Preliminary Phylogeny of Diplostephium (Asteraceae): Speciation Rate and Character Evolution

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PRELIMINARY PHYLOGENY OF *DIPLOSTEPHIUM* (ASTERACEAE): SPECIATION RATE AND CHARACTER EVOLUTION

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Abstract: *Diplostephium* comprises 111 neotropical species that live in high elevation habitats from Costa Rica to Chile. Primarily Andean, the genus seems to have undergone an adaptive radiation indicated by its high number of species, broad morphological variation, and diversification primarily in an ecosystem (páramo) that formed within the last 2–5 my. Internal transcriber spacer (ITS) sequences and several chloroplast markers, *rpoB*, *rpoC1*, and *psbA-trnH* were sequenced in order to infer a preliminary phylogeny of the genus. The chloroplast regions showed no significant variation within the genus. New ITS data were therefore analyzed together with published sequences for generating a topology. Results suggest that *Diplostephium* and other South American genera comprise a polytomy within which a previously described North American clade is nested. Monophyly of *Diplostephium* was neither supported nor rejected, but the formation of a main crown clade using different methods of analysis suggests that at least a good portion of the genus is monophyletic. A Shimodaira-Hasegawa test comparing the topology obtained and a constrained one forcing *Diplostephium* to be monophyletic showed no significant difference between them. Monophyly of some of the previously proposed series of the genus was not supported by the phylogenetic tree. Morphological character mapping results suggest that the high Andean forest tree species are derived from shrubby páramo-puna ancestors, contradicting previous hypotheses about morphological evolution of the genus and documenting an atypical trend of downslope diversification in páramo plants.

Keywords: Adaptive radiation, Andes Cordillera, Astereae, *Diplostephium*, high Andean forest, ITS, morphological evolution, páramo, *psbA-trnH*, puna, *rpoB*, *rpoC1*.

Diplostephium Kunth is a genus of 111 known species (Vargas, 2011), distributed from Costa Rica to northern Chile in high elevation cloud forests (2500–3000 m), páramos (3000–4500 m), and puna habitats (3800–4200 m). The genus is characterized by alternate leaves, white to purplish or bluish ligules, and a pappus formed by two rows of bristles, the outermost one reduced. Morphologically diverse, growth forms in the genus vary from decumbent subshrubs to small trees up to 6 m tall, with leaves that range from 3 mm to 12 cm long. Arrangements of capitula vary from single heads to paniculiform or umbelliform sinflorescences.

Sydney F. Blake compiled the first revision of the genus in 1922, recognizing 40 species. He divided the genus into five series (DENTICULATA, FLORIBUNDA, LAVANDULIFOLIA= DIPLOSTEPHIUM, ROSMARINIFOLIA, and RUPESTRIA) based on leaf and sinflorescence variation. In 1928 he added 15 more species and eliminated the series DENTICULATA and FLORIBUNDA. Later, José Cuatrecasas studied the genus, describing several species and publishing two revisions (Cuatrecasas, 1943, 1969). In the second revision, in which 53 species known from Colombia were treated, he subdivided the genus even further, reinstating series DENTICULATA and FLORIBUNDA, and incorporating seven new series: ANACTINOTA, CORIACEA, CRASSIFOLIA, HUERTASINA, PHYLICOIDEA, SAXATILIA, and SCHULTZIANA. Cuatrecasas (1969: 92) proposed a "phylogenetic" order to these 12 series (Table 1), intuitively polarizing arborescent forms, large leaves, multi-capitulate sinflorescences, and small heads as "atavistic" (i.e., ancestral). These

TABLE 1. Cuatrecasas' circumscription of *Diplostephium* species for Colombia (1963). Series are ordered by morphological polarization of series that contain atavistic (i.e., ancestral) characteristics (top), to series that present derived characteristics (bottom).

Series	Species
DENTICULATA	*D. ochraceum, D. bicolor, D. ellipticum, D. dentatum, D. oblongifolium, D. antioquense, D. tenuifolium, D. tamanum, D. mutiscuanum, D. fosbergii, D. grantii, D. ocanense, D. tachirense, D. leiocladum.*
CRASSIFOLIA	*D. crassifolium.*
CORIACEA	*D. coriaceum.*
HUERTASINA	*D. huertasii, D. juliani.*
FLORIBUNDA	*D. floribundum, D. tolimense, D.farallonense, D. pittierii, D. cinerascens, D. cayambense.*
SCHULTZIANA	*D. juabioyi, D. alveolatum, D schultzii, D. rhododendroides, D. romboidale.*
ROSMARINIFOLIA	*D. lacunosum, D. revolutum, D. violaceum, D. heterophyllum, D. cyparissias, D. rosmarinifolium.*
PHYLICOIDEA	*D. phylicoides.*
RUPESTRIA	*D. rupestre, D. eriophorum, D. weddellii.*
SAXATILIA	*D. saxatile, D. romeroi, D. tergocanum.*
DIPLOSTEPHIUM	*D. micradenium, D. glutinosum, D. apiculatum, D. nevadense, D. glandulosum, D. spinulosum, D. colombianum, D. parvifolium, D. hartwegii.*
ANACTINOTA	*D. anactinotum, D. inesianum.*

character-states are found in series DENTICULATA, CORIACEA, and CRASSIFOLIA, which grow mostly in montane forests. In contrast, character-states such as a shrubby habit, small thick revolute leaves, single capitula, and large heads were considered derived and occur in the páramo and puna species placed in series RUPESTRIA, ANACTINOTA, and DIPLOSTEPHIUM. This hypothesis suggests that extant páramo-puna species were derived from high Andean forest-dwelling ancestors, comparable to other páramo plant radiations such as the Espeletiinae intensely studied by Cuatrecasas (1986). Cuatrecasas' revision of the Colombian species (1969) is considered the last comprehensive study of the genus because of the number of species included and the rearrangement and description of new series. Additionally, Cuatrecasas (1986) hypothesized that the main center of speciation and the origin of the genus were in Colombia probably based this on the fact that more than half of the species of the genus from all the series described including the presumed "ancestral morphotypes" are found in that country. Colombia now has 63 reported species accounting for ca. 57% of the currently

recognized species (Vargas and Madriñán, 2006).

Within the Asteraceae, *Diplostephium* has been placed in the tribe Astereae and the subtribe Hinterhuberinae based on morphology (Nesom, 1994; Nesom and Robinson, 2007), along with other South American genera such as *Floscaldasia* Cuatrec., *Flosmutisia* Cuatrec., *Hinterhubera* Sch. Bip. ex Wedd., *Llerasia* Triana, *Laestadia* Kunth ex Less., and *Oritrophium* (Kunth) Cuatrec. Bonifacino and Sancho (2004) also hypothesized that *Diplostephium* is closely related to the genus *Guynesomia* Bonifacino & Sancho. At the molecular level, there are three published sequences of the genus representing two species (two for the internal transcriber spacer nrDNA marker ITS, and one for the external transcriber spacer ETS). These have been used in studies focusing on the generic relationships within tribe Astereae (Noyes and Rieseberg, 1999; Brouillet et al., 2008; Karaman-Castro and Urbatsch, 2009). Noyes and Rieseberg's study (1999) included one ITS sequence of *Diplostephium rupestre.* Using ITS, Noyes and Rieseberg (1999) revealed a North American crown

clade nested in a Southern Hemisphere grade, suggesting a single origin for the North American Astereae. *Diplostephium* appeared in a South American grade close to the North American clade with low support. Noyes and Rieseberg suggested that the putative ancestors of the North American Astereae were South American taxa, and that the Southern Hemisphere basal grade contains the more ancient lineages for the tribe.

Brouillet et al.'s (2008) study comprised a comprehensive ITS phylogenetic analysis of the Astereae tribe using a considerable sample size of genera. The authors of this paper concluded that the origin of the tribe was probably African, due to the biogeographic pattern of the basal lineages in the topology. Based on these results, they hypothesized that South American species of the tribe are split between the "Paleo South American clade" and the "South American lineages." The Paleo South American clade is nested between the basal lineages and a New Zealand clade, while the South American lineages appear in a more derived clade forming a polytomy with some Australasian lineages and a North American clade. Brouillet et al. used two *Diplostephium* sequences in their study, *D. rupestre* and *D. ericoides*, which were part of a polytomy in the topology along with other South America lineages. Their result suggested that *Diplostephium* is not monophyletic, but phylogenetic support was not shown and was simply mentioned as being non-significant (Brouillet et al., 2008).

Karaman-Castro and Urbatsch (2009) used a subset of the taxa sampled by Brouillet et al. (2008), focusing their study in the *Hinterhubera* group, seven genera of the Hinterhuberinae subtribe in which *Diplostephium* is not included. The ITS region and part of the ETS were used as markers. These authors concluded that these markers do not support the monophyly of the *Hinterhubera* group, nor the monophyly of the Hinteruberinae, a result that agreed with previous phylogenetic studies (Noyes and Rieseberg, 1999; Brouillet et al., 2008).

Here, however, the *Diplostephium* samples, the same used by Brouillet et al. (2008), formed a monophyletic clade in the ITS tree with moderate support. For the ETS phylogenetic analysis, Karaman-Castro and Urbatsch (2009) sequenced one species of *Diplostephium* yielding no evidence about the monophyly of the genus. Additionally, the authors concluded that the ETS topology was poorly resolved in comparison to the ITS.

Diplostephium appears to be a rapidly evolving genus. It is morphologically diverse and has more than 90 species living in the Andean páramos and punas, habitats that were not available until 2–4 mya (van der Hammer and Cleef, 1986). Thus, in order to attempt to reconstruct a molecular phylogentic tree of the genus and test if the genus is monophyletic it is necessary to use markers with high levels of sequence variation. The ITS, the region between the 18S-26S nuclear ribosomal DNA (Baldwin et al., 1995), has been proven to be a valuable marker for phylogenetic reconstruction in the Asteraceae, particularly within the Astereae tribe (Noyes and Rieseberg, 1999; Lowrey et al., 2001; Cross et al., 2002; Brouillet et al., 2008; Karaman-Castro and Urbatsch, 2009). We also tested the chloroplast markers *psbA-trnH*, *rpoB*, and *rpoC1* to see if they might also provide phylogenetically informative variation; those markers have been proposed as DNA barcodes because they have been shown to be variable at the species (Holligsworth et al., 2009).

Our aims in this research were: 1) test additional molecular markers of potential use to reconstruct a phylogeny of *Diplostephium*; 2) generate a preliminary phylogenetic tree of various species currently recognized in *Diplostephium*; 3) analyze the sister group relationships of *Diplostephium* within the Astereae by including a representative sample on the South American lineages; 4) test the monophyly of the genus; and 5) assess Cuatrecasas' evolutionary hypotheses of the genus and the monophyly of his circumscriptions of the series.

MATERIALS AND METHODS

TAXON SAMPLING. The chloroplast regions, *psbA-trnH*, *rpoB*, and *rpoC1* were sequenced using nine, five, and four morphologically divergent species of *Diplostephium* respectively (see Appendix 1 for species names, voucher specimens, and GenBank accession numbers). For the ITS data set we newly sequenced 28 samples of *Diplostephium* and one each of *Laestadia*, *Llerasia*, and *Oritrophium*. These genera were sequenced due to their placement in the Hinterhuberinae (Nesom, 1994; Nesom and Robinson, 2007). Seventy-five additional Astereae sequences from previous studies (Cross et al., 2002; Noyes and Rieseberg, 1999; Karaman-Castro and Urbatsch, 2009) were downloaded from GenBank and included in the analysis. Three of these 70 sequences were from *Diplostephium*. The taxon sampling focused on *Diplostephium* and particularly the South American lineages described in Brouillet et al. (2008). Taking into account this focus, just two samples representing the North American clade (Noyes and Rieseberg, 1999, Brouillet et al., 2008) were included in the analysis. The Genbank sequences generated in this study are listed in Appendix 1, and the sequences downloaded are listed in Appendix 2. The complete ITS matrix (new sequences plus those downloaded from GenBank) contained a total of 101 samples of the tribe Astereae, including 31 samples of *Diplostephium*, which represent 27 species, and 11 of the 12 series proposed by Cuatrecasas. Outgroup taxa were selected taking into account the general phylogenetic tree of the tribe published by Brouillet et al. (2008). *Llerasia* and *Chiliotrichum* Cass. were used as outgroups due to their position in the paleo South American clade (Brouillet et al., 2008), which is basal to the New Zealand clade, the Australasian lineages, the North American clade, and the South American lineages.

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING. Total DNA extractions of all taxa were performed using the Dneasy Plant Mini Kit (Qiagen) from silica gel dried leaves or herbarium specimens. The chloroplast regions were amplified using the polymerase chain reaction (PCR) in a final volume of 25μL, with total DNA and GoTaq® Green Master Mix (Promega) following manufacture's protocols, with addition of BSA at a final concentration of 0.1mg/ml. The thermal profile used for the chloroplast regions was 94°C for 1 min then 40 cycles of 94°C for 30 sec, 50°C for 40 sec, 72°C for 40 sec and a final extension of 72°C for 5 min. The primers used to amplify the chloroplast regions where those of Sang et al. (1997) for *psbA-trnH* and those described in the barcoding protocols from Plant DNA Barcoding working group led by Kew Gardens (http: //www.kew.org/barcoding/ protocols.html) for *rpoB* (2 and 4) and *rpoC1* (1 and 4).

Amplification for the ITS marker using the PCR technique was made in a final volume of 25 μl, with total DNA and GoTaq® Green Master Mix (Promega) following manufacturer's protocols, 0.3 μl of pure Dimethyl Sulfoxide (DMSO), and bovine serum albumen (BSA) at a final concentration of 0.1 mg/ml. The thermal profile used for ITS was 94°C for 1 min, then 30 cycles of 94°C for 1 min, 54° for 1 min, and a final extension 72°C for 1 min. Amplifications for DNA obtained from fresh material were performed using the primers ITS1 and ITS4 in a single reaction (White et al., 1990). Herbarium specimen material was amplified using two reactions with the internal primers ITS2 and ITS3 (White et al., 1990).

PCR products were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega). Direct sequencing using Big Dye Terminator Ready Reaction Mix (Applied Biosystems Inc.) was performed in an ABI PRISM 310 sequencer (Applied Biosystems Inc.) at the sequencing facility of the Universidad de los Andes, Bogota, Colombia. Electropherograms were edited in Geneious 3.5.4 (Drummond et al., 2007). Sequences were aligned in MacClade 4 (Maddison and Maddison, 2000) using pair-wise alignment

and then manually edited. Aligned matrices can be accessed from TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S11212).

PHYLOGENETIC ANALYSIS. Maximum Parsimony (MP) analysis of the ITS dataset was initiated using PAUPRat (Sikes and Lewis, 2001) along with PAUP* 4.0b10 (Swofford, 1998) on the CIPRES online portal (Miller et al., 2009). Two thousand iterations were made using random addition, and tree-bisection-reconnection (TBR) branch swapping, saving one tree per iteration. The set of trees recovered from the parsimony ratchet was used as the starting point for a heuristic search performed in PAUP* 4.0b10 (Swofford, 1998) with the following options: multistate taxa interpreted as polymorphism, all characters equally weighted and unordered, gaps treated as missing, 100 replicates with random sequence addition, TBR branch swapping, and branches collapsed if maximum branch length was zero. Bootstrap support (BS) for MP was performed in PAUP* 4.0b10 (Swofford, 1998) using 1,000 replicates and the same parameters used in the heuristic search.

For the maximum likelihood analysis (ML) the model chosen was calculated in Modeltest 3.7 (Posada and Crandall, 1998). The ITS ML analysis was performed in RAxML 7.0.4 (Stamatakis, 2006) with the General Time Reversible (GTR) model, and rates inv-gamma. ML support was assessed using BS with 100 replicates in the same program with the same parameters.

Bayesian Inference (BI) analysis was performed with Mr. Bayes 1.3.2 (Huelsenbeck and Ronquist, 2001) using 10,000,000 generations, four chains, sample frequency = 1,000 generations, burn-in = 2,500,000 with GTR model, and rates inv-gamma.

A Shimodaira-Hasegawa-test (SH-test, Shimodaira and Hasegawa, 1999) was performed on PAUP* 4.0b10 (Swofford, 1998) to compare statistically the ITS ML tree against a constraint topology where *Diplostephium* was forced to be monophyletic. The constraint tree was obtained by forcing *Diplostephium* to be monophyletic under a

ML analysis with the General Time Reversible (GTR) model, and rates inv-gamma in RAxML 7.0.4 (Stamatakis, 2006). This constraint tree was compared with the phylogenetic tree obtained initially by ML. Tree topologies were considered to be significantly different if p < 0.05. All the above analyses, with the exception of the parsimony ratchet, were performed on the phylocluster computer facility at The University of Texas at Austin.

CHARACTER MAPPING. Character state and habitat reconstruction was performed in MacClade 4 using implicit examination (Maddison and Maddison, 2000), based in a reduced ITS ML topology limited to the clade comprising *Diplostephium* species (clade A). Four characters were reconstructed: habit, leaf size, sinflorescence type, and habitat. In addition, Cuatrecasas' series were coded in order to evaluate their monophyly. For the habit character, the states considered were: subshrub (0–30 cm tall), shrub (0.3–2 m tall), and tree (>2 m tall); for leaf size: small (0–2 cm long), medium (2–5 cm long), and large (>5 cm long); for sinflorescence type: solitary capitula, panicle, raceme, and umbel; finally for the habitat: high Andean forest and páramo (puna was not mapped because species sampled from clade A are not present in this habitat). Data were taken from herbarium specimens deposited in US and COL herbaria.

RESULTS

CHLOROPLAST MARKERS. Sequencing of the chloroplast markers selected was abandoned early in the study due to their low levels of variation. The *rpoC1* region showed two variable sites in the alignment for the four species sequenced, while *rpoB* showed only one mutation in one species from the five sequenced. Finally, *psbA-trnH* showed four variable sites in the matrix of nine species sampled (see Appendix 1 for species sequenced).

ITS PHYLOGENETIC ANALYSIS. As in previous studies (Noyes and Rieseberg,

1999; Brouillet et al., 2008; Karaman-Castro and Urbatsch, 2009), the ITS marker showed sufficient variation to assess the partial backbone phylogenetic tree of the tribe with respect to the group of interest; in contrast, it failed in revealing enough resolution and support for elucidating the relationships between the South American lineages. The aligned matrix of the complete dataset consisted of 559 bp for 101 samples, including the outgroup. Of these, 269 characters were constant, 84 were variable and parsimony-uninformative, and 206 were parsimony-informative. The MP analysis found 42,568 shortest trees of 1,098 steps (see Fig. 1 for strict consensus). The trees had a consistency index (CI) of 0.4487, a retention index (RI) of 0.6501, and a rescaled consistency index (RC) of 0.2919. The ML analysis found one tree of score −5893.165595 (Fig. 2). The topology obtained by the BI analysis 50 percent majority rule consensus is shown in Fig. 3.

Those clades where BS and Posterior Probability (PP) have significant values are consistent among the three topologies obtained by the different analysis methods used. Contradictory positions of taxa between the topologies were the position of the Australasian lineages and the position of some species on the South American lineages. Nonetheless, BS and PP do not support these contradictory positions across the topologies obtained (Figs. 1–3). The MP strict consensus tree (Fig. 1) differs from the ML and BI topologies (Figs. 2 and 3) in the position of the Australasian lineages. While in the MP strict consensus the Australasian lineages appear in a polytomy with South American lineages, in ML they appear as the sister clade of a large polytomy formed by the South American lineages and the North American clade; in BI they appear as a grade in which the large polytomy formed by the South American lineages and the North American clade is nested. None of these three different positions is supported by significant BS values in MP and ML, nor by the PP inferred by BI.

All the species of *Diplostephium* are nested in the large polytomy formed by the South American lineages and the North American clade in ML and BI, while in the MP tree they are nested in the large polytomy formed by the South American lineages, the North American clade, and the Australasian lineages. None of the topologies showed all the species of *Diplostephium* sampled forming a monophyletic group. In MP *D. azureum* is the sister species of a clade formed by *Blakiella bartsiifolia* and *Aztecaster* G.L. Nesom species with a BS less than 50%. The remaining 26 species of *Diplostephium* (30 samples) form a monophyletic group with a BS less than 50%. This monophyletic group includes a clade labeled as A (Fig. 1), recovered also in ML and BI analyses, representing 22 species of *Diplostephium* (24 samples). Nested in clade A is the largest clade of the genus recovered by MP with a BS more than 50%, this group of *Diplostephium* contains 22 samples representing 20 species (BS=58).

In the ML topology, *Diplostephium azureum* is sister to a clade of *Parastrephia* Nutt. species. Again, BS does not support the position of this species. The rest of the *Diplostephium* species form a monophyletic group similar to one described for MP but with the inclusion of *Sommerfeltia spinulosa* as sister species of clade A. The position of *S. spinulosa* on the ML topology is not supported by ML-BS, nor found in the MP and BI topologies. This large clade formed by 30 samples of *Diplostephium* and *S. spinulosa* has a BS support less than 50%. The largest *Diplostephium* monophyletic group with support is clade A with BS=70%.

The BI tree also recovers clade A with a PP=0.95 but does not recover a larger clade with species of *Diplostephium* like MP and ML. As in ML, *D. azureum* appears sister to the clade formed by *Parastrephia* species in the BI topology with a posterior PP=0.59. *Diplostephium glandulosum* appears in the large polytomy with the other South American lineages. *Diplostephium haenkei* also
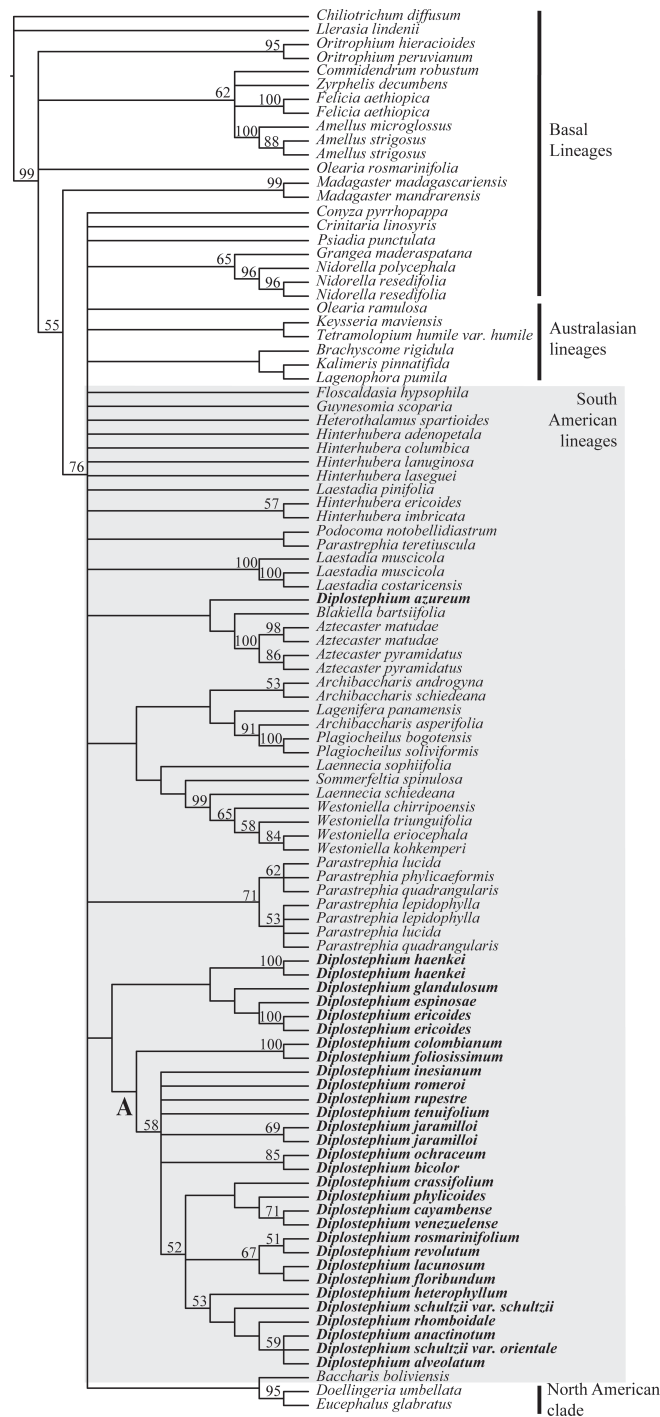
FIG. 1. ITS Maximum parsimony strict consensus of 42,568 trees of 1,098 steps. Bootstrap support values given for nodes with greater than 50% support.

FIG. 2. ITS Single phylogram obtained under maximum likelihood criterion. Bootstrap support values given for nodes with greater than 50% support.
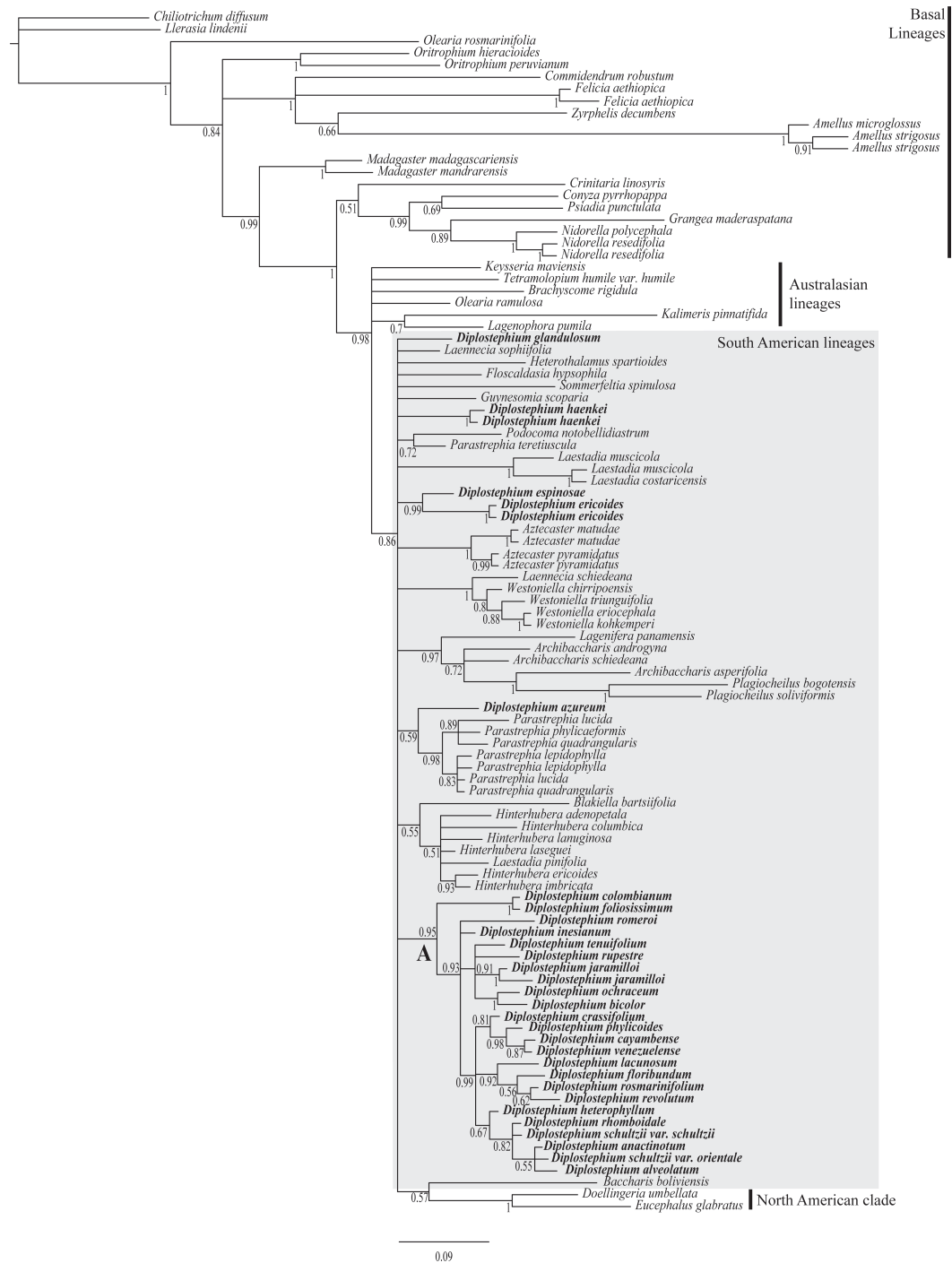
FIG. 3. ITS Bayesian inference topology. Posterior probability values given for nodes greater than 0.5.

appears in the large polytomy with the other South American lineages in which its two samples form a monophyletic clade. *Diplostephium espinosae* and *D. ericoides* form a clade with PP=0.99, a clade that also forms part of the grand polytomy in the topology. Additionally, none of our topologies supports the monophyly of *Laestadia muscicola* and *Diplostephium shultzii* as monophyletic species. *Laestadia muscicola* is paraphyletic, with *L. costaricensis* nested within it, a position with strong support in all the topologies obtained. *Diplostephium schultzii* is polyphyletic, with the two varieties being in different branches inside clade A, both positions have moderate or low support.

CONSTRAINT ANALYSIS. Maximum likelihood analysis with *Diplostephium* forced to be monophyletic obtained a likelihood value of -5895.207338. This topology compared with the initial tree generated by ML (score -5893.165595) was not significantly different from that inferred by the SH-test (p=0.439). This result fails to reject the null hypothesis of the topologies not being significantly different, leading to the premise that the monophyly of *Diplostephium* cannot be rejected by these data.

CHARACTER STATE RECONSTRUCTION TO TEST CUATRECASAS' HYPOTHESES AND SERIES. Because clade A was consistently recovered in all the phylogenetic methods used and presented moderate support in ML and BI, it was used to test the hypotheses proposed by Cuatrecasas about the morphological evolution of *Diplostephium* and its subgeneric subdivision. The morphological reconstruction of characters (Fig. 4a) showed that the hypothetical ancestor of early diverging species of *Diplostephium* in clade A was probably a shrub, and that the tree habit evolved more than once in the genus. Character mapping of leaf size (Fig. 4b), a highly valuable character in the genus associated with habit, suggests that small leaves were ancestral and that medium sized and large leaves evolved at different times in clade A. Likewise solitary capitula (Fig. 4c) can be interpreted as ancestral in the clade A. The

reconstruction of habitat (Fig. 4d) showed a high elevation mountain-living morphotype (páramo or proto-páramo) hypothetical ancestor, rather than a high-mountain forest morphotype for clade A. Taking into account that páramo and puna habitats are correlated with shrubby and sub-shrubby growth forms and that *Diplostephium* species not grouped in clade A are not arborescent, the habitat character mapping suggests that modern arborescent forest species were derived from high elevation ancestors in the genus.

Our results suggest that the series ANACTINOTA, DENTICULATA, FLORIBUNDA, ROSMARINIFOLIA and SCHULTZIANA are not monophyletic when mapped on clade A (Fig. 4a). Even though the series DIPLOSTEPHIUM and RUPESTRIA appear to be monophyletic in clade A, they have representatives in our topologies that are not part of this clade suggesting their non-monophyly. For the series SAXATILIA and HUERTASINA, just one species was sampled for each one and no information about their monophyly was gathered. The series CRASSIFOLIA and PHYLICOIDEA sampled in the study are monotypic, and the series CORIACEA was not sampled. This initial evidence does not support the grouping of species proposed by Cuatrecasas, but the low resolution and support suggest that in order to prove this, a more reliable phylogenetic analysis is needed.

DISCUSSION

The phylogenetic utility of the chloroplast markers used in this study (*psbA-trnH*, *rpoB*, and *rpoC1*) is extremely low with most of the sequences almost identical. For this reason, we do not recommend these makers for the study of recently evolved taxa in the Astereae at the infrageneric level and may not be useful as DNA barcode markers.

The backbone topology obtained in this study in relation to the main groups formerly described within the Astereae agreed with previously published phylogenies (Noyes and Rieseberg, 1999; Brouillet et al., 2008; Karaman-Castro and Urbatsch,
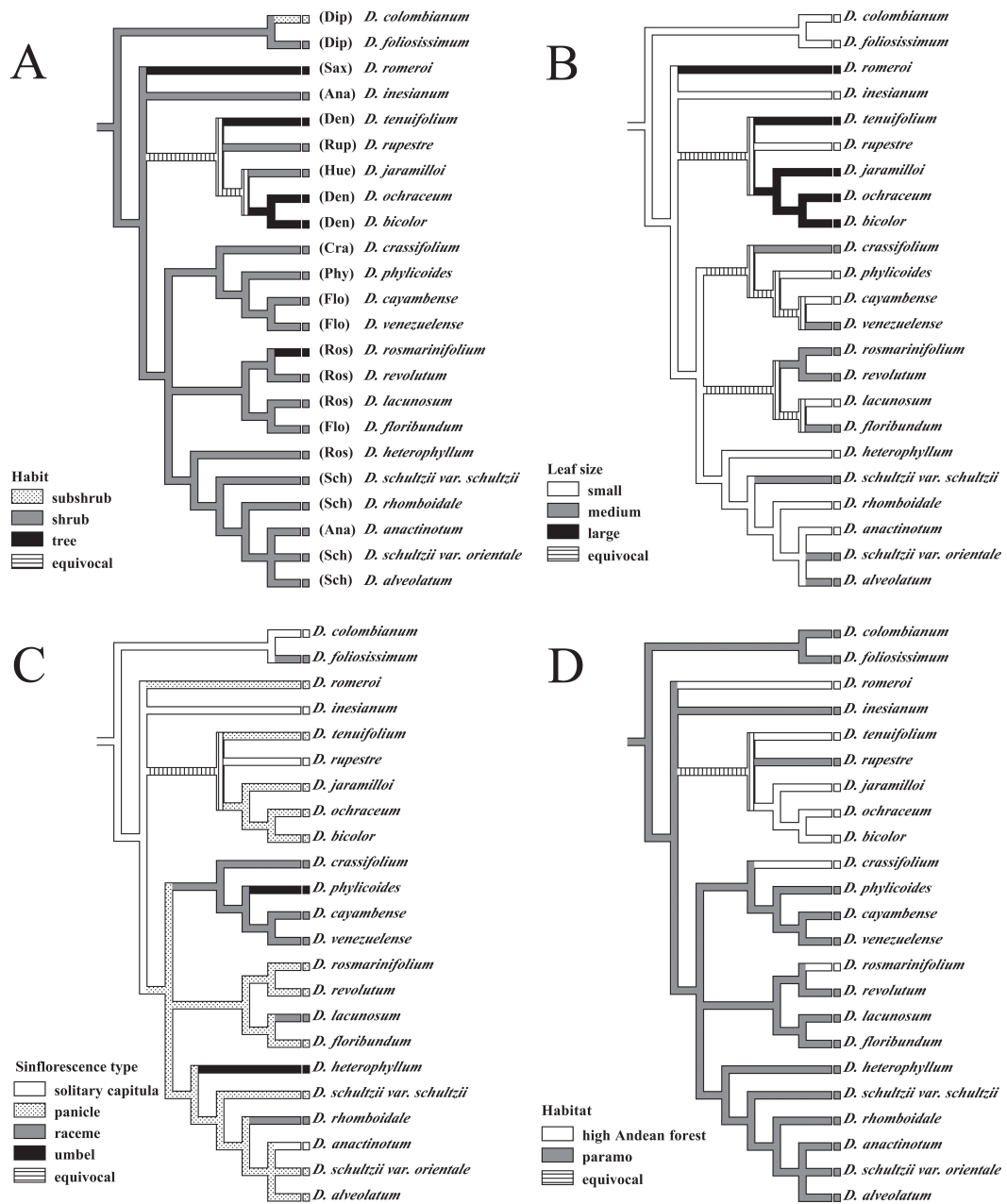
F IG. 4. Clade A character reconstructions. A. Habit and Cuatrecasas' series in parenthesis. Dip: *Diplostephium*, Rup: R UPESTRIA, Sax: S AXATILIA, Ana: A NACTINOTA, Den: D ENTICULATA, Hue: H ERTASINA, Cra: C RASSIFOLIA, Phy: P HYLICOIDEA, Flo: F LORIBUNDA, Ros: R OSMARINIFOLIA, Sch: S CHULTZIANA. B. Leaf size. C. Sinflorescence type. D. Habitat.

2009). All the newly sequenced samples of *Diplostephium* make up part of the polytomy formed by South American lineages and the North American clade. This result confirms

the position of *Diplostephium* in the South American lineages as described by Brouillet et al. (2008), and Karaman-Castro and Urbatsch (2009). The genus *Guynesomia*

also appears in this polytomy along with *Diplostephium* and the other South American lineages, which does not allow acceptance or rejection of the hypothesis of Bonifacino and Sancho (2004) that the two genera are closely related. In this polytomy, the majority of *Diplostephium* samples (24) formed a monophyletic group (clade A, Figs. 1–3) composed of 22 species with a moderate support in ML and BI. The remaining *Diplostephium* samples have contradictory positions between the analyses and no support on the topology. These results do not allow the determination of the monophyly or non-monophyly of *Diplostephium* as a whole. Additionally, the SH-test showed no statistical difference between the topology obtained in the analysis and the topology obtained forcing the genus to be monophyletic under ML.

Even though our results do not allow conclusions about the monophyly of the entire genus, the data suggest that at least part of the genus is monophyletic. Clade A was recovered in all the topologies with BS=70% in ML, and a PP=0.95. This clade represents 80% of the species sampled in this study and contains species from 11 of the 12 series treated by Cuatrecasas (1969) (11 series were sampled). This means that clade A is formed by different morphotype groups described in the genus, and could represent a significant sample of it. If this assumption is correct, we could conclude that even though the traditional genus may not be monophyletic there is a good probability that at least a good portion of it is.

The large number of species in the genus and low molecular variability of ITS present within *Diplostephium* in comparison with that found in other South American taxa, (e.g. *Parastrephia* with four species, *Laestadia* with five, *Blakiella* with one, and *Hinterhubera* with eight) support the hypothesis of a recent adaptive radiation of *Diplostephium* in the Andes. The large morphological variation that evidence character diversification also supports the idea of an adaptive radiation. With 70 species of

Diplostephium found in the páramo, one would expect that the elements that occupy this recently formed habitat would have a similar or younger age. Páramo vegetation is found above 3,000 m and has been shown to appear with the emergence of the Andean cordillera 2–5 mya (van der Hammen and Cleef, 1986). This recent origin, combined with many available niches, explains the radiations seen in many plant taxa now found in these extreme habitats, such as *Espeletia* Mutis ex Humb. and Bonpl. (Rausher, 2002), *Jamesonia* Hook. & Grev. (Sánchez-Baracaldo, 2004), *Valeriana* L. (Bell and Donoghue, 2005), and *Lupinus* L. (Hughes and Eastwood, 2006). Given the signal obtained in this study it is clear that a more powerful phylogenetic tool like the use of high-throughput DNA sequencing, where whole genomes can be obtained in a short period of time, should be implemented to elucidate the phylogenetic relationships of recently evolved taxa.

It is significant that members of the series DIPLOSTEPHIUM (*D. colombianum* and *D. foliosissimum*), characterized by being shrubby and having solitary capitula, are the sister group to the rest of clade A (Fig. 4a). This result, along with the outcome of *Diplostephium* arborescent species being nested in clade A, contradicts the phylogenetic hypothesis proposed by Cuatrecasas (1969) of a forest ancestral morphotype. Additionally, the character reconstructions also indicate that hypothetical proto-páramo dwelling species represent ancestral lineages in clade A. It is important to note that all *Diplostephium* species outside of clade A are predominately shrubby, and their uncertain position does not contradict our previous statements. From the biogeographical point of view, no further evaluation can be made about the Colombian origin hypothesis proposed by Cuatrecasas' (1986) due to the low resolution of our results. While *Diplostephium haenkei,* from southern Peru, appears at the base of *Diplostephium* non-supported clades in MP and ML, a clade formed by *D. colombianum*

(western Colombian Cordillera) and *D. foliosissimum* (northern Peru and Ecuador) is sister to the remainder species in clade A (mostly Colombian) on MP, ML, and BI. These contradictory outcomes leave the former hypothesis about area of origin to be tested in the future.

Taking into account the evidence given, we can conclude that the character mapping does not support Cuatrecasas' hypothesis about the morphological evolution of the genus. Furthermore, we hypothesize that the ancestor of the genus was more likely a páramo-puna-shrub morphotype that originated along the high Andes, with subsequent colonizations to the Andean forest. Evolution of forest tree species derived from páramo ancestors has not been previously described (see van der Hammen and Cleef, 1986). This finding adds a new component to the already complex evolutionary interplay between the páramo flora and the Andean forest.

ACKNOWLEDGEMENTS

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APPENDICES

APPENDIX 1. List of sequences generated in this study. Data is presented in the following order: taxon name, voucher, herbarium and GenBank accession numbers. Accession numbers appear in following order where more than one sequence were produced: ITS, *psbA-trnH*, *rpoB*, and *rpoC1*. Herbaria: ANDES, Herbario Andes, Universidad de los Andes, Bogotá, Colombia; COL, Herbario Nacional Colombiano, Universidad Nacional de Colombia, Bogotá, Colombia; FMB, Herbario Federico Medem Bogotá, Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Villa de Leyva, Colombia; US, United States National Herbarium, Smithsonian Institution, Washington D.C., U.S.A.

Diplostephium alveolatum Cuatrec., *Vargas 183* (ANDES), FJ423515; ***D. anactinotum*** Wedd., *Cuatrecasas 24557* (US), FJ423516, FJ423506; ***D. azureum*** Cuatrec., *Loukuy-Lopez 5295* (US), FJ423517; ***D. bicolor*** S.F. Blake, *Sarria*

462 (COL), FJ423518; **D. cayambense** Cuatrec., *Quenguan 223* (FMB), FJ423519; **D. colombianum** (Cuatrec.) Cuatrec., *Vargas 48* (ANDES), FJ423520, FJ423507; **D. crassifolium** Cuatrec., *Rangel 11293* (COL), FJ423521; **D. espinosae** Cuatrec., *Keating 413* (US), FJ423522; **D. floribundum** (Benth.) Wedd., *Vargas 177* (ANDES), FJ423523; **D. foliosissimum** S.F. Blake, *Smith 12353* (US), FJ423524; **D. glandulosum** Hieron., *Vela 69* (FMB), FJ423525; **D. haenkei** Wedd., *Lewis 88333* (US), FJ423526; *Smith 12525* (US), FJ423527; **D. heterophyllum** Cuatrec., *Vargas 162* (ANDES), FJ423528; **D. inesianum** Cuatrec., *Barclay 6546* (US), FJ423529; **D. jaramilloi** Cuatrec., *Galvis 356* (COL), FJ423530; *Galvis 365* (COL), FJ423531; **D. lacunosum** Cuatrec., *Vargas 46* (ANDES), FJ423532, FJ423508; **D. ochraceum** (HBK) Nees, *Vargas 161* (ANDES), FJ423533, FJ423509, FJ423501, FJ423497; **D. phylicoides** Wedd., *Vargas 166* (ANDES), FJ423534, FJ423510, FJ423502, FJ423498; **D. revolutum** S.F. Blake, *Vargas 45* (ANDES), FJ423535, FJ423511, FJ423503, FJ423499; **D. rhomboidale** Cuatrec., *Vargas 47* (ANDES), FJ423536, FJ423512; **D. romeroi** Cuatrec., *Barclay 6775* (US), FJ423537; **D. rosmarinifolium** Wedd., *Vargas 165* (ANDES), FJ423538, FJ423513, FJ423504, FJ423500; **D. schultzii** Wedd. var. **orientale** Cuatrec., *Vargas 164* (ANDES), FJ423539, FJ423514, FJ423505; **D. schultzii** Wedd. var. **schultzii** Wedd., *Vargas 179* (ANDES), FJ423540; **D. tenuifolium** Cuatrec., *Vargas 184* (ANDES), FJ423541; **D. venezuelense** Cuatrec., *Dorr 7367* (US), FJ423542; **Laestadia muscicola** Wedd., *Zapata 79* (ANDES), FJ423543; **Llerasia lindenii** Triana, *Vargas 185* (ANDES), FJ423544; **Oritrophium peruvianum** (Lam.) Cuatrec. subsp. **lineatum** (Cuatrec.) Cuatrec., *Zapata 65* (ANDES), FJ423545.

APPENDIX 2. List of previously published GenBank ITS sequences used in this paper.

Amellus microglossus DC., DQ478995; **Amellus strigosus** (Thumb.) Less, AF046942, DQ478996; **Archibaccharis androgyna** Blake, DQ478989; **Archibaccharis asperifolia** (Benth.) Blake, DQ478990; **Archibaccharis schiedeana** (Benth) J. D. Jackson, DQ478991; **Aztecaster matudae** (Rzed.) Nesom, DQ479005, DQ479006; **Aztecaster pyramidatus** (B.L.Rob. and Greenm.) Nesom, DQ479007, DQ479008; **Baccharis boliviensis** (Wedd.) Cabrera, DQ478992; **Blakiella bartsiaefolia** (Blake) Cuatrec., DQ479034; **Brachyscome rigidula** (DC.) G. L. Davis, DQ478994; **Chiliotrichum diffusum** (G. Forst.) Kuntze, AF046945; **Commidendrum robustum** DC., AF046943; **Conyza pyrrhopappa** Schultz-Bip. ex A. Rich., AF046953; **Crinitaria linosyris** Less., DQ479043; **Diplostephium ericoides** (Lam.) Cabrera, DQ479003, DQ479004; **Diplostephium rupestre** (Kunth) Wedd., AF046962; **Doellingeria umbellata** (Mill.) Nees, AF046966; **Eucephalus glabratus** (Greene) Greene, DQ479041; **Felicia aethiopica** (Burm.f.) Bol. and W.Dod ex Adams and Salt., AF046941, DQ478997; **Floscaldasia hypsophila** Cuatrec., DQ479009; **Grangea maderaspatana** (L.) Poir., AF046951; **Guynesomia scoparia** (Phil.) Bonif. and G. Sancho, DQ479035; **Heterothalamus spartioides** Hook. and Arn., DQ478993; **Hinterhubera adenopetala** Cuatrec. and Aristeg., DQ479010; **Hinterhubera columbica** Sch. Bip. ex Wedd., DQ479011; **Hinterhubera ericoides** Wedd., DQ479012; **Hinterhubera imbricata** Cuatrec. and Aristeg., DQ479013; **Hinterhubera lanuginosa** Cuatrec. and Aristeg., DQ479014; **Hinterhubera laseguei** Wedd., DQ479015; **Kalimeris pinnatifida** (Maxim.) Kitam., DQ478988; **Keysseria maviensis** (H. Mann) Cabrera, DQ479036; **Laennecia schiedeana** (Less.) Nesom, DQ479038; **Laennecia sophiifolia** (Kunth) Nesom, AF046964; **Laestadia costaricensis** S.F.Blake, DQ479016; **Laestadia muscicola** (Sch. Bip.) Wedd., DQ479017; **Laestadia pinifolia** Kunth, DQ479018; **Lagenifera panamensis** Blake, AF046965; **Lagenophora pumila** Cheeseman, DQ479037; **Madagaster madagascariensis** (Humbert) Nesom, DQ479031; **Madagaster mandrarensis** (Humbert) Nesom, DQ479032; **Nidorella polycephala** DC., DQ478999; **Nidorella resedifolia** DC., AF046952, DQ479000; **Olearia ramulosa** (Labill.) Benth., DQ479033; **Olearia rosmarinifolia** (DC.) Benth., AF497706; **Oritrophium hieracioides** (Wedd.) Cuatrec., AF046946; **Parastrephia lepidophylla** (Wedd.) Cabrera, DQ479019, DQ479020; **Parastrephia lucida** (Meyen) Cabrera, DQ479021, DQ479022; **Parastrephia phylicaeformis** (Meyen) Cabrera, DQ479023; **Parastrephia quadrangularis** (Meyen) Cabrera, DQ479024, DQ479025; **Parastrephia teretiuscula** (Kuntze) Cabrera, DQ479026; **Plagiocheilus bogotensis** (Kunth) Wedd., DQ479001; **Plagiocheilus solivaeformis** DC., DQ479002; **Podocoma notobellidiastrum** (Griseb.) Nesom, AF046954; **Psiadia punctulata** (DC.) Vatke, AF046954; **Sommerfeltia spinulosa** Less., DQ479039; **Tetramolopium humile** (A. Gray) Hillebr. ssp. **humile** var. **humile**, DQ479040; **Westoniella chirripoensis** Cuatrec., DQ479027; **Westoniella eriocephala** (Klatt) Cuatrec., DQ479028; **Westoniella kohkemperi** Cuatrec., DQ479029; **Westoniella triunguifolia** Cuatrec., DQ479030; **Zyrphelis decumbes** (Schltr.) Nesom, DQ478998.