



Phylogenetic relationships of *Discyphus scopulariae* (Orchidaceae, Cranichideae) inferred from plastid and nuclear DNA sequences: evidence supporting recognition of a new subtribe, Discyphinae

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Abstract

The monospecific genus *Discyphus*, previously considered a member of Spiranthinae (Orchidoideae: Cranichideae), displays both vegetative and floral morphological peculiarities that are out of place in that subtribe. These include a single, sessile, cordate leaf that clasps the base of the inflorescence and lies flat on the substrate, petals that are long-decurrent on the column, labellum margins free from sides of the column and a column provided with two separate, cup-shaped stigmatic areas. Because of its morphological uniqueness, the phylogenetic relationships of *Discyphus* have been considered obscure. In this study, we analyse nucleotide sequences of plastid and nuclear DNA under maximum parsimony and maximum likelihood criteria with the aim of clarifying its systematic position and discussing its peculiar morphology in an explicit phylogenetic context. Our analyses failed to support inclusion of *Discyphus* in Spiranthinae, signifying instead that this genus represents an additional isolated lineage of “core spiranthids.” The notable morphological disparity among such major lineages, as compared with the short internal branches subtending them in the molecular trees, would support the hypothesis that *Discyphus* represents a relict from an early radiation that also gave rise to Cranichidinae and Spiranthinae, putatively driven by adaptation to different pollinators given the morphological differences in floral morphology among these taxa.

Key words: ITS nrDNA, *matK-trnK*, Neotropical orchid phylogenetics, Orchidoideae, *rbcl*, Spiranthinae, *trnL-trnF*

Introduction

The genus *Discyphus* Schlechter (1919: 417) was created to include a single species formerly described as *Spiranthes scopulariae* Reichenbach (1854a: 11) and included in subtribe Spiranthinae Lindley (1840: 441) by Schlechter (1920), Balogh (1982), Garay (1982), Szlachetko (1992, 1995), Chase *et al.* (2003) and Salazar (2003b). However, *Discyphus* displays several vegetative and floral morphological peculiarities that cast doubts on its subtribal placement. *Discyphus scopulariae* (Reichenbach 1854a: 11) Schlechter (1919: 417) stands out vegetatively because of its single, sessile, cordate leaf that clasps the base of the inflorescence and lies flat on the substrate (Fig. 1A, B, D). In contrast, most genera of Spiranthinae bear one or more leaves that are petiolate or at least attenuate towards the base, thus never clasping the base of the scape. The only other genus of Spiranthinae with similar leaves is *Nothostele* Garay (1982: 339), which includes two species restricted to the Brazilian Plateau. In the latter, plants produce one or two sessile, round, cordate leaves that lie on the substrate, but the inflorescence and leaves are produced at different times of the year; therefore, a leaf base clasping the scape is impossible (Batista *et al.* 2011). Furthermore, in *Discyphus* the petals are free from the dorsal sepal, but their proximal half is decurrent on the column; in addition, the margins of the labellum are free, whereas in the typical Spiranthinae the petals are adherent to the dorsal sepal, their bases are free from the column and the margins of the labellum are fused to the sides of the column to form a tube leading to the nectary (Garay 1982; Salazar 2003b). Moreover, the column of *Discyphus* has two separate, cup-shaped stigmatic areas (Figs. 1E, 2H), a condition unique among Spiranthinae or of any other genus in Cranichideae Pfeiffer (1874: 901). In Cranichideae, the stigma is usually entire, or, in the few instances when it has two receptive areas, these are never

cup-shaped but flat or with slightly convex lateral surfaces, as in some Cranichidinae Lindley (1840: 441) *s.l.*, such as *Galeoglossum* Richard & Galeotti (1845: 31; Salazar 2009; Salazar *et al.* 2011b), and Goodyerinae Ridley (1907: 12), e.g. in *Anoectochilus* Blume (1825: 411), *Cheirostylis* Blume (1825: 413) and *Hetaeria* Blume (1825: 409; Szlachetko & Rutkowski 2000, Ormerod & Cribb 2003). In those representatives of Spiranthinae in which the stigmatic surface is conspicuously bilobed, such as in *Sauroglossum elatum* Lindley (1840: 480), the receptive stigmatic areas are at least partially confluent, flat and without raised margins (Singer 2002, Salazar 2003b).

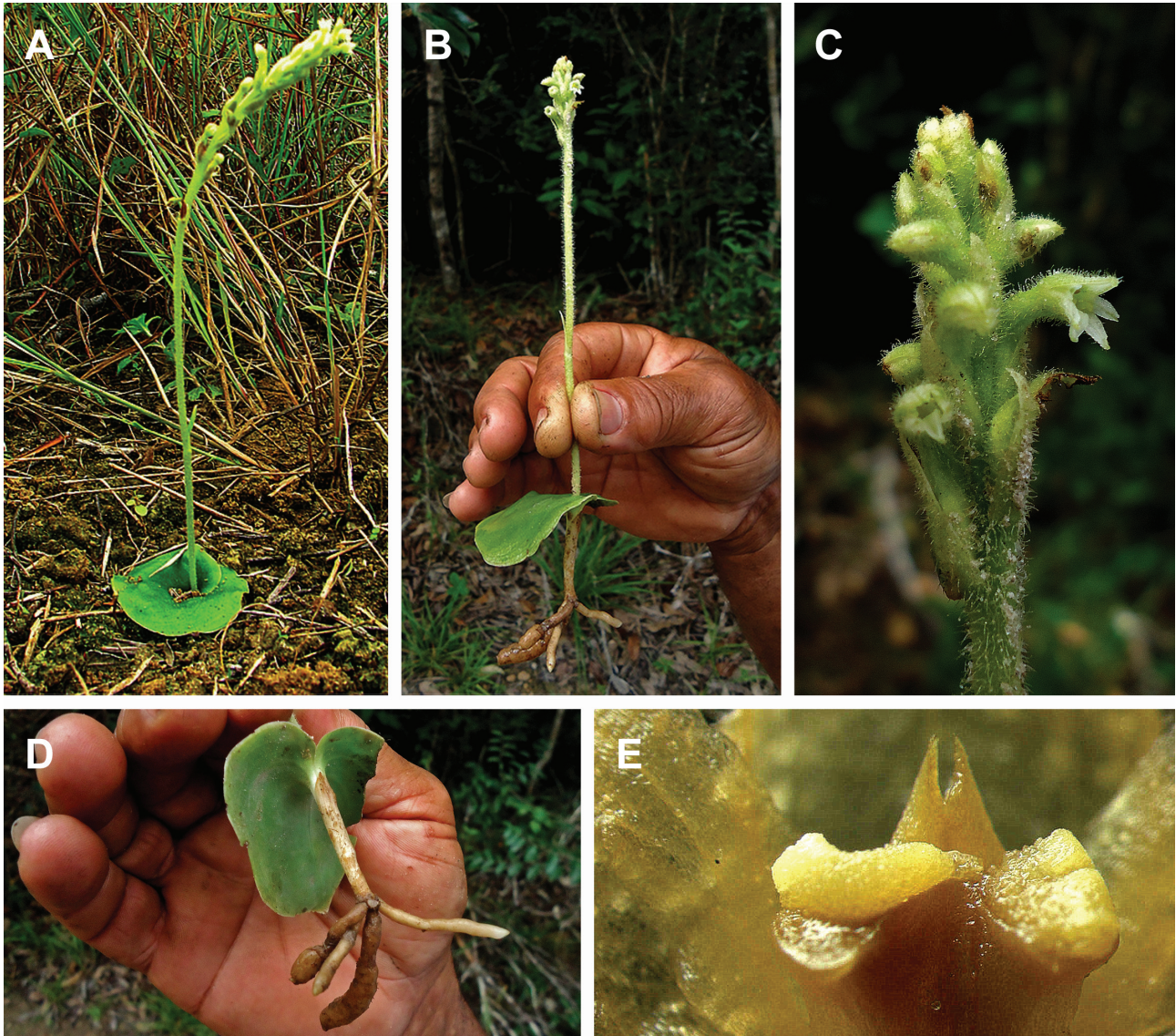


FIGURE 1. *Discyphus scopulariae*. **A.** Flowering plant in situ (Bahia, Brazil, *Popovkin 338A*). **B–E.** Another flowering plant removed from soil (Bahia, Brazil, *Popovkin 900*). **C.** Inflorescence. **D.** Roots and leaf from below. **E.** Close-up of the column apex from below with the pollinarium removed, showing the bifid rostellum remnant and the two stigmatic areas with pollinium fragments presumably deposited by an unrecorded pollinator. Photographers: Alex Popovkin (**A–D**), Isys Souza (**E**).

By virtue of its morphological distinctness, and because the genus remained unavailable for molecular phylogenetic analysis, the phylogenetic relationships of *Discyphus* have been regarded as obscure (Salazar 2003b). A recent rediscovery by the third author of several populations on the northern coast of Bahia, Brazil (*Popovkin 338A, 900, 901, 1516, HUEFS*), made such phylogenetic study a possibility.

In this paper, we assess the systematic position of *Discyphus*, analysing nucleotide sequences of plastid and nuclear DNA in a phylogenetic framework. The plastid sequences analysed were *rbcL* (Chase & Albert 1998), *matK* plus partial *trnK* intron (in which *matK* is embedded; Hilu & Liang 1997) and the *trnL-trnF* region (consisting of the *trnL* intron and the *trnL-trnF* intergenic spacer, or IGS, plus short exon portions; Taberlet *et al.* 1991). The nuclear region analysed was the internal transcribed spacer region of the nuclear ribosomal DNA (nrITS; Baldwin *et al.* 1995). All these DNA regions have been used previously to infer phylogenetic relationships in Spiranthinae (Salazar *et al.*

2003, 2011a; Górnjak *et al.* 2006; Salazar & Ballesteros-Barrera 2010; Batista *et al.* 2011; Salazar & Dressler 2011; Salazar & Jost 2012), other Cranichideae (Figueroa *et al.* 2008; Álvarez-Molina & Cameron 2009; Salazar *et al.* 2009, 2011b; Cisternas *et al.* 2012) and many other orchid clades (review in Cameron 2007). We were particularly interested in clarifying the relationships of *Discyphus* to other Spiranthinae and discussing its morphological peculiarities in light of its inferred phylogenetic position.

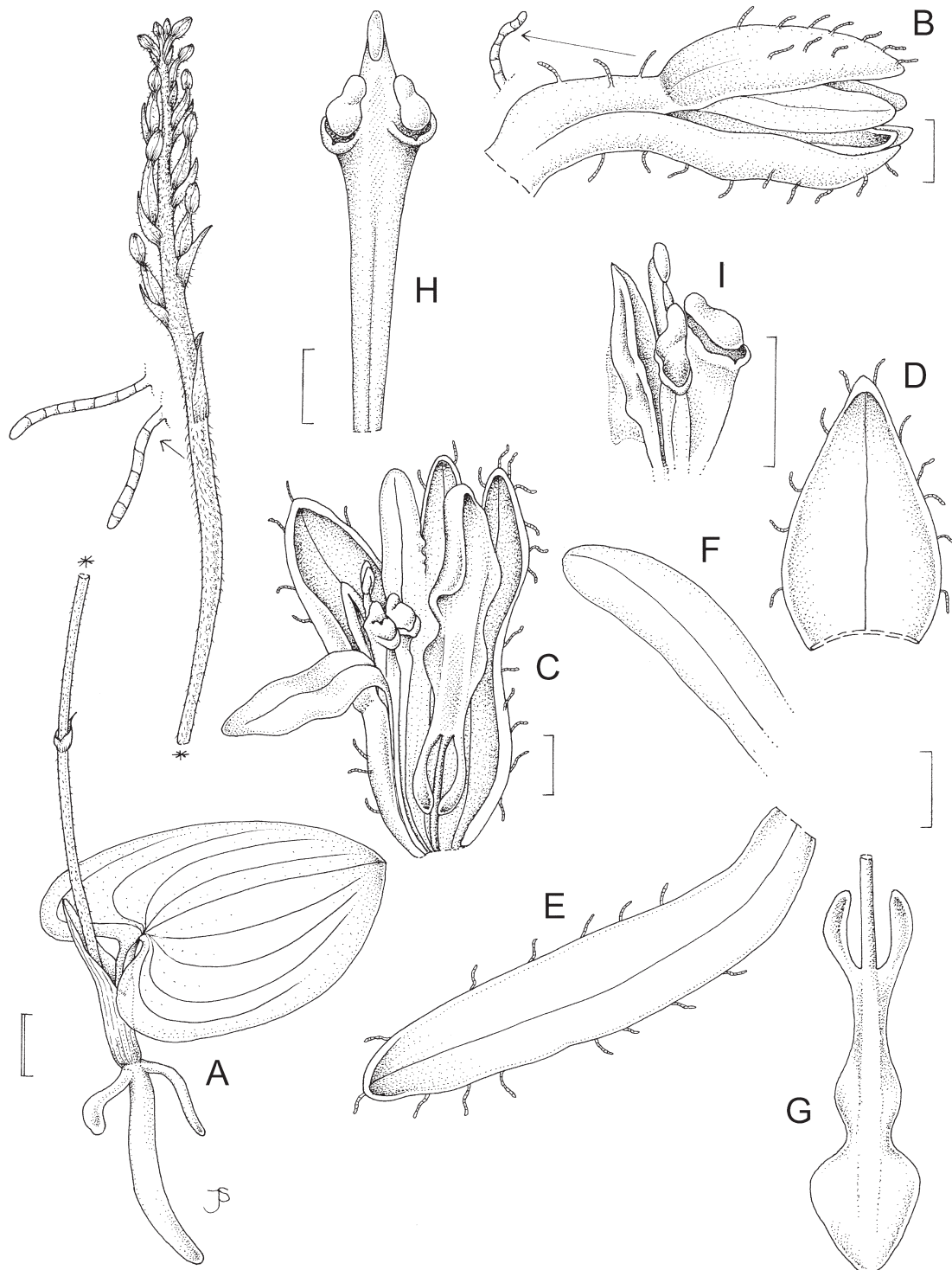


FIGURE 2. *Discyphus scopulariae* (from Coelho de Moraes 2171). A. Habit. B. Flower. C. Flower opened out between dorsal sepal and one lateral sepal. D. Dorsal sepal. E. Lateral sepal. F. Petal. G. Labellum. H. Column, ventral view. I. Column apex, side view. Single bar = 1 mm, double bar = 1 cm. Drawn by Judi Stone and originally published in Pridgeon *et al.* 2003: Fig. 181.1 (reproduced with permission).

Materials and methods

Taxa analyzed:—We analyzed samples of 52 species and 51 genera (Table 1); of these, 24 belong to subtribe Spiranthinae *sensu* Salazar (2003b), and 22 represent all other subtribes of Cranichideae, namely Achlydosinae Clements & Jones in Jones *et al.* (2002: 439), Chloraeinae Pfitzer (1887: 98), Cranichidinae, Galeottiellinae Salazar & Chase in Salazar *et al.* (2002: 172), Goodyerinae, Manniellinae Schlechter (1926: 572) and Pterostylidinae Pfitzer (1887: 97; Kores *et al.* 2001, Salazar *et al.* 2002, 2003, 2009). Our choice of taxa was designed to maximize coverage of genera of Spiranthinae, as far as availability of material permitted. We also included four members of Diurideae (Lindley 1840: 443) Endlicher (1842: 21), a tribe that several molecular phylogenetic analyses have shown to be the sister of Cranichideae (Kores *et al.* 2001, Clements *et al.* 2002, Chase *et al.* 2003, Salazar *et al.*, 2003, 2009, Álvarez-Molina & Cameron 2009, Cisternas *et al.* 2012) and used *Ophrys apifera* Hudson (1762: 340) of tribe Orchideae (Dressler & Dodson, 1960: 35) as a functional outgroup.

Molecular methods:—DNA extraction, amplification, and sequencing were carried out using standard methods and primers described in Salazar *et al.* (2003, 2011a). In all instances, bidirectional sequencing was performed, and the chromatograms were edited and assembled with Sequencher (GeneCodes Corp.). Alignment of *rbcL* was simple because of lack of insertion/deletion (indel) events, but all the other regions were aligned using default settings of the online implementation of the program MAFFT v. 7 (Kato & Standley 2013; <http://mafft.cbrc.jp/alignment/server/>), with minor visual adjustments. Individual indel positions were treated as missing data. The aligned matrix in the Nexus format is available on request from gasc@ib.unam.mx. Sequences for the newly sequenced species have been deposited in GenBank (Table 1).

Phylogenetic analyses:—Our previous phylogenetic analyses of Spiranthinae and Cranichideae were based on the same markers used here (e.g. Salazar *et al.* 2003, 2009, Cisternas *et al.* 2012) and did not find conflicting groups among the separate analyses supported by high bootstrap percentages; moreover, both resolution and overall clade support increased when all data were combined. We therefore opted to analyse all data in combination. We used two phylogenetic methods, namely maximum parsimony (MP) and maximum likelihood (ML), with the aim of comparing results recovered by a method based on explicit models of nucleotide substitution (ML) with another that was not (MP). This approach allowed us to check for possible analytical artefacts, such as “long-branch attraction,” which could mislead MP when different lineages on the tree have dissimilar branch lengths (Felsenstein 1978, Huelsenbeck 1997, Bergsten 2005); there are indeed branch-length inequalities among different species and groups of Spiranthinae as our previous analyses have shown (Salazar & Dressler 2011, Salazar & Jost 2012).

The MP analysis was conducted with the program PAUP* version 4.02b (Swofford 2002) and consisted of a heuristic search with 1000 replicates of random addition of sequences, branch-swapping by “tree bisection-reconnection” (TBR), and the option “MULTREES” (to save multiple trees) activated, saving in memory all most-parsimonious trees (MPTs) found. All characters were treated as unordered and equally weighted. Internal support for clades was assessed by 1000 bootstrap replicates (Felsenstein 1985), each consisting of a heuristic search with 20 random-sequence addition of taxa for the starting trees and the TBR branch-swapping, saving up to 20 MPTs per replicate.

The ML analysis was done with the program RAxML-HPC2 version 7.4.2 (Stamatakis 2006), as implemented in the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal 2.0 (Miller *et al.* 2010). Analysis of 1000 rapid bootstrap replicates (Stamatakis *et al.* 2008) was followed by a search for the tree that maximizes the likelihood function, with the default value of 25 rate categories and estimation of all free model parameters for seven character partitions: first/second codon positions of *rbcL*, third codon positions of *rbcL*, *matK*, *trnK* intron, ITS region, *trnL* intron and *trnL-trnF* IGS. Both the bootstrap searches and the search for the ML tree used the GTRGAMMA model for nucleotides.

TABLE 1. Taxa studied, voucher information and GenBank accessions.

Taxon	Voucher	GenBank accession			
		<i>rbcL</i>	<i>trnL-trnF</i>	<i>matK-trnK</i>	ITS
TRIBE CRANICHIDEAE ENDL.					
Subtribe Achlydosinae M.A.Clem. & D.L.Jones					
<i>Achlydosa glandulosa</i> (Schltr.)	New Caledonia, <i>Clements D-285</i> , CANB	AJ542401	AJ544506	AJ543950	AJ539525
M.A.Clem. & D.L.Jones					

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TABLE 1. (Continued)

Taxon	Voucher	GenBank accession			
		<i>rbcL</i>	<i>trnL-trnF</i>	<i>matK-trnK</i>	ITS
Subtribe Chloraeinae Rchb.f.					
<i>Chloraea magellanica</i> Hook.f.	Chile, <i>Ryan 1</i> , K (spirit)	AJ542403	AJ544504	AJ543948	AJ539523
<i>Gavilea lutea</i> (Pers.) M.N.Correa	Chile, <i>Ryan 3</i> , K (spirit)	AJ542402	AJ544505	AJ543949	AJ539524
Subtribe Cranichidinae Lindl.					
<i>Aa colombiana</i> Schltr.	Colombia, <i>Aldana 2</i> , ANDES	AM778133	AM412731	AM900802	AM419766
<i>Altensteinia fimbriata</i> Kunth	Ecuador, <i>Salazar 6789</i> , MEXU (spirit)	AM778132	AM412737	AM900801	AM419765
<i>Baskervilla colombiana</i> Garay	Colombia, <i>Niessen 5</i> , MEXU (spirit)	AM778157	AM412714	AM900826	AM419791
<i>Cranichis muscosa</i> Sw.	Costa Rica, <i>Pupulin 1792</i> , USJ	AM778143	AM412723	AM900812	AM419777
<i>Galeoglossum thysanochilum</i> (B.L.Rob. & Greenm.) Salazar	Mexico, <i>Tenorio 17900</i> , MEXU	AM778141	AM412725	AM900810	AM419775
<i>Gomphichis caucana</i> Schltr.	Colombia, <i>Díaz 159</i> , ANDES	AM778136	AM412736	AM900805	AM419770
<i>Ponthieva guatemalensis</i> Rchb.f.	Central America (cultivated specimen), <i>Salazar s.n.</i> , MEXU	AM778152	AM412713	AM900821	AM419786
<i>Porphyrostachys pilifera</i> Rchb.f.	Peru, <i>Whalley s.n.</i> , K (photograph)	AJ542411	AJ544496	AJ543942	AJ539514
<i>Prescottia plantaginea</i> Lindl.	Brazil, <i>Salazar 6350</i> , K (spirit)	AJ542414	AJ544493	AJ543939	AJ539511
<i>Pterichis habenarioides</i> Schltr.	Colombia, <i>Aldana 12</i> , COL	AJ542416	AJ544491	AJ543937	AJ539509
<i>Stenoptera ecuadorana</i> Dodson & C.Vargas	Ecuador, <i>Salazar 6357</i> , K (spirit)	AJ542413	AJ544494	AJ543940	AJ539512
Subtribe Discyphusinae Salazar & van den Berg					
<i>Discyphus scopulariae</i> (Rchb.f.) Schltr.	Brazil, <i>Popovkin 338A</i> , UEFS	LK391733	LK391734	LK391835	LK391732
Subtribe Galeottiellinae Salazar & M.W.Chase					
<i>Galeottiella sarcoglossa</i> (A.Rich. & Galeotti) Schltr.	Mexico, <i>Jiménez 2334</i> , AMO	AJ542407	AJ544500	AJ543945	AJ539518
Subtribe Goodyerinae Klotzsch					
<i>Dossinia marmorata</i> (Lindl.) E.Morr.	Tropical Asia (cultivated specimen), <i>Munich Bot. Gard. 94/1190</i> , M	AJ542405	AJ544502	AJ543947	AJ539521
<i>Goodyera pubescens</i> (Willd.) R.Br.	USA, <i>Chase 212</i> , NCU	AF074174	AM419815	AJ543954	AJ539519
<i>Ludisia discolor</i> (Ker-Gawl.) A.Rich.	Tropical Asia (cultivated specimen), <i>Salazar 6354</i> , K (spirit)	AJ542395	AJ544466	AJ543911	AJ539483
<i>Pachyplectron arifolium</i> Schltr.	New Caledonia, <i>Chase 529</i> , K	AJ542404	AJ544503	AJ310051	AJ539522
<i>Platylepis polyadenia</i> Rchb.f.	Madagascar, <i>Salazar 6352</i> , K (spirit)	AJ542406	AJ544501	AJ543946	AJ539520
Subtribe Manniellinae Schltr.					
<i>Manniella cypripedioides</i> Salazar, T.Franke, Zapfack & Benkeen	Cameroon, <i>Salazar et al. 6323</i> , YA	AJ542409	AJ544498	AJ543943	AJ539516
Subtribe Pterostylidinae Pfitz.					
<i>Pterostylis curta</i> R.Br.	Australia, <i>Chase 572</i> , K	AJ542400	AJ544507	AJ543951	AJ539526
Subtribe Spiranthinae Lindl.					
<i>Aulosepalum tenuiflorum</i> (Greenm.) Garay	Mexico, <i>Salazar 6017</i> , MEXU	–	–	AJ543919	–
	Mexico, <i>Salazar et al. 6150</i> , MEXU	AJ542433	AJ544474	–	AJ539591
<i>Beloglottis costaricensis</i> (Rchb.f.) Schltr.	Mexico, <i>Soto 8129</i> , MEXU	AJ542432	AJ544475	AJ543920	AJ539492
<i>Coccineorchis cernua</i> (Lindl.) Garay	Panama, <i>Salazar et al. 6249</i> , MEXU (spirit)	AJ542422	AJ544485	AJ543930	AJ539502
<i>Cyclopogon epiphyticus</i> (Dodson) Dodson	Ecuador, <i>Salazar 6355</i> , K	AJ542425	AJ544482	AJ543927	AJ539499

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TABLE 1. (Continued)

Taxon	Voucher	GenBank accession			
		<i>rbcL</i>	<i>trnL-trnF</i>	<i>matK-trnK</i>	ITS
<i>Deiregyne diaphana</i> (Lindl.) Garay	Mexico, <i>Salazar et al. 6172</i> , MEXU	AJ542440	AJ544467	AJ543912	AJ539484
<i>Dichromanthus cinnabarinus</i> (La Llave & Lex.) Garay	Mexico, <i>Linares 4469</i> , MEXU	AJ542438	AJ544469	AJ543914	AJ539486
<i>Eltroplectris calcarata</i> (Sw.) Garay & H.R.Sweet	Brazil, <i>Soares s.n.</i> , K (photograph)	AJ519446	AJ519452	AJ519450	AJ519448
<i>Eurystyles borealis</i> A.H.Heller	Mexico, <i>Soto 9149</i> , AMO	AJ542427	AJ544480	AJ543925	AJ539497
<i>Funkiella hyemalis</i> (A.Rich. & Galeotti) Schltr.	Mexico, <i>Salazar et al. 6128</i> , MEXU	AJ542429	AJ544478	AJ543923	AJ539495
<i>Lankesterella gnoma</i> (Kraenzl.) Hoehne	Brazil, <i>Warren s.n.</i> , K (spirit)	–	FN556168	FN556173	FN556163
<i>Mesadenella petenensis</i> (Standl. & L.O.Williams) Garay	Mexico, <i>Salazar 6069</i> , MEXU	AJ542421	AJ544486	AJ543931	AJ539503
<i>Mesadenus lucayanus</i> (Britt.) Schltr.	Mexico, <i>Salazar 6043</i> , MEXU	AJ542436	AJ544471	AJ543916	AJ539488
<i>Nothostele acianthiformis</i> (Rchb.f. & Warm.) Garay	Brazil, <i>Viana 767</i> , BHCB	–	FN868836	FN868833	FN868838
<i>Odontorrhynchus variabilis</i> Garay	Chile, <i>Wallace 130/85</i> , CANB	AJ542426	AJ544481	AJ543926	AJ539498
<i>Pelexia adnata</i> (Sw.) Poit. ex Spreng.	Mexico, <i>Salazar 6012</i> , MEXU	AJ542423	AJ544484	AJ543929	AJ539501
<i>Pteroglossa roseoalba</i> (Rchb.f.) Salazar & M.W.Chase	El Salvador, <i>Salazar 6023</i> , MEXU	–	FN868837	FN868834	FN868839
<i>Sacoila lanceolata</i> (Aubl.) Garay	Brazil, <i>Da Silva 874</i> , MG Panama, <i>Förther 2545</i> , M	AJ542441 –	AJ544529 –	AJ543933 –	– AJ539504
<i>Sarcoglottis acaulis</i> (J.E.Sm.) Schltr.	Trinidad, <i>Salazar 6356</i> , K (spirit)	AJ542424	AJ544483	AJ543928	AJ539500
<i>Schiedeella llaveana</i> (Lindl.) Schltr.	Mexico, <i>Salazar 6073</i> , MEXU Mexico, <i>Salazar 6105</i> , MEXU	– AJ542437	AJ544470 –	– AJ543915	– AJ539487
<i>Sotoa confusa</i> (Garay) Salazar	Mexico, <i>Hernández 3320</i> , MEXU	–	FN641876	HE575506	FN641865
<i>Spiranthes spiralis</i> (L.) Cheval.	United Kingdom, <i>Bateman s.n.</i> , K (spirit)	AJ542434	AJ544473	AJ543918	AJ539490
<i>Stenorrhynchos glicensteinii</i> Christenson	Mexico, <i>Salazar 6090</i> , MEXU	AJ542420	AJ544487	AJ543532	AJ539505
<i>Svenkoeltzia congestiflora</i> (L.O.Williams) Burns-Bal.	Mexico, <i>Salazar 6143</i> , MEXU	AJ542431	AJ544476	AJ543921	AJ539493
TRIBE DIURIDEAE ENDL.					
Subtribe Acianthinae (Lindl.) Schltr.					
<i>Acianthus caudatus</i> R.Br.	N.A.	–	–	–	AF347976
<i>Acianthus exsertus</i> R.Br.	Australia, <i>Chase 565</i> , K	AF074101	AJ409373	AJ309993	–
Subtribe Caladeniinae Pfitzer					
<i>Microtis parviflora</i> R.Br.	Australia, <i>Chase 553</i> , K Australia, 'MA21', CANB	AF074194 –	AJ409428 –	AJ310045 –	 DQ104550
Subtribe Diuridinae Lindl.					
<i>Diuris sulphurea</i> R.Br.					
Subtribe Cryptostylidinae Schltr.					
<i>Cryptostylis subulata</i> (Labill.) Rchb.f.	Australia, <i>Chase 554</i> , K Australia, <i>Chase 332</i> , K	AJ542398 AF074140	AJ544509 AJ409395	AJ543952 AJ310015	AJ539527 AF348015
TRIBE ORCHIDEAE DRESSLER & DODSON					
Subtribe Orchidinae Dressler & Dodson					
<i>Ophrys apifera</i> Huds.	United Kingdom, <i>Chase 536</i> , K	AJ542396	AJ544511	AJ543953	AJ539529

Results

The MP analysis found 24 MPTs with a length of 6383 steps, CI (excluding uninformative characters) of 0.41 and RI of 0.60. The strict consensus of the 24 MPTs is shown in Fig 3. The maximum likelihood tree (log ML score = -40655.206328) recovered by the ML analysis is shown in Fig 4. On both trees, bootstrap percentages (BP) are indicated under the branches (in the following indicated as “MP/ML”). The MP and ML analyses recovered similar patterns of relationship, including monophyletic Cranichideae *s.l.* (BP 100/100), which include, in the ascending branching order, Chloraeinae (BP 100/100), [Achlydosinae-Pterostylidinae] (BP 72/98), Goodyerinae (BP 100/100) and a “core spiranthid” clade (BP 100/100) encompassing Galeottiellinae, Manniellinae and a group consisting of all the representatives of Cranichidinae *s.l.* and Spiranthinae (BP 99/100). Relationships within the last group differ between the MP and ML analyses only in that in the strict consensus from the MP there is a polytomy formed by *Discyphus*, a high-Andean clade encompassing *Stenoptera* Presl (1827: 95) through *Aa* Reichenbach (1854b: 18; clade “a” in Fig. 3), a group with [*Prescottia* Lindley (in Hooker 1824: 115)-*Galeoglossum*] sister to “core” Cranichidinae (*Pterichis* Lindley (1840: 444) through *Ponthieva* Brown (1813: 199; clade “b”), and strongly supported Spiranthinae to the exclusion of *Discyphus* (clade “c”; BP 100). In contrast, in the ML tree *Discyphus* is sister, with BP < 50, to a monophyletic but weakly supported Cranichidinae *s.l.* (BP < 50) and these, in turn, are sisters to strongly supported Spiranthinae (excluding *Discyphus*; BP 100; Fig. 4). In both the MP and ML analyses, relationships within Spiranthinae and the various clades of Cranichidinae match closely those found in previous molecular phylogenetic analyses (e.g. Salazar *et al.* 2003, 2009, 2011a, b, Álvarez-Molina & Cameron 2009, Salazar & Ballesteros-Barrera 2010, Batista *et al.* 2011, Salazar & Dressler 2011, Salazar & Jost 2012).

Discussion

Both our MP and ML analyses failed to provide support for inclusion of *Discyphus* in Spiranthinae but recovered the same strongly supported four clades of “core spiranthids” that have been consistently found in previous studies (Salazar *et al.* 2003, 2009, Álvarez-Molina & Cameron 2009; Figs. 3, 4). As in those studies, in our analyses relationships among the main clades of core spiranthids and now also *Discyphus* are not unequivocally resolved. Salazar *et al.* (2009) proposed that the lack of clear patterns of support for the relationships among these clades, in contrast with the strongly supported relationships at lower and higher levels of the phylogenetic tree, might indicate a rapid morphological differentiation or a slower local rate of molecular evolution. Likewise, Álvarez-Molina & Cameron (2009: 1036) suggested that the presence of short branches along the spine of a cladogram indicates a rapid radiation of taxa. The marked among-clade distinctness in floral morphology, as compared with the relative homogeneity within each clade, would lend support to the hypothesis of a rapid differentiation, likely fuelled by adaptation to different types of pollinators. For instance, the most obvious features distinguishing Cranichidinae *s.l.* (clades a + b, Figs. 3, 4) from Spiranthinae are the non-resupinate, wide-open flowers of the former, in contrast with the resupinate, tubular flowers of the latter, and both flower orientation and perianth aperture would indicate important differences in their pollination mechanisms. Pollination in Spiranthinae involves introduction of the mouthparts or the whole anterior part of the head or body of the pollinator as it probes deeply into the floral tube to access nectar (e.g. Catling 1993, Singer & Coccuci 1999, Singer & Sazima 1999, 2000, Benítez-Vieira *et al.* 2006, Salazar *et al.* 2011a, Singer 2002, Figueroa *et al.* 2012). In *Prescottia*, the only genus of Cranichidinae *s.l.* for which natural pollination had been studied, pollinators (pyralid moths) partially introduce their proboscis into the calceolate labellum, but there is no floral tube because sepals and petals are revolute (Singer & Sazima 2001). Most other members of Cranichidinae *s.l.*, and particularly “core” Cranichidinae such as *Cranichis* Swartz (1788: 120), *Ponthieva* and *Pterichis*, have a widely open perianth (cf. Pridgeon *et al.* 2003, Salazar *et al.* 2009). Pollination in these groups has not been documented in detail, but casual *in situ* observations of various dipterans visiting flowers of *Ponthieva fertilis* (Lehmann & Kraenzlin in Kränzlin 1899: 498) Salazar (in Salazar *et al.* 2009: 416) and *P. racemosa* (Walter 1788: 222) Mohr (1901: 460) indicated that their flowers function differently from those of Spiranthinae, with the insects probing the labellum of the flowers with their mouth parts as they stand on the other perianth parts (G. A. Salazar, pers. obs.). The relatively short, partially diverging sepals and petals of *Discyphus scopulariae* (Fig. 1B, C) display a half-way stage between the wide-open flowers of Cranichidinae *s.l.* and the tubular flowers of genuine Spiranthinae.

Szlachetko (1992) pointed out some similarities in labellum morphology between *Discyphus* and *Coccineorchis* Schlechter (1920: 434), most notably the swollen retrorse basal auricles (Fig. 2C, G), but this feature also occurs in other members of Spiranthinae, such as *Sarcoglottis* Presl (1827: 95; Salazar 2003b), a distant relative of both

Coccineorchis and *Discyphus* (Figs. 3, 4) as well as in *Cybebus* Garay (1978: 15; not available for molecular study), and therefore it likely evolved independently several times. Schlechter (1920), Balogh (1982) and Szlachetko (1995) grouped *Discyphus* with a heterogeneous assortment of genera, including *Beloglottis* Schlechter (1920: 364), *Hapalorchis* Schlechter (1919: 30), *Spiranthes* Richard (1817: 20) and *Galeottiella* Schlechter (1920: 360), the last now in a monogeneric subtribe (Salazar *et al.* 2002, 2003; Salazar 2003a) because of their bifid rostellum remnant (Fig. 1E); however, this feature occurs in distantly related groups both in and outside Spiranthinae (e.g., in various Goodyerinae, such as *Goodyera* Brown (1813: 197) and *Platylepis* Richard (1828: 34) and should not be viewed as evidence of a close relationship.

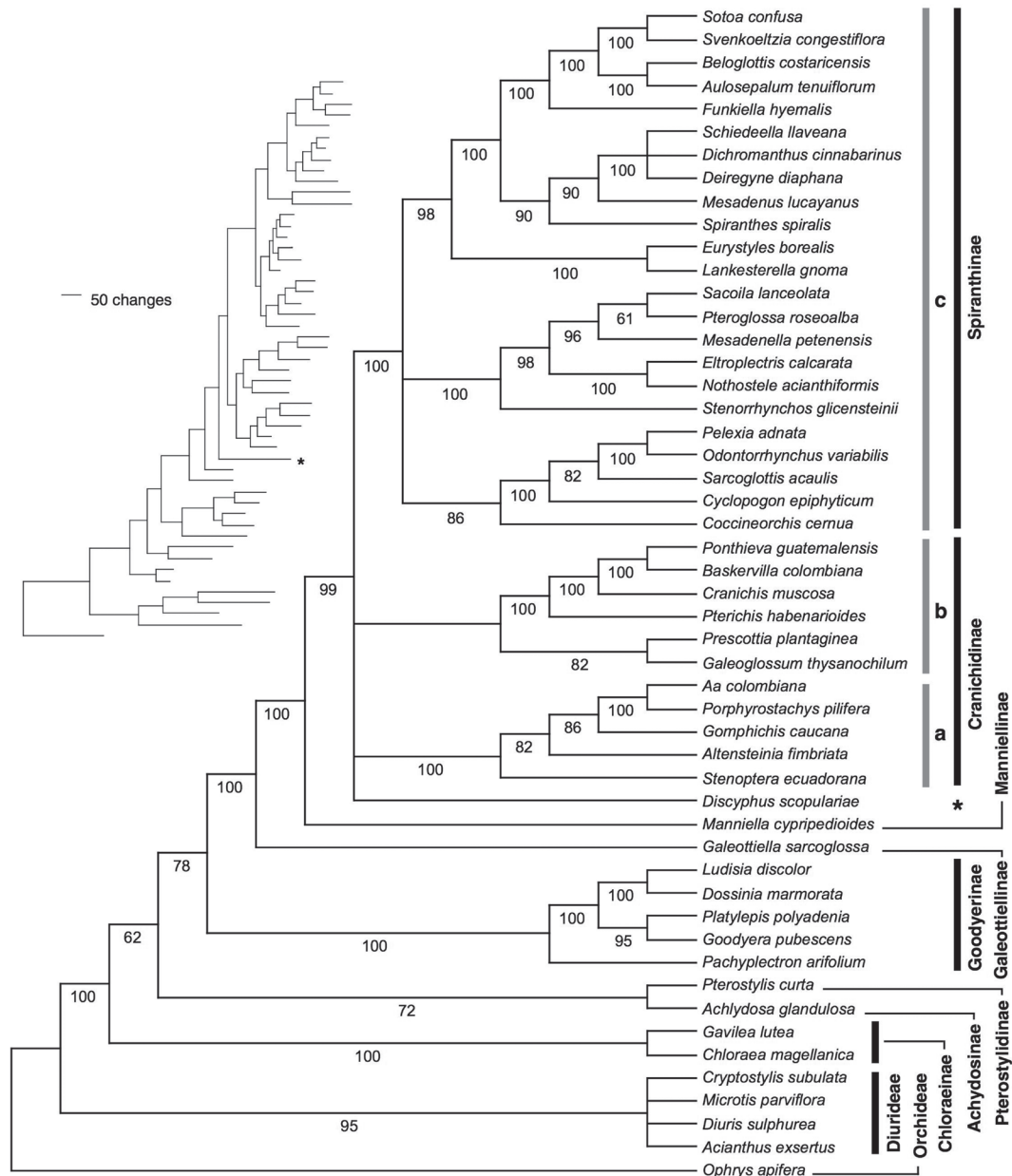


FIGURE 3. Phylogenetic relationships in Spiranthinae inferred from nuclear (ITS) and plastid (*rbcl*, *matK-trnK*, *trnL-trnF*) DNA sequences by maximum parsimony (MP). The main tree is the strict consensus of 24 most parsimonious trees (MPTs) recovered by the analysis; numbers under branches are bootstrap proportions (from the MP bootstrap analysis). The inset on the upper left hand is one of the 24 MPTs with branches drawn proportional to branch length. The major clades referred to in the text are marked as follows: a, *Stenoptera* clade; b, *Prescottia* clade; c, “core” Cranichidinae; d, Spiranthinae (excluding *Discyphus*). The position of *Discyphus* is indicated by an asterisk (*).

All of the above shows that *Discyphus* does not fit into any of the previously identified main clades of core spiranths, appearing instead to represent an isolated lineage that diverged together with Cranichidinae and Spiranthinae from their common ancestor relatively rapidly, as shown by the comparatively few nucleotide substitutions along their

subtending branches in the molecular tree in contrast with their noticeable morphological disparity. Neither the DNA data here analysed nor morphology support the inclusion of *Discyphus* in Spiranthinae. Since our ML analysis grouped *Discyphus* with Cranichidinae (with low bootstrap support), an option would be to include *Discyphus* in Cranichidinae. However, in our view the genetic and structural distinctness of *Discyphus* supports recognition of a distinct subtribe, which is little disruptive to the established taxonomy and better reflects the complex diversification history of the whole group; it also avoids the loss of evolutionary information that would result from merging morphologically and functionally distinctive groups such as *Discyphus* and Cranichidinae. Even though *Discyphus* presently includes only one species, both its phylogenetic position and morphological uniqueness indicate that it is a relict from the early divergence that also gave rise to the subtribes Spiranthinae and Cranichidinae, which, with their approximately 470 and 215 species, respectively (Pridgeon *et al.* 2003), represent the major diversification of terrestrial orchids in the Neotropics. Therefore, we opt here for placing *Discyphus* in a subtribe of its own.

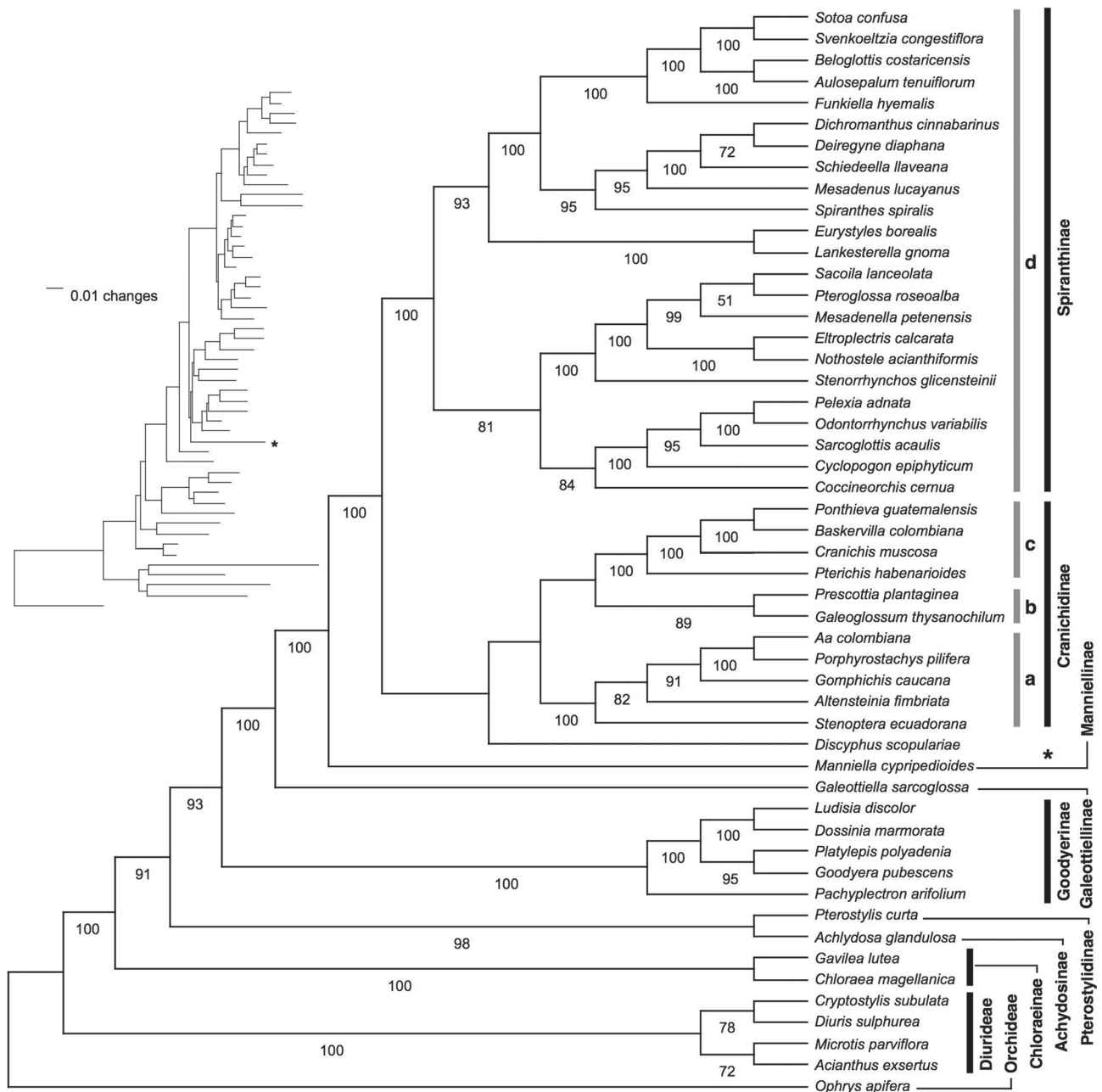


FIGURE 4. Phylogenetic relationships in Spiranthinae inferred from nuclear (ITS) and plastid (*rbcL*, *matK-trnK*, *trnL-trnF*) DNA sequences by maximum likelihood (ML). The main tree is the ML tree; numbers under branches are bootstrap proportions from the ML bootstrap analysis. The inset on the upper left hand is the ML tree with branches drawn proportional to branch lengths. The major clades referred to in the text are marked as follows: a, *Stenoptera* clade; b, *Prescottia* clade; c, “core” Cranichidinae; d, Spiranthinae (excluding *Discyphus*). The position of *Discyphus* is indicated by an asterisk (*).

Taxonomy

Discyphinae Salazar & van den Berg, *subtribus nova*.

Type: *Discyphus scopulariae* (Reichenbach 1854a: 11) Schlechter (1919: 417)

Acaulescent, deciduous geophytes with fasciculate roots; a single orbicular, cordate leaf lying on the substrate and clasping the base of the scape; inflorescence densely glandular-pubescent; flowers campanulate, resupinate, petals free from the dorsal sepal but adnate to the proximal half of the column, labellum free (i.e., its margins not adhering to the sides of the column); column provided with two separate receptive areas, those concave and with raised margins (“cup-shaped”).

This subtribe consists of a monospecific genus distributed from Panama, northern Venezuela and Trinidad to eastern Brazil (Szlachetko 1992, Salazar 2003b).

Discyphus Schlechter (1919: 417). Type species: *Discyphus scopulariae* (Rchb.f.) Schltr.

Dikylikostigma Kraenzlin (1919: 321). Type species: *Dikylikostigma preussii* Kraenzlin (1919: 321)

Discyphus scopulariae (Rchb.f.) Schltr.

Basionym: *Spiranthes scopulariae* Rchb.f. Type: VENEZUELA. Caripe, Moritz 626 (holotype W-R!).

Homotypic synonym: *Gyrostachys scopulariae* (Rchb.f.) Kuntze (1891: 664).

Heterotypic synonyms: *Dikylikostigma preussii* Kraenzl. Type: VENEZUELA. La Victoria, Preuss 1626 (B, destroyed); *Spiranthes rotundifolia* Cogniaux (1906: 542). Type: BRAZIL. Bahia, Salzmann 538 (G); *Cyclopogon rotundifolius* (Cogn.) Schlechter (1920: 394)

Discyphus scopulariae is terrestrial in savannas and riparian forests from near sea level to about 800 m. Full descriptions and additional information on *Discyphus* can be found in Foldats (1969), Garay (1982), Szlachetko (1992) and Salazar (2003b).

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