotoa*, they have a more or less one-sided raceme with bright yellow flowers probably pollinated by hummingbirds (Fig. 7H). *Sotoa confusa* (Garay) Salazar and *Svenkoeltzia congestiflora* (L.O.Williams) Burns-Bal. have been placed by taxonomists in various versions of *Funkiella* (e.g. Garay, 1982; Szlachetko, 1993b; Szlachetko *et al.*, 2005), but their phylogenetic position precludes their inclusion in *Funkiella* (Fig. 3). An as-yet undescribed species recently discovered in the Chihuahuan Desert (north-eastern Mexico) 'blurs' the morphological and genetic distinction between *Sotoa* and *Svenkoeltzia*, and ongoing phylogenetic studies might result in the merging of these two small genera (G. A. Salazar & T. J. Hernández-López, unpubl. data).

Beloglottis (Fig. 7I) is widespread in the mainland Neotropics (Mexico to Bolivia) and occurs in moist to wet tropical forests and cloud forests, usually living as a lithophyte or epiphyte. As in previous molecular analyses (e.g. Salazar *et al.*, 2003, 2011a, 2016), in the present study *Beloglottis* is strongly supported as the sister of *Aulosepalum* (Fig. 7J). Szlachetko (1996) considered the distinctive Guiana Highland/Andean genus *Helonoma* as a synonym of *Beloglottis*. No material of *Helonoma* suitable for DNA analysis has been available to us, but such an approach is unsustained on morphological and ecological grounds. *Helonoma* spp. occur in the highly specialized, wet, oligotrophic environments on top of Guiana Highland tepuis and Andean tepui habitats of Colombia, Ecuador and Peru in the case of *H. peruviana* (Szlach.) Salazar, Dueñas & Fern.-Alonso (formerly *Wallnoeferia peruviana* Szlach.; Dueñas & Fernández-Alonso, 2009). Indeed, *Helonoma* is similar to the Guiana Highland endemic, monospecific *Aracamunia* in its rhizomatous habit, roots covered by silvery pubescence, few-flowered raceme, flowers provided with a long mentum (and correspondingly long column foot), partially fused sepals and spatulate petals partially adnate to the sepals. *Aracamunia liesneri* Carnevali & I.Ramírez is distinctive, however, in the clavate, glandular processes arising from the leaf axils, which have been suggested to be compatible

with carnivory (Carnevali & Ramírez in Steyermark & Holst, 1989; Salazar, 2003b). *Aracamunia* has not been available for molecular study.

Aulosepalum (Fig. 7J) occurs from Mexico to Costa Rica, and its species inhabit predominantly tropical deciduous and semi-deciduous forests, oak–coniferous forests and xerophilous scrub (Salazar 2003b, 2005a). *Aulosepalum* has been the subject of taxonomic contention (see discussion under *Aulosepalum* and, especially, *Deiregyne* in Salazar, 2003b). Garay's (1982) concept of *Aulosepalum* required only a few adjustments, such as inclusion of *A. pyramidale* (Lindl.) M.A.Dix & M.W.Dix (placed by Garay in his *Kionophyton*) and an additional species, *A. riodelayense* (Burns-Bal.) Salazar, to match the strongly supported monophyletic group identified in this study. González & Szlachetko (1995) segregated *A. pyramidale* and *A. riodelayense* in their new genus, *Gracielanthus* R.González & Szlach., which is polyphyletic (Fig. 3). Other than their shorter floral tubes and correspondingly shorter labellum bases, these two species fit well with the rest of *Aulosepalum*. To further complicate nomenclature, Rutkowski, Mytnik & Szlachetko (2004) treated *Aulosepalum* as a subgenus of their concept of *Deiregyne* (which corresponds to Garay's *Aulosepalum*, as argued by Catling, 1989; Salazar, 2003b), but soon after they changed their minds and considered *Aulosepalum* as a monospecific, distinct genus (Szlachetko *et al.*, 2005). The details of this nomenclatural fiasco are beyond the focus of the present paper and will be dealt with in another contribution (G. A. Salazar, R. Chalqueño & S. A. Adachi, unpubl. data).

Spiranthes (Fig. 3, clade 3) is strongly supported in our analyses. Dueck, Aygoren & Cameron (2014) thoroughly assessed phylogenetic relationships in this genus based on a nearly complete sample of the *c*. 36 currently accepted species using several plastid and nuclear DNA regions. Our results, based on a limited sample of taxa (14 species), agree in most details with theirs, placing *S. romanzoffiana* plus *S. lucida* as the sister of the rest, matching the 'mainly western North American clade' of Dueck *et al.* (2014) Our analysis also recovers an Old World group, including *S. aestivalis* (Poir.) Rich., *S. sinensis* (Pers.) Ames and *S. spiralis* (L.) Chevall., that is sister to their 'midwestern and eastern North American clade', and the relationships in the latter are congruent with their results, too. Of particular interest was the inclusion, in our analysis, of Mesoamerican *S. graminea* Lindl. and *S. nebulorum* Catling & V.R.Catling (Fig. 7K), which were unavailable to Dueck *et al.* (2014). Based on cytogenetic and morphological similarities, e.g. to *S. praecox* (Walter) S.Watson, Dueck *et al.* (2014) suggested that *S. graminea* could belong in the primarily western North American clade, but our results place *S. graminea* plus *S. nebulorum* as sister to the rest minus the western clade. Both our molecular results and our ancestral area reconstruction agree with the hypothesis posed by Dueck *et al.* (2014) that *Spiranthes* is derived from Mesoamerican ancestors (Figs 3, 10; Supporting Information, Fig. S8).

Catling (1983) reviewed the pollination mechanisms of several northern North American *Spiranthes* spp. Except for auto-pollinating and apomictic races that occur in various species, flowers of Spiranthes are pollinated by several kinds of bees, mainly of Apidae (bumblebees, *Bombus* spp., and honey bees, *Apis mellifera*) but also members of Halictidae, Megachilidae and Andrenidae. In species pollinated by *Bombus* and megachilids, e.g. *S. lacera* (Raf.) Raf. and *S. romanzoffiana*, nectar accumulates at the bottom of the floral tube. The bees land on the lowermost open flowers and crawl upward on the raceme, probing the flowers for nectar. The viscidium in these species is comparatively long and rigid, adhering to the dorsal surface of the bee's galea. However, in *S. lucida*, pollinated by halictid bees, the bees visit many flowers that they reach by flight. In this species, nectar accumulates on the ventral surface of the column and the oval viscidium is attached to the clypeus (see Catling, 1983; Salazar, 2003b). Likewise, *S. spiralis*, distributed in Ireland, southern Britain, central Europe and the Mediterranean, is pollinated by *Bombus* and *Apis* (e.g. Darwin, 1877; reviewed by Jacquemyn & Hutchings, 2010).

The last main group in the *Spiranthes* clade (Fig. 3, clade 4) includes an assortment of genera centred in Mexico/northern Central America and the Caribbean. *Physogyne* (Fig. 7L) and *Pseudogoodyera* (Fig. 8A) were only recently included in a molecular phylogenetic analysis (Salazar *et al.*, 2016). *Physogyne* includes two or three species restricted to steep slopes and rocky outcrops in tropical deciduous forest and its ecotones with warm pine–oak forest on the Pacific slope of Mexico. *Pseudogoodyera* consists of two species, one of them, *P. pseudogoodyeroides* (L.O.Williams) R.González & Szlach., widespread on the Atlantic slope of Mexico south to Belize and the other, *P. wrightii* (Rchb.f.) Schltr., endemic to Cuba. Both species live in small soil pockets on karstic outcrops in areas of moist, semi-evergreen tropical forests. *Pseudogoodyera pseudogoodyeroides* and *Physogyne gonzalezii* (L.O.Williams) Garay were both placed in *Pseudogoodyera* by Burns-Balogh (1986b), but she oddly included *Physogyne sparsiflora* (C.Schweinf.) Garay in *Schiedeella*. Both these genera are only rarely collected and little is known of any aspect of their biology; further study is required to determine whether they should be merged in a single genus.

Our results show that, as currently delimited, *Mesadenus* is polyphyletic: 'core' *Mesadenus*, i.e. the clade that includes *M. polyanthus* (Rchb.f.) Schltr. (the

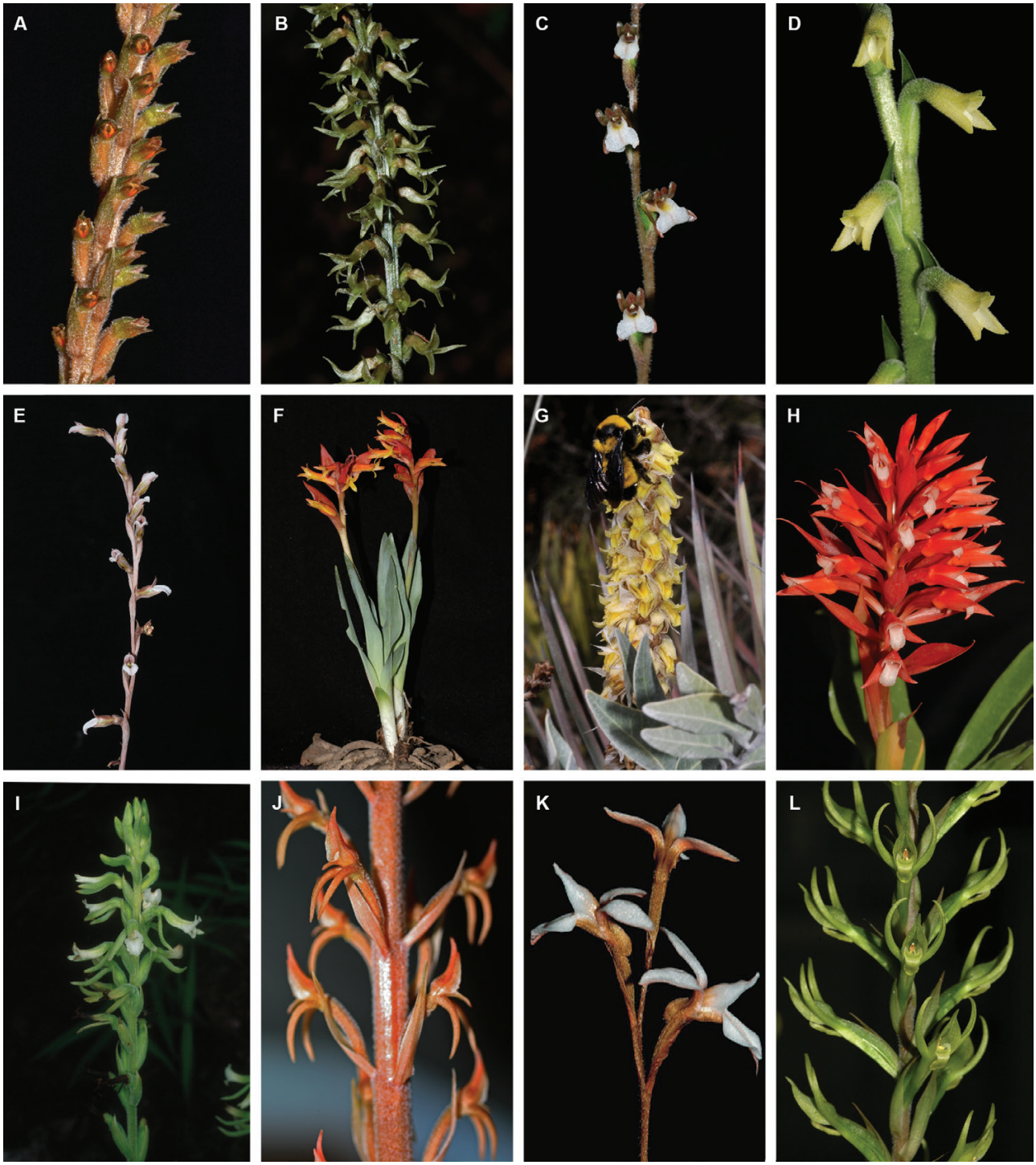
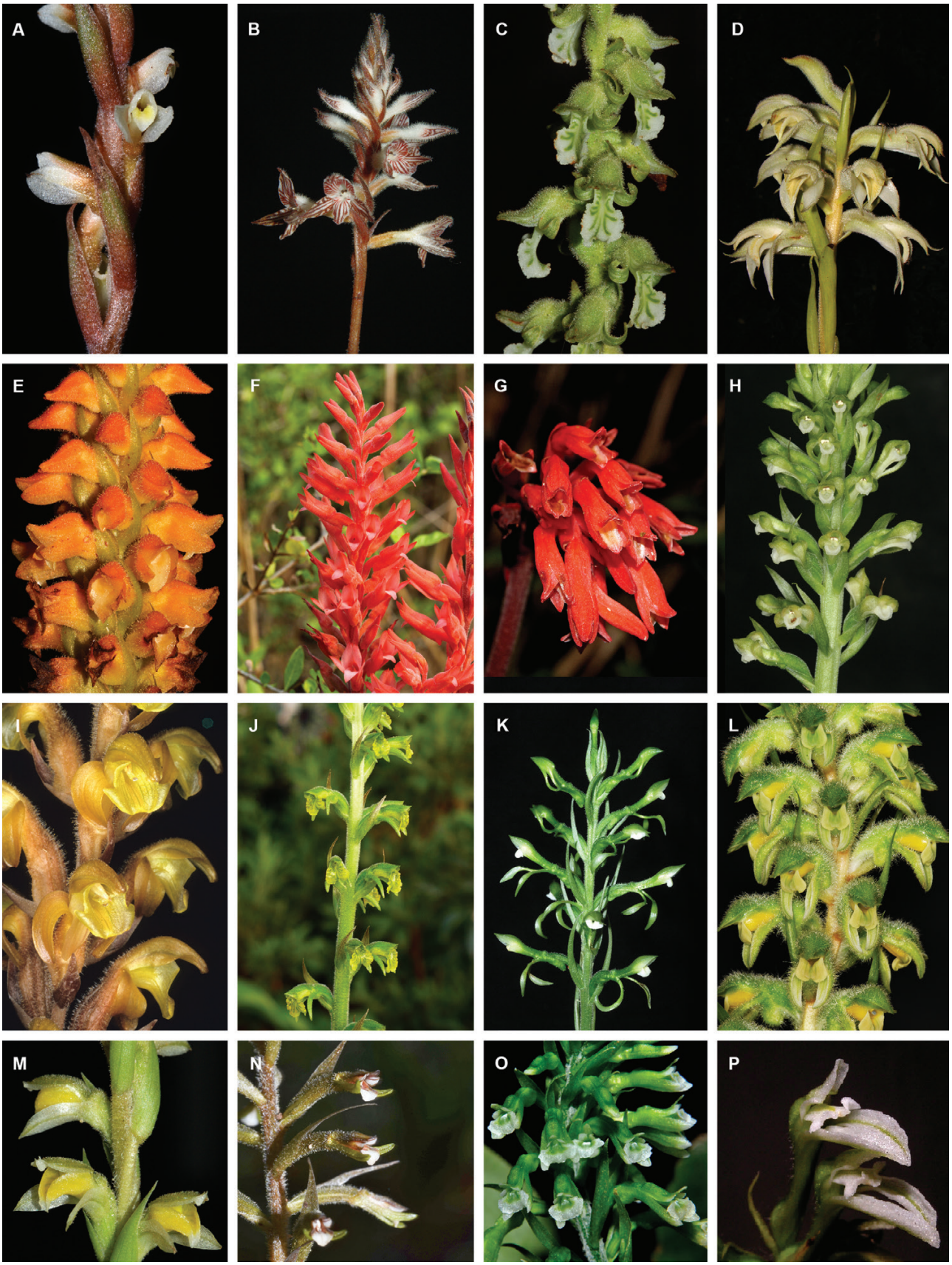


Figure 8. Inflorescences of selected members of Spiranthinae (continuation). A. *Pseudogoodyera pseudogoodyeroides* (Mexico, *Francke s.n.*). B. *Mesadenus polyanthus* (Mexico, *Salazar 7370*). C. *Greenwoodiella micrantha* var. *garayana* (Mexico, *Salazar et al. 7420*). D. *Kionophyton sawyeri* (Mexico, *Salazar 7252*). E. *Schiedeella transversalis* (Mexico, *Salazar 6873*). F. *Dichromanthus yucundaa* (Mexico, *García-Mendoza & Franco 8744*). G. *Deiregyne densiflora* (Mexico, *Reyes s.n.*). H. *Stenorrhynchos glicensteinii* (El Salvador, *Salazar & Linares 7532*). I. *Thelyschista ghillanyi* (Brazil, *van den Berg 1435*). J. *Buchtienia ecuadorensis* (Peru, *Simpson s.n.*). K. *Nothostele acianthiformis* (Brazil, *Viana 767*). L. *Eltroplectris triloba* (Brazil, *Batista 3293*). Photographers: Gerardo A. Salazar (A–F, H), Jerónimo Reyes (G), Cássio van den Berg (I), Phillip Simpson (J), João A. N. Batista (K, L).

type species; Fig. 8B), is weakly supported as sister to *Greenwoodiella* in a strongly supported subgroup of the *Spiranthes* clade (Fig. 3, clade 4). However, Brazilian *M. glaziovii* does not group with Mexican, Caribbean and Central American representatives of the genus, being weakly (BP 68 in our combined ML analysis; Fig. 4) to strongly supported (BP 100 in our combined MP analysis; Supporting Information, Fig. S5) as belonging in the *Stenorrhynchos* clade. *Mesadenus* is distinctive in its tiny flowers with all perianth segments similar in shape and colour (the labellum at most slightly wider than the other perianth parts) and the viscidium positioned on the centre of the ventral surface of the pollinarium (e.g. Salazar, 2003b: fig. 191.1). However, small flowers and a central viscidium have evolved in distantly related groups of Spiranthinae, such as *Funkiella minutiflora* (see earlier; Fig. 2L). The central position of the viscidium could be correlated with the shortening of the rostellum to match the reduction of the flower as a whole, but this hypothesis will be explored further elsewhere (G. A. Salazar & J. A. N. Batista, unpubl. data). On the other hand, most *Greenwoodiella* spp. were placed formerly in *Schiedeella*, but our results, and the previous study of Salazar *et al.* (2016), confirm that *Schiedeella* as interpreted by most previous taxonomists is polyphyletic. *Mesadenus* and *Greenwoodiella* differ in vegetative and floral attributes that have been discussed elsewhere (Salazar *et al.*, 2016).

Kionophyton, the clade that includes the type species of *Schiedeella*, *S. transversalis* (A.Rich. & Galeotti) Schltr. (see discussion in Salazar *et al.*, 2016), *Dichromanthus* and *Deiregyne* are all strongly supported genera. Burns-Balogh (1986a) placed *Kionophyton sawyeri* (Standl. & L.O.Williams) Garay (Fig. 8D) in monotypic *Greenwoodia* Burns-Bal. but *K. seminuda* (Schltr.) Garay in *Stenorrhynchos* section *Mesadenella* (Pabst & Garay) Burns-Bal.; our results confirm that these two morphologically similar species belong together. Our sample of *Schiedeella s.s.* includes *S. transversalis* (Fig. 8E), *S. crenulata* (L.O.Williams) Espejo & López-Ferrari, *S. affinis* (C.Schweinf.) Salazar and *S. durangensis* (Ames & C.Schweinf.) Garay. *Schiedeella affinis* has been treated as a member of *Mesadenus* (Garay, 1982) and *Brachystele* (e.g. Burns-Balogh, 1986b; Szlachetko *et al.*, 2005) based on its minute flowers, which are among the smallest in Spiranthinae, but similarity is probably due to simplification resulting from extreme size reduction. Vegetatively and eco-geographically *S. affinis* fits well with other *Schiedeella*.

Dichromanthus (Fig. 8F) *sensu* Salazar *et al.* (2002, 2011a) and Salazar & García-Mendoza (2009) and *Deiregyne sensu* Garay (1982) form a strongly supported sister-pair with similar habitat preferences, vegetative morphology and overall floral structure. The main differences are flower colour, relative length of the floral tube and the structure of the rostellum and viscidium, all these related to differences in pollination syndrome (see Salazar *et al.*, 2011a). In *Dichromanthus cinnabarinus* (Lex.) Garay, the rostellar remnant is soft, pliable and notched/hollow as a result of the apical portion of the rostellum being removed together with the viscidium, whereas in *D. aurantiacus* (Lex.) Salazar & Soto Arenas, *D. michuacanus* (Lex.) Salazar & Soto Arenas and *D. yucundaa* Salazar & García-Mend. (Fig. 8F) the viscidium is sheath-like and its removal leaves a relatively hard (but flexible), pointed rostellar remnant. However, in all four species the flowers are tubular, and the labellum has a narrow, conduplicate basal channel with submarginal nectar glands (see Salazar *et al.*, 2011a: figs 4, 5). *Dichromanthus* spp. display an ornithophilous pollination syndrome, except for *D. michuacanus*, which is pollinated by bumblebees (Salazar *et al.*, 2011a; Figueroa *et al.*, 2012). *Deiregyne* spp. display a mellitophilous pollination syndrome, and there are some occasional field observations of bumblebees pollinating their flowers, as in *D. densiflora* (C.Schweinf.) Salazar & Soto Arenas (Fig. 8G). *Deiregyne* has had an unnecessarily complex taxonomic story caused by undue concern over interpretation of imprecise generic definitions, such as the original formulation of *Deiregyne* by Schlechter (1920), which encompassed a heterogeneous assortment of species currently placed in *Deiregyne* (*sensu* Garay, 1982), *Schiedeella* (*sensu* Salazar *et al.*, 2016) and *Aulosepalum* (*sensu* Garay, 1982; Salazar, 2003b). As delimited by Garay (1982), who was the first to lectotypify the genus, *Deiregyne* only required the transfer of a few species described since, usually as members of *Oestlundorchis* Szlach. (a synonym of *Deiregyne*; e.g. Soto *et al.*, 2007) and a species segregated by Garay (1982) in monotypic *Dithyridanthus* Garay to achieve monophyly (Salazar & Ballesteros-Barrera, 2010). *Deiregyne* spp. are relatively homogeneous in habit and floral structure, and they share diaphanous floral bracts with dark veins that permit recognition of the genus at a glance even from herbarium specimens (Fig. 8G; see Hágsater *et al.*, 2005: figs 445–450). Balogh (1981, 1982) and Burns-Balogh (1986b) placed most *Deiregyne* spp. (as interpreted here) in *Schiedeella*, whereas her concept of *Deiregyne* (Burns-Balogh, 1986b, 1988) is equivalent to Garay's (1982) circumscription of *Aulosepalum* (see above). Recently, Szlachetko & Kolanowska (2013) contributed further to this nomenclatural mayhem, proposing conservation of *Deiregyne sensu* Burns-Balogh based on recycling of Szlachetko's (1995b) old argument on interpretation of the imprecise original diagnosis of the genus by Schlechter (1920).



Stenorrhynchos *clade*

This clade was recovered consistently by our analyses with varying degrees of support (Fig. 4; Supporting Information, Figs S1–S6) and most previous molecular phylogenetic studies of Spiranthinae (e.g. Salazar *et al.*, 2003, 2011a, 2016; Batista *et al.*, 2011; Borba *et al.*, 2014). Like the *Spiranthes* clade (see earlier), the *Stenorrhynchos* clade displays several pollination syndromes, including halictid bee pollination (*Mesadenella*: Singer, 2002), hummingbird pollination (*Stenorrhynchos*: Siegel, 2011; *Sacoila*: Singer & Sazima, 2000) and butterfly pollination (*Pteroglossa* spp.: Pansarin & Ferreira, 2015). The taxonomy of this natural group has been confounded by floral homoplasy, as taxonomists focused only on floral characters are easily misled by convergent features due to similar pollination syndromes such as adaptation to hummingbird pollination (van der Pijl & Dodson, 1966; Salazar *et al.*, 2003, 2011a; Batista *et al.*, 2011). Many species in this major clade have a narrowly pointed, stiff rostellum remnant, with some noticeable exceptions, such as *Buchtienia* Schltr. and *Thelyschista* (see below).

As noted earlier, '*Mesadenus*' *glaziovii* is sister to the rest of the *Stenorrhynchos* clade, which includes three main clades (Fig. 4). *Stenorrhynchos s.s.* (clade 5; see Salazar *et al.*, 2011a) is strongly supported as monophyletic and includes about seven species that florally are nearly indistinguishable from one another but exhibit distinctive vegetative attributes, ecological preferences and distributions (Salazar, 2003b; Christenson, 2005; Salazar *et al.*, 2011a). Next, two morphological 'oddballs', *Thelyschista* and *Buchtienia*, form a grade with a group that includes the remainder of this major clade. Monospecific *Thelyschista* has green sepals and white labellum and petals, the latter forming a narrow, somewhat recurved floral tube with the dorsal sepal and the labellum (Fig. 8I); its column is distinctive in the tridentate rostellum with the massive viscidium wedged between the three teeth (Salazar, 2003b: fig. 207.1). On the other hand, *Buchtienia* (Fig. 8J) includes four species with greenish to pinkish or brownish flowers, vegetatively and florally reminiscent of *Eltroplectris*, except for lack of a spur and their peculiar column structure. The column in *Buchtienia* is abruptly expanded laterally from a narrow base and somewhat sigmoid when seen from one side, with a shortly oblong, pliable rostellum that, after removal of the pollinarium, ends in a membranaceous, emarginate lamina (Salazar, 2003b; de Fraga, Meneguzzo & Saddi, 2015). This contrasts with the straight, clavate column ending in a bristle-like, hard rostellum/rostellum remnant that is common in the *Stenorrhynchos* clade.

The other taxa of the *Stenorrhynchos* clade form a weakly supported group consisting of two subclades. The first of these (Fig. 4, clade 6) is strongly supported (BP 99) and includes *Nothostele acianthiformis* (Fig. 8K) sister to *Eltroplectris* (Fig. 8L), in agreement with the analysis of Batista *et al.* (2011), who discussed in detail similarities and differences between these two genera and chose to maintain them separate. The second subclade is weakly supported (BP 67; Fig. 4, clade 7) and in turn encompasses two weakly supported groups. One of these (BP 63) includes the species of *Mesadenella* (Fig. 9A) and some *Pteroglossa* (Fig. 9B). Of these, Szlachetko (in Rutkowski *et al.*, 2008) segregated *P. roseoalba* (Rchb.f.) Salazar & M.W.Chase to *Callistanthos* Szlach. and both *P. euphlebia* (Oliv. ex Rchb. f.) Garay and *P. glazioviana* (Cogn.) Garay to *Cogniauxiocharis* (Schltr.) Szlach. The other one (BP 65) consists of two major groups: the first with *Lyroglossa grisebachii* (Cogn.) Schltr. (Fig. 9D) sister to a species pair of *Pteroglossa macrantha* (Rchb.f.) Schltr. (type species of *Pteroglossa*; Fig. 9C) and *Sacoila hassleri* (Cogn.) Garay (BP 95), and a second including *Skeptrostachys* (Fig. 9E) plus *Sacoila lanceolata* (Fig. 9F; BP 67). Thus, *Mesadenella*, *Sacoila* and *Pteroglossa* are non-monophyletic. Szlachetko (in Rutkowski *et al.*, 2008) created an additional monospecific genus, *Lyrochilus*, for *Pteroglossa hilariana* (not sampled by us), which according to him is similar in habit to *Lyroglossa* and in floral structure to *Pteroglossa*. Salazar (2003b) proposed that distinguishing *Eltroplectris* from *Pteroglossa*, each including about ten species, has been problematic because of use of

Figure 9. Inflorescences of selected members of Spiranthinae (continuation). A. *Mesadenella petenensis* (Mexico, *Jiménez-Machorro 3002*). B. *Pteroglossa euphlebia* (Brazil, *Guimarães 191*). C. *Lyroglossa grisebachii* (Brazil, *Batista 1821*). D. *Pteroglossa macrantha* (Argentina, *Singer s.n.*). E. *Skeptrostachys gigantea* (Brazil, *Batista Bianchetti 3352*). F. *Sacoila lanceolata* (Mexico, *Amith 1922*). G. *Coccineorchis cernua* (Peru, *Edquen s.n.*). H. *Sauroglossum elatum* (Brazil, *Smidt 1007*). I. *Sarcoglottis cerina* (El Salvador, *Batlle s.n.*). J. *Odontorrhynchus chlorops* (Argentina, *Rodríguez s.n.*). K. *Pelexia funckiana* (Mexico, *Figueroa 6*). L. *Pelexia hirta* (Ecuador, *Tobar 15*). M. *Brachystele cyclochila* (Brazil, *Batista et al. 2225*). N. *Cyclopogon epiphyticum* (Ecuador, *Salazar et al. 9764*). O. *Cyclopogon ovalifolius* (Peru, *Morón s.n.*). P. *Veyretia rupicola* (Brazil, *van den Berg 1477*). Photographers: Gerardo A. Salazar (A, I, K, N), Leonardo R. S. Guimarães (B), João A. N. Batista (C, E, M), Rodrigo B. Singer (D), Jonathan Amith (F), José D. Edquen (G), Eric C. Smidt (H), Juan J. Rodríguez (J), Francisco Tobar (L), Érica Morón (O), Cássio van den Berg (P).

inconsistent characters, such as the degree of adnation of the spur to the ovary (e.g. Szlachetko, 1995c). This is corroborated by the recent discovery of a new Peruvian species closely related to *P. macrantha* that completely lacks a spur (Damián & Salazar, 2017). Szlachetko & González (1996b) transferred *E. triloba* (Lindl.) Pabst and several other species to their new genus, *Ochyrella* Szlach. & R.González, but our study indicates that *E. triloba* is closely related to *E. calcarata* (Sw.) Garay & H.R.Sweet, the type species of *Eltroplectris*, and that neither the generic limits of Szlachetko and co-workers nor those accepted by Salazar (2003b) for *Eltroplectris* and *Pteroglossa* represent natural groups. Salazar (2003b) argued that the short, ventrally channelled column, narrowly triangular rostellum, concave anther and marginal, completely adnate nectar glands of *Lyroglossa* are reminiscent of those of *Pteroglossa*, and such morphological similarity is consistent with their close relationship to one another revealed by this study. Salazar (2003b) also stressed the morphological similarity of *Sacoila* to *Skeptrostachys*, which agrees with their close relationship revealed by our analysis. Nevertheless, considerable work remains to be done on a critical reassessment of generic limits in this major clade, which will probably result in a reduction of the number of genera.

Pelexia *clade*

Our combined analyses weakly support the association of *Coccineorchis* with the rest of the *Pelexia* clade (ML BP 61, Fig. 5; MP BP 69; Supporting Information, Fig. S5). *Coccineorchis* is not closely related to *Stenorrhynchos* and its close kin, despite similar overall flower shape and the presence in both genera of a hard, bristle-like rostellum sheathed by the viscidium. These features, together with the somewhat nodding, dense raceme of bright yellow to red tubular flowers are suggestive of hummingbird pollination in *Coccineorchis*, which has not been corroborated in the field (Fig. 9G; Salazar *et al.*, 2011a).

Sauroglossum elatum (Fig. 9H) diverges next (BP 68), and its position agrees with previous molecular phylogenetic analyses that have included this species and a few other representatives of the *Pelexia* clade (Borba *et al.*, 2014; Salazar *et al.*, 2016). *Sauroglossum* is polyphyletic, as Andean *S. corymbosum* does not group with south-eastern Brazilian/Argentinian *S. elatum*, the type species of that genus. Flowers of *S. elatum* have a short rostellum with a ventrally adhesive viscidium that, upon removal of the pollinarium, leaves a notch at the apex of the column (Singer, 2002; Salazar, 2003b). Singer (2002) studied the reproductive biology of *S. elatum*, demonstrating protandry and pollination by noctuid moths.

The remainder of the *Pelexia* clade forms three major groups, the first of which is *Sarcoglottis* (Fig. 2M–T; Fig. 5, clade 8). This mostly South American genus includes a derived, strongly supported Mesoamerican subclade (*S. corymbosa* to *S. cerina*; BP 92; Fig. 9I). *Sarcoglottis sceptrodes* Schltr. also occurs in Mesoamerica, but it is closer to Caribbean/northern South American *S. acaulis* (Sm.) Schltr. and Andean *S. speciosa* C.Presl (the latter the type species of *Sarcoglottis*). The recent segregates *Zhukowskia* (Schltr.) Szlach., R.González & Rutk. and *Potosia* (Schltr.) R.González & Szlach., typified by *Sarcoglottis smithii* (Rchb.f.) Schltr. and *S. schaffneri* (Rchb.f.) Ames, respectively, are nested in the Mesoamerican subclade and are thus phylogenetically untenable and taxonomically superfluous.

Traditionally, *Sarcoglottis* was distinguished from *Pelexia* by characters of the nectary, which in the former supposedly is completely fused with the ovary, with neither a prominent spur nor a clearly visible line of adnation (e.g. Garay, 1982), whereas in the latter the nectary is prominent and chin-like, saccate or spurred. However, there is substantial variation in this feature in *Sarcoglottis* (Fig. 2M–P), and reliance on this single character has led some taxonomists to create new genera, such as *Zhukowskia*, to accommodate the 'intermediate' species (see Szlachetko *et al.*, 2000). *Potosia*, on the other hand, was first created as a section of *Pelexia* by Schlechter (1920) and recently raised to generic level, without any meaningful discussion supporting such a decision, in a minimal paper published in a journal of invertebrate zoology (Mytnik, 2003). Subsequently, Mytnik-Ejsmont & Rutkowski (2006) attempted [sic] 'to verify a legitimacy of distinguishing particular genera within the subtribe Cyclopogoninae', including among others *Pelexia*, *Sarcoglottis*, *Potosia* and *Zhukowskia*. For this, they conducted phenetic analyses of (mostly floral) morphological characters, but because they used the genera as terminals, their analyses provided no evidence on generic limits and composition; at most, their phenograms depict overall morphological similarities among genera that were arbitrarily delimited beforehand.

Sarcoglottis is the strongly supported sister of a clade that includes non-monophyletic *Pelexia* that has nested in it several species assigned to other genera (Fig. 5, clade 9). A first group (clade 10) includes Andean *Pelexia weberbaueriana* (Kraenzl.) Schltr., *Sauroglossum corymbosum* and *Odontorrhynchus chlorops* (Rchb.f.) Garay (Fig. 9J); the oldest available generic name for such a group is *Synassa* Lindl., typified by *Synassa corymbosa* Lindl. (=*Sauroglossum corymbosum*). Rutkowski *et al.* (2008) revived *Synassa* but only to include the type species and the morphologically similar *Sauroglossum aurantiacum*

(C.Schweinf.) Garay, but our results show that this group is more diverse than previously thought. It is noteworthy that Schweinfurth (1951) originally described *Sauroglossum aurantiacum* as a variety of *Pelexia weberbaueriana*, which is consistent with the close genetic relationship found here between the latter and *S. corymbosum*.

Excluding the aforementioned *P. weberbaueriana*, the remaining *Pelexia* spp. are found in a strongly supported clade in which most *Brachystele* spp. and *Odontorrhynchus variabilis* are nested (Fig. 5, clades 11–13). Two clades of *Pelexia* were consistently recovered, largely corresponding to Schlechter's (1920) sections ['*Eu*-']*Pelexia* (Fig. 9K) and *Pachygenium* (Fig. 9L). The latter is distinguished by its oblanceolate-spathulate leaves that attenuate basally and comparatively fleshy flowers with a usually saccate spur, versus the distinctly petiolate, obliquely ovate leaves and somewhat membranaceous flowers with cylindrical, retrorse spur of the former. Szlachetko *et al.* (2001) raised section *Pachygenium* to generic rank. Our results are consistent with recognition of those two clades as distinct genera, although some features that Szlachetko *et al.* (2001) cited to differentiate *Pachygenium* from *Pelexia s.s.* (e.g. the attributes of the rostellum, viscidium and stigma) do not hold true as distinguishing characters. However, these clades diverge significantly in ecological preferences and overall floral morphology. Species of section *Pachygenium* usually inhabit open grasslands, rocky fields and forest savannas; their flowers (Fig. 9L) have a broadly channelled labellum and diurnal perfume and are pollinated by bumblebees, at least *P. eckmanii* (Kraenzl.) Schltr. (Dressler 1981, 1993) and *P. oestrifera* (Rchb.f. & Warm.) Schltr. (Singer & Sazima, 1999). In contrast, species of section *Pelexia* (or *Pelexia s.s.*) inhabit forests and its narrow flowers (Fig. 9K) are apparently odourless, but no information is available on their natural pollination.

Like *Pelexia*, *Brachystele* (Fig. 9M) is polyphyletic. *Brachystele guayanensis* is deeply embedded among *Cyclopogon* spp. as sister to *Veyretia* in a different main subclade of the *Pelexia* clade (Fig. 6, clade 17). Conversely, Chilean *Odontorrhynchus variabilis* is nested in 'core' *Brachystele* (Fig. 5, clade 12) and morphologically is barely distinguishable from *B. unilateralis* (Poir.) Schltr., the type species of *Brachystele*. Rutkowski *et al.* (2008) placed *Brachystele* and *Sauroglossum* in their polyphyletic version of Spiranthinae and *Odontorrhynchus* in polyphyletic 'Stenorrhynchidinae.' Core *Brachystele* shows geographical structure: western South American species *B. unilateralis* and *O. variabilis* form a strongly supported group and south-eastern South American *B. subfiliformis* (Cogn.) Schltr. to *B. cyclochila* (Kraenzl.) Schltr. form another clade (although with BP < 50 in our combined ML analysis). Our analyses are not decisive about whether *Brachystele* is closer to *Pelexia s.s.* or to *Pachygenium*, although *Brachystele* is similar in habitat preference and overall distribution to *Pachygenium*, both centred in open habitats in south-eastern South America (Supporting Information, Fig. S8). Moreover, pollination by native and introduced bumblebees has been reported for *Brachystele unilateralis* (Sanguinetti & Singer, 2014). All this suggests a close relationship between *Pachygenium* and *Brachystele*.

Cyclopogon (Fig. 6) is perhaps the taxonomically most challenging genus of Spiranthinae. Taxonomists have recognized several genera, most of them segregated from *Cyclopogon s.l.* (except *Veyretia*; see below), based on single floral attributes, such as whether the lateral sepals are partially connate to form 'a distinct sepaline tube', on the basis of which Garay (1978, 1982) treated *Cyclopogon* as monospecific and moved all other species to *Beadlea* Small. Similarly, Garay (1982) created *Stigmatosema* Garay to include two former *Cyclopogon* spp. in which the apex of the rostellum remnant is 'sulcate' (i.e. it has the lateral margins upturned), and about a dozen additional species have been subsequently transferred to, or described as, *Stigmatosema*. Szlachetko (1994b) segregated *Cocleorchis* Szlach., with deflexed ('revolute') rostellum margins, and *Warscaea* Szlach., with a broad and short rostellum that upon removal of the viscidium is deeply notched. However, *Cyclopogon* (*Cocleorchis*) *dressleri* Szlach. (of which *C. sarcoglottidis* Szlach., the type species of *Cocleorchis*, is considered here as a synonym) is strongly supported by our analyses as the sister of *Cyclopogon ovalifolius* C.Presl, the type species of *Cyclopogon*, demonstrating the meaninglessness of segregating genera based on minor floral attributes in this florally labile clade.

The sister group of the rest in *Cyclopogon s.l.* is a strongly supported, small subclade including *C. variegatus* Barb.Rodr. to *C. olivaceus* (Rolfe) Schltr. (Fig. 6, clade 14), which is distinctive in its dark brownish- to purplish-green leaves, often dotted with white or pink, and a relatively simple labellum (i.e. not abruptly expanded into an apical lobe or epichile; Fig. 9N). Should *Cyclopogon* be divided into sections, the name available for this clade is section *Beadlea* (Small) Burns-Bal. Next, there is a large group that includes many species with homogeneous floral morphology (Fig. 6, clade 15), in which *C.* ('*Warscaea*') *apricus* (Lindl.) Schltr. is sister to the rest. The third main clade of *Cyclopogon* (Fig. 6, clades 16 and 17) includes an assortment of species that have been attributed to several genera, namely *Cyclopogon* [*C. micranthus* (Barb.Rodr.) Schltr., *C. ovalifolius*, *C. elatus* (Sw.) Schltr., *C. luteo-albus* (A.Rich. & Galeotti) Schltr., *C. obliquus* (J.J.Sm.) Szlach., *C. saccatus* (A.Rich. & Galeotti)

Schltr. and *C. truncatus* (Lindl.) Schltr.], *Stigmatosema* [*C. inaequilaterus* (Poepp. & Endl.) Schltr., *Cocleorchis* (*C. dressleri*), '*Brachystele*' *guayanensis* and *Veyretia*.

Veyretia spp. were formerly included in *Sarcoglottis* section *Aphylla* Burns-Bal. (Burns-Balogh, 1983). Szlachetko (1995a) segregated *Veyretia* from *Sarcoglottis* mainly based on the presumed absence of leaves at flowering time (although leaves can be present or absent at flowering time; instead of flat and broad as in *Sarcoglottis*, they are grass-like and convolute; Hoehne, 1945; Salazar, 2003b) and the bifurcate nectar chamber. The strongly supported embedded position of *Veyretia* in *Cyclopogon s.l.* in our trees is unexpected, but the vegetative and floral peculiarities of the species of *Veyretia* probably represent derived modifications of the otherwise conservative, symplesiomorphic vegetative and floral morphology of the *Cyclopogon* clade. The association of '*Brachystele*' *guayanensis* with *Veyretia* is also surprising at first glance, given the obvious difference in the appearance of the minute flowers of the former. However, '*B.*' *guayanensis* shares with *Veyretia* a preference for open grassland habitats and, upon close examination, it is evident that its flowers have the two-chambered nectary of *Veyretia* (although more shallowly so in *B. guayanensis*, in proportion to its noticeable reduction in flower size). As in the *Stenorrhynchos* clade, much work remains to be done to sort out generic limits in this group. Such work ideally should include detailed morphological and developmental comparative studies, coupled with observations on natural pollination, to achieve a better understanding of structural homology and functionally driven homoplasy.

Floral morphological characters as phylogenetic and taxonomic markers

As indicated above, Burns-Balogh & Robinson (1983) carried out a cladistic analysis of floral morphological characters for the '*Pelexia* alliance', including *Cyclopogon*, *Pelexia* and *Sarcoglottis* (*Veyretia* spp. were then included in *Sarcoglottis* section *Aphylla*). Some characters used by Burns-Balogh & Robinson (1983) exhibit continuous variation (e.g. flowers erect or horizontal, pollinarium oblong vs. wishbone-shaped, position of the sepals relative to the labellum), and their coding in discrete states is questionable. Hence, discussion here is restricted to discrete characters. Burns-Balogh & Robinson (1983) identified several putative synapomorphies for the *Pelexia* alliance, including apiculate anther, oblong, truncate or shallowly notched rostellum remnant (Fig. 2O, R, T) and apical viscidium held between the apices of the pollinia and located on the dorsal side of the rostellum (Fig. 2Q, S), thus corresponding to the 'wedge-type' viscidium of Greenwood (1982). However, an apiculate anther is not exclusive to *Cyclopogon*, *Pelexia* and *Sarcoglottis* but is also present in *Brachystele*, *Odontorrhynchus* and *Sauroglossum*, and hence is a putative synapomorphy of the *Pelexia* clade except *Coccineorchis* (Salazar, 2003b). On the other hand, the truncate rostellum remnant and a wedge-type viscidium are structurally and functionally linked because the viscidium corresponds to the distal portion of the rostellum and when it is detached it leaves a straight or somewhat concave zone of rupture that produces the 'truncate or shallowly notched rostellum remnant' (Fig. 2T). Both attributes are absent in the species of *Sauroglossum*, *Odontorrhynchus* and *Brachystele* but present in *Sarcoglottis*, *Pelexia s.s.*, *Pachygenium*, *Cyclopogon s.l.* and *Veyretia*. In all these genera the pollination mechanism involves release of the viscid matter by the viscidium when its dorsal surface is pressed by the underside of the labrum of their pollinators (several types of bees) when they extend their mouthparts to probe the flower for nectar (Singer & Coccuci, 1999; Singer & Sazima, 1999; field observations not available for *Veyretia*). Another unique trait of the wedge-type viscidium is that it is located between the divergent apices of the pollinia, which is linked to the aforementioned pollination mechanism, since the labrum could not contact the dorsal surface of the viscidium if the apices of the pollinia were parallel and connivent over the dorsal surface of the viscidium, as in other Spiranthinae (Fig. 2Q, S; cf. Greenwood, 1982). The fact that those three features of the rostellum and viscidium are always present together strongly suggests that they are linked functionally, and their use as independent characters in a cladistic analysis is unadvisable because they 'overweigh' as three characters what is actually one. The same reasoning is applicable to the suite of co-occurring characters that characterize the hummingbird pollination syndrome evolved convergently in the *Pelexia*, *Stenorrhynchos* and *Spiranthes* clades (Salazar *et al.*, 2011a).

Burns-Balogh & Robinson (1983) identified two synapomorphies supporting a clade formed by *Pelexia* and *Sarcoglottis* (the latter including *Veyretia* as section *Aphylla*), i.e. subulate (slender, long and pointed) basal nectar glands in the labellum (Fig. 2N, P) and a non-basal position of the entrance of the stylar channel in the stigma. The relationships recovered by our analyses suggest that possession of subulate nectar glands is a putative synapomorphy of the clade that includes *Sarcoglottis* plus *Pelexia s.l.* and most species of *Brachystele* and *Odontorrhynchus*, with subsequent reversals (secondary losses) in clades 10 and 12 (Fig. 5). The second putative synapomorphy of *Pelexia* and *Sarcoglottis* according to Burns-Balogh & Robinson (1983), a non-basal position of the entrance of the stylar channel, is based on an incorrect interpretation of the homology of the structures

concerned. According to Burns-Balogh & Robinson (1983), in *Pelexia* and *Sarcoglottis* the entrance to the stylar channel is located 'above the stigmatic area [. . .] at the base of the sterile rostellum', whereas in *Cyclopogon*, *Spiranthes* and other Spiranthinae it is located 'in an area between the two [fertile, or receptive] stigmatic lobes, sometimes very near the base of the lobes'. Obviously, they considered that the often bilobed or bipartite receptive stigmatic surface of Spiranthinae represents the lateral lobes of the stigma, with the 'sterile rostellum' representing the median lobe. However, micromorphological and developmental studies conducted by Rasmussen (1982), Kurzweil (1988), Figueroa *et al.* (2012) and Figueroa (2014) clearly showed that, in Spiranthinae and other Cranichideae, the median carpel develops before the lateral carpels, enlarges considerably and gives rise to both the receptive stigmatic area(s) and a non-receptive portion that bears the viscidium. The last, non-receptive portion conforms to the original definition of rostellum by Richard (1817) and upheld by, among others, Vermeulen (1959), Dressler (1993), Kurzweil (1988, 1998) and Salazar *et al.* (2011a), which is the one followed here (see Rasmussen, 1982, for a different interpretation). Those developmental studies also showed that the lateral carpel apices (corresponding to the lateral stigma lobes of most plants, but not Cranichideae) arise fused as a transverse ridge adjacent to the base of the receptive area of the median stigma lobe and appear to contribute little to the receptive surface itself. Therefore, the supposed lateral stigma lobes of Burns-Balogh & Robinson (1983) are part of the receptive portion of the median lobe. Moreover, in all flowers of *Cyclopogon*, *Pelexia*, *Sarcoglottis* and *Veyretia* that we have examined the entrance to the stylar channel is located near the base of the stigmatic area. Therefore, the entrance of the stylar channel in the *Pelexia* clade is always located at the confluence of the three carpel apices and interpretation of the alternative condition as a synapomorphy for a *Sarcoglottis–Pelexia* clade to the exclusion of other genera is unsustained.

The cladistic analysis of '*Deiregyne*' *sensu* Burns-Balogh (1988; =*Aulosepalum* Garay as interpreted here), based on 25 floral attributes and one vegetative attribute, will be discussed elsewhere against the framework of a detailed study of the phylogenetic relationships of *Aulosepalum* based on a multilocus molecular analysis and comparative morphological observations (G. A. Salazar, R. Chalqueño & S. A. Adachi, unpubl. data). On the other hand, the six vegetative and 43 floral features used in the phenetic and cladistic analyses of genera for which monophyly was not assessed (because the genera were used as terminals) by Rutkowski *et al.* (2008), briefly described in their appendix 1, deserve more careful discussion than the focus of the present work permits. However, a perusal of their definition of the characters and their states reveals many potential problems, including, among others: (1) character redundancy in, for instance, characters 5 (leaf petiole narrow = no/yes) and 6 (leaf petiole gradually transforming into the blade = no/yes), which are clearly a single attribute that was scored twice; likewise, characters describing the apex of the rostellum, e.g. 39 (rostellum bilobed = no/yes), 44 (rostellum furculate = no/yes), 45 (rostellum subulate = no/yes), 46 (rostellum tridentate = no/yes), represent an 'inflation' of the weight assigned to one and the same structure; (2) gradual attributes arbitrarily made discrete, e.g. character 13 (most of the spur united to the ovary, but with a free top = no/yes) and 14 (spur united with ovary basally only, mostly free = no/yes); and (3) autapomorphic attributes, which are uninformative about relationships among the genera and irrelevant in this context for generic delimitation, because the genera were delimited a priori, e.g. characters 2, 4, 9, 10, 18, 34 and 36. Overall, there is a lack of discussion backed by detailed comparative and developmental evidence from studies conducted by those authors or referred to the literature, a requisite for any solid interpretation of structural homology and evolution. These issues will have to be addressed in monographs of the major clades and monophyletic genera, as applicable. There is little point in trying to make sense of the complex evolution of floral morphology in reference to artificial major groups ('subtribes') and arbitrarily delimited genera such as those recognized by Rutkowski *et al.* (2008).

Biogeographical considerations

Our ancestral area analysis indicates a Neotropical origin for Spiranthinae, with eastern South America attaining the highest probability as the area of origin of the subtribe. Migrations associated with secondary diversification in Mesoamerica and subsequently North America/Eurasia are indicated for the *Spiranthes* clade, and again in Mesoamerica for several subclades of the *Pelexia* and *Eurystyles* clades (Fig. 10; Supporting Information, Fig. S8). Previous hypothetical scenarios about historical biogeography of Spiranthinae such as those in Rutkowski *et al.* (2008) are hardly comparable because they are based on groupings that, according to our results, do not represent clades. However, as noted earlier, both our phylogenetic and biogeographical results agree with the proposal of Dueck *et al.* (2014) that *Spiranthes*, a predominantly temperate North American/Eurasian clade, is derived from Mesoamerican ancestors.

Cyclopogon obliquus has a puzzling distribution and a tortuous taxonomic history, having been described

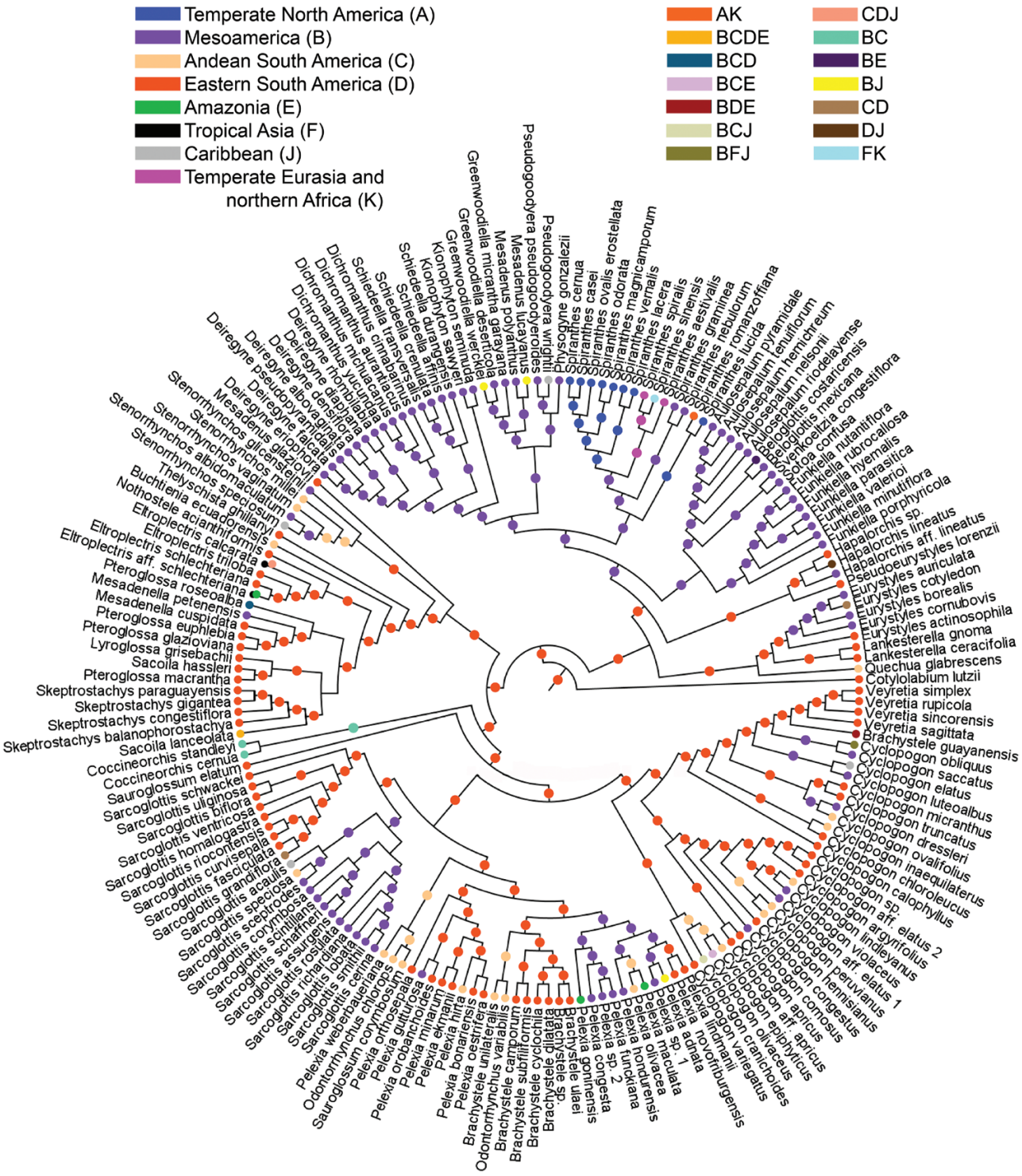


Figure 10. Ancestral area reconstruction under the BMM method with RASP. The most probable ancestral areas of clades are indicated as coloured circles. For simplicity, outgroups and areas not directly relevant to Spiranthinae were trimmed (see the full set of reconstructed probabilities in Supporting Information, Fig. S8).

originally from a plant found at the Buitenzorg (Bogor) Botanic Garden in Java, Indonesia, and assigned then to the 'catch-all' genus *Spiranthes s.l.* Later it was re-described twice, once as *Manniella hongkongensis* S.Y.Hu & Barretto (Hu & Barretto, 1976) from a plant found, as the name implies, on the island of Hong

Kong, and the other as *Pelexia hameri* Garay on the basis of a specimen from El Salvador, Central America (Garay, 1978). The species has been found since in other locations in both hemispheres, including Samoa, Sri Lanka, the West Indies (Guadeloupe and Cuba), Nicaragua, Costa Rica (see Blanco, 2002, and references therein) and Mexico (Soto *et al.*, 2007). Nevertheless, as for all its close relatives, this species is most likely of Neotropical origin and its first discovery in an Asian botanical garden, associated with a cultivated plant of the Neotropical family Cyclanthaceae, suggests an accidental introduction into south-eastern Asia, where it appears to be spreading (cf. Comber, 1990; Cribb & Ormerod, 1999).

FINAL CONSIDERATIONS AND FUTURE WORK

This study represents the most thorough phylogenetic analysis of Spiranthinae conducted to date and is based on an intensive effort to sample the structural, ecological and geographical diversity displayed by this subtribe worldwide. Our generic sample is nearly complete according to the genera recognized in the phylogenetic classification of the orchid family by Chase *et al.* (2015). Genera that have not been available for molecular analysis include mostly monospecific taxa, such as *Cybebus*, restricted to Andean Ecuador and Colombia, and *Degranvillea*, a mycoheterotrophic taxon endemic to lowland, seasonally dry tropical forests in French Guiana (Determann, 1985). *Cybebus* shares some vegetative characters with the *Pelexia* clade, including rhizomatous habit and long-petiolate leaves with several sunken longitudinal veins (reminiscent of plants of *Coccineorchis* and some *Pelexia s.s.*), but the small, stiff rostellum remnant suggests a possible relationship to the *Stenorrhynchos* clade. Overall appearance of its flowers is reminiscent of members of *Eltroplectris* and *Pteroglossa*, but no spur is present (Salazar, 2003b). On the other hand, *Degranvillea* shows some floral features in common with the *Pelexia* clade, including the narrowly conical sepaline spur, subulate nectar glands of the labellum and truncate rostellum with terminal viscidium (as in some species of *Sauroglossum* and *Pelexia s.s.*), but its highly modified vegetative organs do not offer any obvious clue about its phylogenetic affinities. Lastly, *Aracamunia* and *Helonoma* are two distinctive genera highly specialized to the wet, oligotrophic environments of tepuis of the Guayana Highlands and the Andean tepuis. As mentioned earlier, *Helonoma* was sunk in *Beloglottis* by Szlachetko (1996), but these two groups are too distinctive morphologically and ecologically to accept such an idea. Resolution of this issue will have to wait until samples suitable for DNA analyses become available.

The phylogenetic framework generated by this study, which includes many clades not only supported by the DNA sequences but also by structural, ecological and distributional data (e.g. *Funkiella s.l.*; see earlier and Fig. 2A–L), provides an objective basis for subsequent macroevolutionary analyses, including detailed morphological and developmental studies and systematic monographs of natural taxa. Those parts of the phylogenetic tree that still lack clear resolution or support, such as the relationships among some of the major clades of Spiranthinae and interspecific relationships in species-rich genera (i.e. *Cyclopogon*, *Pelexia*, *Sarcoglottis*) will benefit from further work aimed at increasing the sample of both taxa and characters. Affordable access to genome-scale data offers a promising possibility, although difficulty of accessing restricted, rare taxa is a problem likely to be solved only through involvement of local researchers and students in the regions where the diversity of this subtribe is concentrated. The results presented here offer a framework for designing future collecting efforts and focusing monographic work.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Taxa studied, voucher information and GenBank accession numbers.
Figure S1. Strict consensus from the MP analysis of plastid DNA sequences. Numbers above branches indicate bootstrap percentages > 50.
Figure S2. Maximum likelihood tree from the ML analysis of plastid DNA sequences. Numbers above branches indicate bootstrap percentages > 50.
Figure S3. Strict consensus from the MP analysis of nuclear DNA sequences. Numbers above branches indicate bootstrap percentages > 50.
Figure S4. Maximum likelihood tree from the ML analysis of nuclear DNA sequences. Numbers above branches indicate bootstrap percentages > 50.
Figure S5. Strict consensus from the MP analysis of combined plastid and nuclear DNA sequences. Numbers above branches indicate bootstrap percentages > 50.
Figure S6. Maximum likelihood tree from the ML analysis of combined plastid and nuclear DNA sequences. Numbers above branches indicate bootstrap percentages > 50.
Figure S7. Maximum likelihood phylogram from the ML analysis of combined plastid and nuclear DNA sequences. Branch lengths are drawn proportional to the amount of character change.
Figure S8. Ancestral area reconstruction under the BMM method with RASP. Ancestral areas with different probabilities are indicated as portions of coloured rings. The most probable ancestral areas are indicated by letters at the centre of each ring.