Geography shapes the phylogeny of frailejones (Espeletiinae Cuatrec., Asteraceae): a remarkable example of recent rapid radiation in sky islands

Mauricio Diazgranados Corresp., Janet C. Barber

1 Natural Capital and Plant Health Department, Wakehurst Place, Royal Botanic Gardens, Kew, Ardingly, West Sussex, United Kingdom
2 Department of Biology, Saint Louis University, Saint Louis, Missouri, United States
3 Missouri Botanical Garden, Saint Louis, Missouri, United States

Corresponding Author: Mauricio Diazgranados
Email address: M.Diazgranados@kew.org

Background. The páramo ecosystem, located above the timberline in the tropical Andes, has been the setting for some of the most dramatic plant radiations, and it is one of the world’s fastest evolving and most diverse high-altitude ecosystems. Today 144+ species of frailejones (subtribe Espeletiinae Cuatrec., Asteraceae) dominate the páramo. Frailejones have intrigued naturalists and botanists, not just for their appealing beauty and impressive morphological diversity, but also for their remarkable adaptations to the extremely harsh environmental conditions of the páramo. Previous attempts to reconstruct the evolutionary history of this group failed to resolve relationships among genera and species, and there is no agreement regarding the classification of the group. Thus, our goal was to reconstruct the phylogeny of the frailejones and to test the influence of the geography on it as a first step to understand the patterns of radiation of these plants. Methods. Field expeditions in 70 páramos of Colombia and Venezuela resulted in 555 collected samples from 110 species. Additional material was obtained from herbarium specimens. Sequence data included nrDNA (ITS and ETS) and cpDNA (rpl16), for an aligned total of 2954 bp. Fragment analysis was performed with AFLP data using 28 primer combinations and yielding 1665 fragments. Phylogenies based on sequence data were reconstructed under maximum parsimony, maximum likelihood and Bayesian inference. The AFLP dataset employed minimum evolution analyses. A Monte Carlo permutation test was used to infer the influence of the geography on the phylogeny. Results. Phylogenies reconstructed suggest that most genera are paraphyletic, but the phylogenetic signal may be misled by hybridization and incomplete lineage sorting. A tree with all the available molecular data shows two large clades: one of primarily Venezuelan species that includes a few neighboring Colombian species; and a second clade of only Colombian species. Results from the Monte Carlo permutation test suggests a very strong influence of the geography
on the phylogenetic relationships. Venezuelan páramos tend to hold taxa that are more distantly-related to each other than Colombian páramos, where taxa are more closely-related to each other. **Conclusions.** Our data suggest the presence of two independent radiations: one in Venezuela and the other in Colombia. In addition, the current generic classification will need to be deeply revised. Analyses show a strong geographic structure in the phylogeny, with large clades grouped in hotspots of diversity at a regional scale, and in páramo localities at a local scale. Differences in the degrees of relatedness between sympatric species of Venezuelan and Colombian páramos may be explained because of the younger age of the latter páramos, and the lesser time for speciation of Espeletiinae in them.
Geography shapes the phylogeny of frailejones (Espeletiinae Cuatrec., Asteraceae): a remarkable example of recent rapid radiation in sky islands

Mauricio Diazgranados¹ and Janet C. Barber²,³

¹ Natural Capital and Plant Health Department, Wakehurst Place, Royal Botanic Gardens, Kew, Ardingly, West Sussex, United Kingdom
² Department of Biology, Saint Louis University, Saint Louis, Missouri, United States
³ Missouri Botanical Garden, Saint Louis, Missouri, United States

Corresponding author: Mauricio Diazgranados (m.diazgranados@kew.org)
ABSTRACT

Background. The páramo ecosystem, located above the timberline in the tropical Andes, has been the setting for some of the most dramatic recent rapid plant radiations, and it is one of the world’s fastest evolving and most diverse high-altitude ecosystems. Today 144+ species of frailejones (subtribe Espeletiinae Cuatrec., Asteraceae) dominate the páramo. Frailejones have intrigued naturalists and botanists, not just for their appealing beauty and impressive morphological diversity, but also for their remarkable adaptations to the extremely harsh environmental conditions of the páramo. Previous attempts to reconstruct the evolutionary history of this group failed to resolve relationships among genera and species, and there is no agreement regarding the classification of the group. Thus, our goal was to reconstruct the phylogeny of the frailejones and to test the influence of the geography on it as a first step to understand the patterns of radiation of these plants.

Methods. Field expeditions in 70 páramos of Colombia and Venezuela resulted in 555 collected samples from 110 species. Additional material was obtained from herbarium specimens. Sequence data included nrDNA (ITS and ETS) and cpDNA (rpl16), for an aligned total of 2954 bp. Fragment analysis was performed with AFLP data using 28 primer combinations and yielding 1665 fragments. Phylogenies based on sequence data were reconstructed under maximum parsimony, maximum likelihood and Bayesian inference. The AFLP dataset employed minimum evolution analyses. A Monte Carlo permutation test was used to infer the influence of the geography on the phylogeny.

Results. Phylogenies reconstructed suggest that most genera are paraphyletic, but the phylogenetic signal may be misled by hybridization and incomplete lineage sorting. A tree with all the available molecular data shows two large clades: one of primarily Venezuelan species that includes a few neighboring Colombian species; and a second clade of only Colombian species. Results from the Monte Carlo permutation test suggests a very strong influence of the geography
on the phylogenetic relationships. Venezuelan páramos tend to hold taxa that are more distantly-related to each other than Colombian páramos, where taxa are more closely-related to each other.

**Conclusions.** Our data suggest the presence of two independent radiations: one in Venezuela and the other in Colombia. In addition, the current generic classification will need to be deeply revised. Analyses show a strong geographic structure in the phylogeny, with large clades grouped in hotspots of diversity at a regional scale, and in páramo localities at a local scale. Differences in the degrees of relatedness between sympatric species of Venezuelan and Colombian páramos may be explained because of the younger age of the latter páramos, and the lesser time for speciation of Espeletiinae in them.

**INTRODUCTION**

The mechanisms of evolution and drivers of diversity in tropical ecosystems are not well understood, and most existing studies have focused on lowland taxa and ecosystems. The Andes are one of the most topographically and climatically complex orographic systems (Killeen et al. 2007; Särkinen et al. 2012) and they are a renowned hotspot for biodiversity (Brooks et al. 2006; Young et al. 2015). Above the timberline in the tropical Andes, the páramos dominate the landscape. They are considered the world’s most diverse high-altitude ecosystem (Luteyn 1999; Rangel-Ch. 2000; Sklenár et al. 2005), and one of the fastest evolving ecosystems (Madriñán et al. 2013). With an estimated age of 2–4 million-years (Hooghiemstra & Van der Hammen 2004), the páramos emerge on top of the Andean cordilleras and massifs, rising in some instances to the snow line above 4700 m. In a few cases, they appear in small humid mountain refugia with poorly drained soils surrounded by high Andean forest. Whether they are isolated by deep Andean valleys or by dense forest, biogeographically páramos function like islands and are often referred to as “sky islands”.
Cuatrecasas (1934) classified the páramos in three zones based on the type of vegetation and the altitudinal gradient: 1) subpáramos or low páramo, have thickets and shrubby vegetation (~3000–3600 m); 2) páramo proper, are open grasslands of heliophilous plants (~3600–4200 m); and 3) superpáramo, the highest plant-life zone of these neotropical mountains, with soil affected by frequent frost and scarce psychrophilic vegetation (~4200–4800 m). The páramos have been the stage for great diversification of several groups of organisms, including amphibians, mosses, and vascular plants (Diazgranados 2015; Fernández-Alonso 2002; Madriñán et al. 2013; Rangel-Ch. 2000), in a short period of time (less than 4 my BP; Cuatrecasas 1986; Hooghiemstra & Van der Hammen 2004; Madriñán et al. 2013; Van der Hammen & Cleef 1986). Thus, they are an ideal system to understand rapid adaptive radiations and speciation mechanisms in sky islands.

Frailejones (the name used in this work to refer to all species within subtribe Espeletiinae Cuatrec. (Asteraceae: Millerieae Lindl.)) are the most representative and iconic plants of the páramo. They are all 144+ species distributed in eight genera (Fig. 1): Carramboa Cuatrec. (4 spp.), Coespeletia Cuatrec. (8), Espeletia Mutis ex Humb. & Bonpl. (72), Espeletiopsis Cuatrec. (23), Libanothamnus Ernst (11), Paramiflos Cuatrec. (1), Ruilopezia Cuatrec. (24) and Tamania Cuatrec. (1) (Diazgranados 2012a; Diazgranados & Morillo 2013; Diazgranados & Sanchez 2013). In reality there are many undescribed taxa, for instance the first author is currently describing 14 new species discovered during the course of his field and herbarium work. Despite the interest in the frailejones, there is considerable controversy about the classification within the subtribe, and many of the genera were not resolved as monophyletic in earlier studies (Panero 2007; Rauscher 2002; Sklenár et al. 2005). The subtribe has enormous morphological variation (Fig. 1), including numerous synapomorphies: spiral leaf phyllotaxis; obpyramidal to prismatic shape of the glabrous and epappose cypselae; fertile female ray flowers and functionally male disc flowers; pluriseriate involucre and persistent pales of the receptacle; thick and woody stems; xeromorphic structure; specialized life-forms; and a static chromosome number (n=19).
Frailejones are widely distributed and abundant in the high Andean forest and páramos of Colombia (88 spp.) and Venezuela (68 spp.); only one species occur in northern Ecuador, with an isolated population in the Sierra de Llanganates (Diazgranados 2012a; Diazgranados 2013). Although there are some species endemic to the high Andean forests, and a few that can succeed at altitudes as low as 1300 m or as high as 4780 m, most of the taxa (104 spp.) are found between 3200–3400 m of altitude (Diazgranados 2012a). There are three apparent centers of species diversity: Mérida (with 44 spp.) in Venezuela, Santander–Norte de Santander (41 spp. combined) and Boyacá (45 spp.) in Colombia (Cuatrecasas 1986; Diazgranados 2012a).

Local endemism at the specific level is extremely high (ca. 90%), possibly as a result of island-like radiation on a continental scale (Hughes & Eastwood 2006). Most of the species are gregarious and often represent more than 40% of plant cover (Luteyn 1999; Rangel-Ch. 2000). Seeds lack a pappus or other disseminating device and are dispersed mainly by gravity. According to Cuatrecasas (2013), wind or animals may disperse the cypsela short distances but never more than 1–3 m from the parent plant, although light rains and small streams may disperse them longer distances. Bees (principally species of Bombus, Colletes and Apis) are the most frequent pollinators of Espeletiinae and no long-distance pollinators are known (Berry & Calvo 1989; Berry & Calvo 1994; Fagua & Gonzalez 2007; Sobrevila 1988). Hence, both pollination and seed dispersal suggest that there is a strong isolation by distance among different páramos, which are normally separated by several kilometers of areas unsuitable for frailejones species.

The subtribe has been recently circumscribed within the tribe Millerieae Lindl., as part of the Heliantheae Alliance (Baldwin 2009; Baldwin et al. 2002; Panero 2007). There is no agreement regarding Cuatrecasas’ classification within the subtribe. The monophyly of the subtribe was confirmed based on nrDNA ITS with 14 frailejones species and 51 outgroups (Rauscher 2002). According to this work the closest relatives are Rumfordia DC., Ichthyothere
Mart. and *Smallanthus* Mack.

Speciation of the group started very recently, most likely during the late Pliocene or early Pleistocene (2–4 my BP) (Cuatrecasas 1986; Hooghiemstra & Van der Hammen 2004; Torres et al. 2013; Van der Hammen & Cleef 1986), and it is likely an ongoing process. Due to this remarkable diversity, which appears to have evolved over a relatively short period of time, the group has been considered a classic example of rapid radiation in the tropics (Cuatrecasas 1986; Monasterio & Sarmiento 1991; Rauscher 2002), although this hypothesis has never been rigorously tested. Expansions (with reconnection) and contractions (with isolation) of the páramo ecosystem during Pleistocene glaciations and inter-glaciations could have played a major role in the radiation and dispersion of these taxa (Cuatrecasas 2013).

Two general hypotheses for the radiation of frailejones can be proposed:

1) Upward migration → horizontal migration/expansion → speciation

Ancestors colonized the high altitudes of the mountains finding available niches and subsequently expanded their distributions occupying different areas of their fundamental ecological niche. In this scenario, distant high-altitude species would be more closely related to each other than to geographically closer lower altitude species and overall morphology would reflect synapomorphies rather than convergence.

2) Horizontal migration/expansion → upward migration → speciation

Ancestors of the different genera migrated horizontally (i.e. at the same altitude), probably during the Pleistocene glaciations, followed by vertical migrations (upward in altitude) with subsequent allopatric speciation events. In this scenario, phylogenetic clustering (with closely distributed species more related to each other) would be more common; morphology with frequent homoplasy would be the dominant mode of evolution.

Of course it is possible that it was not one or the other but rather a combination of the two previous hypotheses. With more than 22–24 known Pleistocene glaciations and inter-glaciations it
is plausible that radiation could have followed this pattern, with downward migration, hybridization and introgression during the glaciations, and upward migration and allopatric speciation during the inter-glaciations. A well-resolved phylogeny can provide information to test these hypotheses. Although there have been some previous attempts to reconstruct the evolutionary history of this group based on morphological (Cuatrecasas 1976; Cuatrecasas 2013) and molecular data (Rauscher 2002; Rauscher 2000; Sánchez 2005b), relationships among genera and species remain largely unresolved. We present here the most complete phylogeny to date for frailejones and discuss Cuatrecasas’ generic classification and the influence of geography in shaping the evolution of this group, in a first attempt to understand the processes underlying this spectacular radiation.

MATERIALS & METHODS

Taxon sampling
During major expeditions beginning in 2007, ca. 70 páramo locations of Colombia and Venezuela were visited to photograph, collect and geo-reference frailejones species. A total of 555 samples (MDC 3537–4092) from ca. 110 species were collected (Table 1), following standard protocols for herbarium and molecular analyses (voucher are in ANDES, COL, HECASA and to be distributed (Thiers [continuously updated])). Collections were made under permits No. 2698 of 09/23/2009 and No. 2 of 02/03/2010 (Ministerio de Ambiente, Colombia), and IE-126 (Venezuela, authorized by Petr Sklenář). Notable collections included 14 new taxa (two already published: Coespeletia palustris Diazgr. & Morillo and Espeletiopsis diazii Diazgr. & L.R.Sánchez), the first report of the genus Ruilopezia for Colombia (with R. cardonae (Cuatrec.) Cuatrec.), several new reports for localities, numerous putative hybrids, a few species previously
thought to be extinct, and the identification of several critically endangered species (Diazgranados 2012a; Diazgranados 2013; Diazgranados 2015; Diazgranados & Morillo 2013; Diazgranados & Sanchez 2013). Additional material was obtained from specimens at MO, US and F. *Espeletia pycnophylla* Cuatrec., the sole species found in Ecuador, was collected in the south of Colombia near the border with Ecuador, eliminating the need for field work in Ecuador.

**DNA purification**

DNA extraction from frailejones is particularly complex because of the abundant indumentum of the leaves and the high concentration of terpenes and other metabolites. Extractions from pubescent tissue yield degraded DNA. Adult leaves with old indumentum are sometimes contaminated by fungi. Therefore, young developing leaves from the center of the rosette were use for this purpose, shaving the tissue part of interest. We highly recommend shaving the leaves *in situ*. An initial set of 16 species was used for high quality large scale extractions with the CTAB protocol (Doyle & Doyle 1987) followed by purification via cesium chloride gradients. Subsequently the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) was used, optimizing the Qiagen protocol (2006) to obtain comparable quality. Modifications to the manufacturer’s protocol included the following: 1) after shaving and grinding the leaf fragments under liquid nitrogen, ground tissue is incubated for 24 hours with 400 μl of buffer AP1, 4 μl of RNase, 60 μl of 2-β-mercaptoethanol, 60 μl of proteinase-k and 5 μl of sodium dodecyl sulfate (SDS) at 20% (w/v), inverting the tubes a few times during incubation; 2) after addition of buffer AP2, the mix is incubated on ice for one hour; and 3) when indument was not totally removed, a double amount of reagents was used. After checking for DNA quality and protein content through spectrophotometry, extracted DNA was cleaned using the following procedure: 1) incubation for 30 minutes at 37°C with 0.1% (w/v) SDS, 20 μl/ml of proteinase-k; 2) precipitation by incubation for 30 minutes at –20°C with 5 μl 3M of NaAc and 100 μl of EtOH 95%; 3) centrifugation at
14000 rpm for 15 min; 4) removal of the upper phase by inversion and lyophilization; and 5)
resuspension with 50 μl of AE buffer. The ingroup included DNA extracted from 240 samples,
representing all eight genera and 140 species (including various new taxa being described, and
*Tamananthus crinitus* V.M.Badillo); the outgroup comprised 40 samples from 26 species of the
following genera: *Ichthyothera, Polynamia, Rumfordia* and *Smallanthus*. Available Genbank
sequences (14 ingroup and 51 outgroup sequences for ITS generated by Rauscher; 2002) were
compared to the sequences obtained here.

**PCR amplifications for sequence data**

A combined approach of using DNA sequence data from three lines of evidence (cpDNA, nrDNA
and AFLPs) was used in this project. Preliminary screening of chloroplast and nuclear regions
was performed with 24 samples from species covering the entire geographic range of the
subtribe. Sánchez (2005b) explored 18 chloroplast regions based on Shaw’s recommendations
(2005; 1998) for 24 frailejones species, but none of the amplified regions was of utility to
establish well-supported relationships at generic and species levels. We screened seven additional
regions reported as highly variable (Shaw et al. 2005; Shaw et al. 2007; Timme et al. 2007a):
*rpoB-trnY, ndhC-trnV, trnL-rpl32, rpl16, rps16, ycf6-psbM and trnG*. The variable region *trnS–
trnG* has an inversion in Asteraceae that prevents amplification (Jansen & Palmer 1987). Initial
screenings showed *rpl16* as promising, with 29 haplotypes in 47 sequences. Therefore this region
was amplified for phylogenetic reconstructions, using the primers by Small et al. (1998): F71 (5’-
GCTATGCTTAGTGAGACTCGTTG-3’) and R1516 (5’-
CCCTTCATTCTTCCTCATGTTG-3’).

The nuclear ribosomal internal transcribed spacer (nrITS) has been used widely to
reconstruct phylogenies in several groups of Asteraceae (Blöch 2010; Friar et al. 2008;
Gruenstaeudl et al. 2009; Keeley et al. 2007; Morgan et al. 2009; Schilling & Panero 2011; Vaezi
Rauscher (2000) conducted extensive work in the Espeletiinae with nrITS, using 169 accessions from 15 ingroup and 51 outgroup species. He found a level of variation between 0–4.5%, with no detected intraspecific variation in numerous species. He cloned all accessions and found that 20% of these had only one polymorphic nucleotide position (two haplotypes), while 25% had nucleotide polymorphisms at more sites, suggesting a certain level of within-individual variation. However, he reported that variants typically coalesced within the population and/or species, with no effect on phylogenetic inference; furthermore, several species yielded exactly the same sequences. To build upon Rauscher’s work, ITS1–5.8S–ITS2 (ITS, hereafter) was also used, with the universal primers: ITS-1AF (5’-TCCTTCCGCTTATTGATATGC-3’) and ITS-4R (5’-GGAAGTAAAAGTCGTAACAAGG-3’) (White et al. 1990). We tested for the presence of pseudogenic ITS regions as a consequence of incomplete concerted evolution based on the identification of the conserved 5.8S motifs (CGATGAAGAACGTAGC, GAATTGCAGAATCC and TTTGAACGCA) and a GC% comparison across all the sequences (Harpke & Peterson 2008a; Harpke & Peterson 2008b). No pseudogenic copies were found.

Additional nuclear DNA regions, including single copy genes, were screened without success, either because of very low variability (e.g. gapC), high complexity and multiple bands (e.g. leafy, pepC), or unsuccessful amplifications (e.g. waxy). However, the external transcribed spacer region (ETS) of the nuclear 18S-26S ribosomal repeat showed fairly good phylogenetic resolution power. Numerous studies in related groups have used ETS for reconstructing phylogenies (Baldwin & Markos 1998; Clevinger & Panero 2000; Ekenäs et al. 2007; Garcia et al. 2011; Masuda et al. 2009; Mavrodiev et al. 2008; Moore et al. 2012; Morgan et al. 2009; Schilling & Panero 2011; Soltis et al. 2008; Timme et al. 2007b; and others; Wahrmund et al. 2010). Timme et al. (2007b) reported a large region of one to five subrepeats (each of ~250 bp) in the ETS for Helianthus L., with intraspecific variation, evidenced by multiple bands during gel
electrophoresis. Although we did not find multiple bands in frailejones, we did find interspecific variation in the number of subrepeats. The length of this region in Espeletiinae is between ~1450–2500 bp. Therefore, in addition to external primers, internal primers were used: ETS1f (5’-CTTTTTGTGCATAATGTATATATAGGGGG-3’), ETS2f (5’-CTGAGCCCCACTTCGTTTGC-3’), 11r (5’-CAAACAAACACCACCTCATGCACC-3’), and 18S2l (5’-TGACTACTGGCAGTCAACCAG-3’) (Timme et al. 2007b). Three additional internal primers were developed for this study: ETS3f (5’-GASCTGACGAAGTACCCATGA-3’), ETS4f (5’-CTCAATGGGCCACAACATC-3’) and 10r (5’-CGGGTGGCTAATTGTTGG-3’).

All polymerase chain reactions (PCR) were carried out in a final volume of 25 μl. Reactions were prepared with reagents from the GoTaq DNA polymerase kit (Promega, Madison, WI, USA), with 0.5 μl of template DNA (~10–100 ng), 5 μl of 5X Green GoTaq Reaction Buffer, 2.5 μl of MgCl$_2$ (25 Mm), 1.5 μl dNTPs (10 mM), 0.5 μl of each primer (10 mM), 0.25 μl of GoTaq DNA polymerase, 1.5 μl of DMSO and 0.5 μl of bovine serum albumin (BSA; 0.1 μg/μl).

Amplifications were performed using Eppendorf (Westbury, New York, USA) Mastercycler gradient ep thermal cyclers. Cycler programs were: for rpl16: denaturation at 94°C for 2 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 65°C for 4 min, followed by a final extension at 65°C for 10 min; for ITS: denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 20 s, annealing at 54°C for 35 s, and extension at 72°C for 45 s, followed by a final extension at 72°C for 7 min; and for ETS: denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1.5 min, followed by a final extension at 72°C for 5 min PCR products were checked on 1% agarose gels before being cleaned with the QIAquick PCR purification kit (Qiagen, Valencia, California, USA). PCR products were sent for sequencing to Macrogen Inc. (Seoul, South Korea).

Consensus sequences were assembled in Sequencher™ 4.2, and aligned manually using Se-Al
Concatenation of matrices and final editing of nexus files were conducted in Mesquite 2.75 (Maddison & Maddison 2011). Sequences are available in GenBank, with accession numbers: ETS: KY231675–KY231818, ITS: KY231383–KY231532 and rpl16: KY231533–KY231674. GenBank accession numbers and voucher information per sample are provided in Table S1.

**AFLP amplifications and genotyping**

In addition to sequence data, amplified fragment-length polymorphisms (AFLPs) were used to infer relationships among the species, and particularly to resolve polytomies or nodes with low or no support by sequence data. AFLPs are variable markers typically used for fingerprinting, estimating relatedness, genetic mapping and more recently for reconstructing phylogenies (Avise 2004; McKinnon et al. 2008; Meudt & Clarke 2007; Vos et al. 1995). Numerous phylogenetic studies in recently radiated groups have used AFLPs (Jabaily 2009; Koopman et al. 2008; McKinnon et al. 2008; Schmidt-Lebuhn 2007; Schmidt-Lebuhn et al. 2009; Worley et al. 2009). These markers have also been used successfully in phylogenetic reconstructions in Asteraceae (Koopman 2005; Koopman et al. 2001). Acquisition of AFLP data followed a modified version of the protocol by Trybush et al. (2006). DNA was digested for 1 h at 37°C in 30 µL of total volume with 0.25 µl of EcoRI, 0.5 µl of MseI, 3 µl of 1x NEB buffer 4 (New England Biolabs, Ipswich, MA), 1.5 µg of BSA and 2 µl of template DNA. Each pair of EcoRI and MseI adaptors was annealed after incubation for 10 min at 65°C, and allowed to cool very slowly for 4 h. Ligation included the 30 µl digestion mix plus a 10 µl mix containing 1.5 µl of T4 DNA ligase (60 U), 1 µl of ATP, 1 µl of 1x NEB buffer 4, 0.5 µl of the annealed EcoRI adaptors, 5.0 µl of the annealed MseI adaptors and 1 µl of ddH₂O. The ligation reaction was incubated at 37°C for 4 h 15 min. The digested-ligated mix was 10-fold diluted. Pre-amplifications were prepared in a total volume of 10 µl, with 2 µl of 5X Green GoTaq Reaction Buffer, 1 µl of MgCl₂, 0.25 µl of dNTPs (10
mM), 0.05 μl of each pre-selective primer (EcoRI primer +A and MseI primer +C), 0.6 μl of DMSO, 0.2 μl of BSA 1X, 2.5 μl of the digested-ligated template DNA, 0.1 μl of GoTaq DNA polymerase and 2.35 μl of ddH2O. Thermocycling was performed with a first step of enzyme denaturation at 65°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. PCR products with pre-amplified DNA were 20-fold diluted. Selective amplifications were performed in 10 μl reactions with the same quantities and reagents as for the pre-selective PCR, except for the addition of 0.07 μl of each selective primer (EcoRI primer +3b and MseI primer +3b) and only 1 μl of pre-amplified DNA. Selective reactions included denaturation at 95°C for 15 min, 13 cycles of 30 s at 94°C, 1 min at 65°C with a ramp temperature of –0.7°C/cycle, and 1 min at 72°C; then 25 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C; and a final extension of 10 min at 72°C.

WellRED D2-, D3- and D4-PA fluorescent primers (Sigma-Aldrich, St. Louis, MO) were used for selective amplifications. In total, 36 primer combinations were tested following recommendations for Helianthus (Applied Biosystems 2007). Five combinations failed to amplify (selective 3b for EcoRI-selective 3b for MseI: ACT-CAC, ACC-CAG, ACT-CAG, AAC-CAT and ACT-CAT), and three primer combinations showed only weak amplification (ACG-CAA, ACG-CAC and ACT-CAA). The remaining 28 primer combinations were used for genotyping (Table 2). Results from each step were checked by electrophoresis on 1.5% agarose gels. Each 96-well plate contained 81 samples, of which 12 (~15%) were replicated. In addition, 12 samples were repeated between plates and where possible, multiple samples were included per species. Genotyping was performed on a Beckman-Coulter CEQ8000 sequencer, multiplexing the reactions with the three WellRED dyes, in a final volume of 10 μl. Scoring was conservative, with only the strongest allele peaks scored. Alleles not consistent between replicated samples or between different runs for the same sample were discarded. Minimum bin (peak) width was set to 1 base (b). Bins of < 60 b were eliminated. To determine minimum fragment size and minimum
intensity threshold, multiple partitions were tested by measuring homoplasy and other tree
descriptors (see Table 3). Since a preponderance of phylogenetic signal was found in sizes
between 60 and 100 b, the minimum size of 60 b was retained. The minimum intensity threshold
with phylogenetic signal was established at 1000 relative fluorescent units (RFU). From an initial
total of 5551 fragments, only 1665 were retained. Samples with poor amplifications were
discarded. In total, 118 ingroup and 20 outgroup taxa were genotyped.

Phylogenetic reconstruction

Phylogenies based on sequence data were reconstructed under maximum parsimony (MP),
maximum likelihood (ML) and Bayesian inference (BI). The AFLP dataset employed minimum
evolution (ME) analyses. Trees were rooted with *Smallanthus*, *Ichthyothere* and *Rumfordia*
species, based on Rauscher (2002). MP analyses were performed in PAUP* version 4.0b10
(Swofford 2002). For all analyses, gaps were treated as missing data and ambiguous positions
were excluded. Full heuristic searches were configured with MAXTREES set to 100,000, with
the tree-bisection-and-reconnection (TBR) branch swapping algorithm, 10 random additions, and
holding 1 tree at each step. Non-parametric bootstrap searches were estimated with 100,000
replicates and MAXTREES set to 1. For ML and BI reconstructions, evolutionary models were
selected using the AIC criterion in JModelTest 0.1.1 (Posada 2008), which uses the Phyml
algorithm to test 88 models in large phylogenies by maximum likelihood (Guindon S 2003). ML
reconstructions were performed in Garli 2.0.1019 (Zwickl 2006) at the the CIPRES Science
Gateway V. 3.1 (http://www.phylo.org/sub_sections/portal/). The candidate model of evolution
selected via AIC by JModelTest was established for the ML searches without optimization,
allowing Garli to infer parameter values for each model. Each data set was analyzed in eight
independent runs. Bootstrap analyses were performed with 120 replicates and 8 independent runs.
The CIPRES portal allows a maximum of 100 replications×runs, therefore each bootstrap
analysis was performed in 10 tasks of 12×8. Bayesian inference was obtained in MrBayes v3.1.2 implemented also at the CIPRES portal. As with ML analyses, models of evolution were tested for each partition. Settings included 2 runs, 8 independent chains, temperature factor of 0.05, 50,000,000 generations sampling every 5,000 trees, and discarding the first 8,000 trees as burn-in. Data sets containing AFLPs were run for 150,000,000 generations with a temperature factor of 0.005 and a stopping rule when convergence reached 0.01, sampling every 5,000 trees and discarding the first 17,500 trees. ME analyses were performed in PAUP* with the Nei-Li distance (fragments), a starting tree by neighbor joining, TBR branch swapping and MulTrees on. Bootstrap searches had 10,000 replicates. Partitions (ETS, ITS, rpl16 and AFLPs) were analyzed both separately and combined. One-tailed Shimodaira-Hasegawa (SH) tests (Shimodaira & Hasegawa 1999) were conducted in PAUP* to compare between topologies, and incongruence length difference (ILD) tests (Farris et al. 1995), implemented as partition homogeneity in PAUP*, were used to assess combinability of partitions. The ILD test was run with 100 replications and 10 random additions. Bootstrap support values and posterior probabilities were summarized with the function Sumtrees implemented in DendroPy (Sukumaran & Holder 2010). Concatenation of support values was performed with the function Sumlabels in the same program. FigTree v1.3.1 (Rambaut 2009) was used to edit the trees. A Monte Carlo permutation test with 1000 permutations, as implemented in GenGIS (Parks et al. 2009), was used to test for the influence of the geography on the phylogeny. Two additional analyses were run to further verify the resulting topologies: an unrooted phylogenetic network (split network) on the ETS-ITS-rpl16 data set, with the NeighborNet algorithm as implemented in SplitsTree4 (Huson & Bryant 2006); and a haplotype network based on rpl16, with equal weighting on transversions/transitions, the median-joining (MJ) network algorithm, and "Connection cost" distance method, built with Network 4.6.1.1. (Fluxus Technology Ltd. 2012). The split network displays simultaneously all the possible networks, without giving any hierarchy. Haplotype
networks, despite phylogenetic approaches, use autapomorphic characters (unique mutations) for the calculations.

**RESULTS**

Field collections included ~75% of the recognized taxa. During the development of this project 17 new species were published by other researchers (Díaz-Piedrahita & Rodriguez-Cabeza 2008; Díaz-Piedrahita & Rodriguez-Cabeza 2010; Díaz-Piedrahita & Rodriguez-Cabeza 2011; Díaz-Piedrahita et al. 2006; Diazgranados & Morillo 2013; Diazgranados & Sanchez 2013). Most of these newer taxa remain unsampled in the field by us, and fragments from herbarium specimens could not be amplified due to the preservation techniques used when they were collected. A few species known from only a single collection have not been found in decades (e.g. *Espeletia canescens* A.C.Sm., *E. marnixiana* S.Díaz & Pedraza, *E. miradorensis* (Cuatrec.) Cuatrec., *E. tapirophila* Cuatrec., *E. tillettii* Cuatrec., *Espeletiopsis trianae* (Cuatrec.) Cuatrec., *Ruilopezia usubillagae* Cuatrec., etc.); furthermore, those species were collected in areas that are currently politically unstable or with very difficult access. *Tamananthus crinitus*, included in the subtribe by Panero (2007), is known from only one poorly preserved specimen, glued to the herbarium sheet (US). DNA amplifications for *T. crinitus* were unsuccessful, but amplifications for AFLPs were fruitful. Two recognized hybrids (Diazgranados 2012a) with clean sequences were included in most of the data sets: *Espeletiopsis ×bogotensis* (Cuatrec.) Cuatrec. (= *Espeletiopsis corymbosa* Bonpl. × *Espeletia grandiflora* Bonpl.), and *Espeletiopsis ×cristalinensis* (Cuatrec.) Cuatrec.

Success in DNA purification varied from taxon to taxon. In general, we found that extractions are particularly difficult from species with leaves that are highly tomentose (e.g. *Espeletia paipana* S.Díaz & Pedraza) or extremely coriaceous (*Espeletiopsis jimenez-quesadae*...
Sequences were not successfully obtained from all samples for all markers. Therefore, combined data sets vary in the number of the species to secure the maximum possible coverage. Statistics for all data sets are shown in Table 3 and sequences used for reconstructions are shown in Table 1S. The chloroplast region showed very little interspecific and intergeneric variation within the subtribe. Only 35 (3.6%) characters were informative, including outgroups. Within the subtribe (after excluding uninformative characters), many species even from different genera share exactly the same sequence in the alignment. Trees from rpl16 (not shown) had little resolution, with only a few clades recovered in the strict consensus tree: one clade including the Venezuelan species and one clade comprising the Colombian species (including *Espeletia pycnophylla*, present in both Colombia and Ecuador).

Most ITS sequences were clean and easily readable, showing only one band in gels and none or only a few double peaks; these positions were coded using IUPAC notations. Noisy sequences were re-amplified, and subsequently discarded if the problem persisted. For our data sets, including outgroups (13 species), 173 (26.7%) characters were informative; excluding outgroups, only 90 (13.9%) characters were informative. The ingroup for ITS included 119 species of frailejones (84.4%). Resolution based on ITS was better than rpl16 but still insufficient to resolve the phylogeny (tree not shown). The subtribe was recovered as monophyletic, in agreement with Rauscher (2002). Clades comprising Colombian and Venezuelan species were also recovered, but with low support. In the ML tree the following genera were recovered in clades with low support: *Carramboa* species, *Libanothamnus* species including *E. ×cristalinensis*, most of the *Ruilopezia* species including the monotypic *Tamania*, and two clades of Colombian *Espeletiopsis* nested within a big clade of Colombian *Espeletia*.

ETS proved to be much more informative than ITS. However, amplification was difficult due to the internal repeats. Outgroups have a very large indel (~700 bp) of repeats in the 5’ end. In *Carramboa* species, this indel is reduced to ~560 bp, whereas in most of the remaining
Venezuelan species (except for the *Libanothamnus* taxa) the indel is interrupted by four or five conserved regions of ~28 bp, so that the large indel is replaced by 5-6 smaller indels of ~84 bp. These indels disappear in most of the Colombian species and *Libanothamnus*. A second section (~300 bp) downstream with two additional large indels extends from there. This second section is very difficult to amplify due to the previous repeats. Therefore, this entire section was excluded from the analyses. Sequences in the 3’ end are, by contrast, highly conserved. The complete ETS region varies from ~1450–2500 bp. The final alignment did not include the far 3’ end because of low variability. Instead, the region between primers ETS1f and 11r (including internal primers) was used (1324 characters in alignment). Coverage included 151 sequences comprising 119 species. As with ITS, samples with multiple bands or noisy sequences were discarded. Contig assemblage included as many as 12 single stranded sequences, and samples were often re-amplified to verify sequences. Bootstrap analyses for the ML tree for ETS produced 64 nodes with support >60%, and 25 nodes with support >90%.

The best ME tree for the AFLP data had a score of 3.39528, and the length of the shortest ME tree was 10,490 steps. The data set showed a very large amount of homoplasy (HI: 0.841), but phylogenetic signal was evident. A phylogeny based on AFLPs resolves the subtribe as monophyletic, although with support values <50 (Fig. 2). *Tamanathus crinitus*, included as part of the ingroup, is placed within the outgroup, supporting the exclusion of this genus from the subtribe (Diazgranados 2012a). A number of clades have good support; however, there is no support for the backbone of the tree. An unsupported clade of four species, two from Colombia and two from Venezuela, is sister to the rest of the Espeletiinae. This result could be a product of homoplasy or of incomplete amplifications for those samples due to DNA quality. The tree shows two large reciprocally monophyletic clades with distinct geographic structure. One clade contains two smaller clades: one with primarily Venezuelan species and a few neighboring Colombian species; and other containing northern Colombian species (Fig. 2). Within the Venezuelan clade,
Carramboa badilloi (Cuatrec.) Cuatrec. (three samples of two varieties) appears to be monophyletic. Carramboa rodriguezii (Cuatrec.) Cuatrec. falls in a sister clade but with no support. Carramboa trujillensis (Cuatrec.) Cuatrec. is grouped with a sympatric species, Libanothamnus griffini (Ruiz-Terán & López-Fig.) Cuatrec. with high posterior probability (PP). Tamania appears to be sister to a few Ruilopezia species. Most of the Libanothamnus species form a clade containing some nodes with fair support. The smaller clade of northern Colombian taxa comprises species restricted to Santander and Norte de Santander, two states bordering Venezuela, with the exception of two species (Espeletia frontinoensis Cuatrec. and E. praefrontina Cuatrec.) from the extreme northwest of Colombia. The second large clade within the subtribe contains only Colombian species, from Boyacá down to the limits with Ecuador. Most of the species within this clade belong to Espeletia, except for seven species of Espeletiopsis and the monotypic Paramiflos. Some species (e.g. Espeletia lopezii Cuatrec., E. argentea Bonpl., Espeletiopsis pleiochasia (Cuatrec.) Cuatrec.) show strong support for their monophyly.

The partition homogeneity test (ILD test) suggested some incongruence between the nuclear ribosomal regions (p=0.01), and between nuclear ribosomal and chloroplast regions (p=0.14). However, topologically these trees are almost identical, with only a few conflicting positions (Fig. 3 and 4). In both trees combining sequence data, the monophyly of the subtribe is strongly supported with a clear geographic structure, in general agreement with the phylogeny based on AFLPs. The tree based on the three regions (ETS-ITS-rpl16) showed increased support for most nodes, and more resolution than the trees based solely on nrDNA data or on individual partitions. In this tree, numerous clades are well supported. Coespeletia species (except for C. moritziana (Sch.Bip. ex Wedd.) Cuatrec.) fall in the same clade along with the sympatric Espeletia semiglobulata Cuatrec. and E. cuniculorum Cuatrec., all from the superpáramos of Mérida (Venezuela). The tree recovers a clade containing Espeletiopsis pannosa (Standl.)
Cuatrec. and *E. angustifolia* (Cuatrec.) Cuatrec., two Venezuelan species with sericeous silvery indumentum and white-purplish flowers, and *Ruilopezia floccose* (Standl.) Cuatrec., another species with silvery indumentum. Three similar *Ruilopezia* species (*R. marcescens* (S.F.Blake) Cuatrec., *R. lindenii* (Sch.Bip. ex Wedd.) Cuatrec. and *R. leucactina* (Cuatrec.) Cuatrec.) are grouped in a clade separated from the rest of the species of the genus. *Espeletia schultzii* Wedd. forms a well-supported clade with *E. aristeguietana* Cuatrec. and *E. jajoensis* Aristeg. Another clade of very similar species is recovered: *Espeletia ulotricha* Cuatrec., *E. nana* Cuatrec. and *E. marthae* Cuatrec. The latter species are all very small plants from rocky páramos, with simplification of synflorescence, small leaves and rectangular sheaths. Most of the *Ruilopezia* species form a clade, with *Tamania* nested within. The monophyly of *Libanothamnus* is supported, although the clade contains *R. cardonae*, the southernmost species of the genus, and *Espeletiopsis ×cristalinensis*. The Colombian clade is only well-supported by posterior probability. A clade with most of the species of *Espeletiopsis* is recovered. *Paramiflos* is nested within a small *Espeletiopsis* clade. Several small clades geographically meaningful are also supported.

A tree reconstructed with all the available molecular data (sequence data and AFLPs) loses some resolution, and some nodes with very low support collapse (Fig. 5). However, the base of the Espeletiinae clade is more resolved, showing two large clades: one of primarily Venezuelan species that includes a few neighboring Colombian species; and a second clade of only Colombian species, depicting a shallower resolution. Despite some loss of resolution, the geographic structure is still obvious even in the Colombian clade. Furthermore, the topology is congruent with trees generated only with sequence data, and all the small clades described previously are retained. Both the split network based on ITS-ETS-rpl16 (Fig. S1) and the haplotype network with rpl16 (Fig. S2) show this striking geographic structure. It is particularly interesting to note in the haplotype network that one cluster of haplotypes show only Colombian
taxa, while the other cluster includes all the Venezuelan species, a few Colombian species and some haplotypes shared by taxa from the two countries. Lastly, a Monte Carlo permutation test performed with the ML reconstruction suggests a very strong influence of the geography on the phylogenetic relationships (p=0.00, Fig. 6).

**DISCUSSION**

**Molecular markers**

Evolutionary relationships within numerous recent rapid radiations in animals and plants are still obscure because of the low phylogenetic signal of conventional markers. An exhaustive screening of 25 chloroplast regions for frailejones has been carried out in this work and by Sánchez (2005a), with unsatisfactory results. The only chloroplast region to show usable variation was rpl16 and therefore it was selected to investigate sequence variability throughout the entire subtribe. With a length of 962 bases when aligned, only 3.5% were informative characters, which was insufficient to resolve the phylogeny. ITS was more variable, but only large clades were recovered but with low support. ETS turned out to be a much more informative region. According to Baldwin and Markos (1998), ETS is part of the same transcription unit as the ITS region and consequently should not be regarded as an independent line of phylogenetic evidence for comparison with ITS results. These authors affirmed that ETS can fulfill the need for additional nucleotide characters to augment the phylogenetic signal of ITS in young angiosperm clades.

The partition homogeneity test (ILD test) suggested some levels of incongruence between these ITS and ETS, and between the nuclear ribosomal and chloroplast markers used (p=0.01 and 0.14, respectively). Results from this test, however, can be affected by the disparity in levels of homoplasy between the data sets, as reported by Dolphin et al. (2000). Numerous papers have discussed the limitations of the ILD to measure incongruence among data partitions (Barker &
Lutzoni 2002; Dowton & Austin 2002; Planet 2006; Quicke et al. 2007). Imbalance between partitions is evident in this case (Table 3). Excessive type I error for the ILD test as a measure of combinability has been reported and a critical value of 0.001 has been proposed (Cunningham 1997). Therefore, the ILD test should not be used as a “hard” method to decide about combinability, but as an approach to explore congruence. On the other hand, the utility of topological tests is questionable when trees from some partitions recover only a few clades with low support and no support whatever for the remaining groups (e.g. rpl16).

The tree based on all available molecular evidence preserves the geographic structure for the most part, retaining nodes with high support. Moreover, it resolves a split at the base of the subtribe, showing two clear clades containing Venezuelan and Colombian species. Both the split network (Fig. S1) and the haplotype network (Fig. S2) show similar results. Results of this project indicate that increasing the amount of data can help to increase the phylogenetic signal, at least for shallow phylogenies of rapid radiated groups. Data from the latest high-throughput sequencing technologies should facilitate deeper exploration into the origin and evolution of such groups.

**Hybridization and evolution of frailejones**

Hybrid speciation and reticulate evolution are common processes in plants, and have been reported widely for Asteraceae (Mallet 2007; Moodya & Rieseberg 2012; Nolte & Tautz 2010). It may take several million years after the split of a species pair before the capacity to hybridize is completely lost (Nolte & Tautz 2010). Thus, species of hybrid origin in young groups may be more common than previously thought. A species of hybrid origin maintains allelic combinations that contribute to the spread and stabilization of the hybrid lineage, and it is generally recognized as a species through time (Mallet 2007). Hybridization does not necessarily involve polyploidy in closely related taxa; there are other mechanisms that facilitate rapid hybrid speciation in
sympatric or parapatric species, such as transgressive segregation and ecological hybrid speciation (e.g., sexual selection; (Seehausen 2004).

Hybridization is a frequent natural process across the Espeletiinae and it has been reported in a number of sources. Diazgranados (2012a) states that when two or more sympatric or parapatric species of frailejones occur, hybridization usually happens and he has documented this with a listing of the 33 published hybrids. In addition, individuals that hypothetically have introgressed with three species have been documented and numerous hybrid zones have been reported for the páramos of Mérida (Morillo & Briceño 2007; Rauscher 2000). It is also possible that a few putative species with unknown populations are hybrids. Cuatrecasas (2013) recognized the putative hybrid origin of a few taxa, and Rauscher (2002) confirmed eight different natural hybrid crosses involving 12 species. Hybridization has been proposed as an important mechanism in adaptive radiations (Seehausen 2004) and it appears that it is important in the Expeletiinae as well.

The rapid radiation of frailejones could be explained by hypotheses of both allopatric speciation and hybrid swarm origin. The high altitudes of the tropical Andes provided multiple underutilized niches (i.e. fitness peaks) for colonization. If a colonizing species contained sufficient variation at functional loci, it could express multiple fitness peaks simultaneously. If only subsets of these peaks were effectively utilized, different populations of functional genotypes could have emerged rapidly by multiple events of ecological speciation (Schluter 2000). Glaciations would have increased the probability of secondary contact between related species or divergent populations, generating a ‘syngameon’ scenario. Under these conditions, if hybrids had no ecological disadvantage and more functional gene combinations than the parental species, niche partitioning could have favored rapid speciation in sympatric species. Inter glaciations would later segregate populations, favoring allopatric speciation.
Intermediacy or conflicting positions in the phylogenies can be explained by hybridization events or incomplete lineage sorting in recent radiations (Knowles & Chan 2009; Knowles & Carstens 2007). The very large amount of homoplasy found in the AFLPs data (HI=0.841) can be explained as well by the homogenization of hybrid parental genomes at each AFLP locus (Seehausen 2004).

**Generic relationships**

Most of the genera proposed by Cuatrecasas (1976; 1995) are at least partially supported in all trees. *Carramboa* is monophyletic in most trees (Figs. 3–5) except the AFLP tree (Fig. 2), where *C. rodriguezii* is placed (without support) in a different clade that includes a sympatric species, *R. marcescens*. Hybridization occurs frequently between *Carramboa* and other sympatric species (Morillo & Briceño 2007; Rauscher 2000) and it is possible that the very small and patchy populations of this species are hybridizing with *R. marcescens*.

The *Carramboa* clade is subtended by *Ruilopezia josephensis* and *Espeletia weddellii*, both placements with no support (Figs. 3–4). The first species is likely a hybrid (Mavárez, com. pers., based on new genomic evidence), with scattered individuals in only one locality of the Páramo de San José, in sympatry with *Carramboa badilloi* and various species of *Ruilopezia*. *Espeletia weddellii* is a species with a somehow variable morphology that hybridizes frequently (e.g. with *E. marthae* or *E. schultzii*). Therefore its conflicting position with lack of support might be explained by the effect of introgression.

In the trees based on sequence data (Figs. 3–4) and all the available molecular evidence (AME; Fig. 5), *Libanothamnus* is paraphyletic because it includes *Espeletiopsis ×cristalinensis* (not included in the total evidence analysis) and *Ruilopezia cardonae*. However, the former species is a hybrid between *Libanothamnus neriifolius* and *Espeletia aristeguietana* (*Diazgranados 2012a*). With respect to the latter, *R. cardonae* is the southernmost of the
Ruilopezia species, found in an area of less than 1 km² in the Tamá massif. This páramo is separated from the closest páramo in Venezuela by a distance of ca. ~40 km. In between, the Táchira depression forms a deep valley of unsuitable conditions for any Espeletiinae. It is plausible that Ruilopezia cardonae, along with two or three Libanothamnus species, could have crossed this depression during a cold Pleistocene period. Since then, R. cardonae has been evolving isolated from its congeneres, and probably hybridizing with L. tamanus (Cuatrec.). Interestingly, both species share some morphological characteristics, such as very similar leaf tomentum and venation. Ongoing or past hybridization between these species can explain the position of R. cardonae within the Libanothamnus clade.

In the AME reconstruction (Fig. 5), three Coespeletia species form a clade with Espeletia cuniculorum, whereas in the nrDNA (Fig. 3) and sequence data (Fig. 4) trees, E. cuniculorum and E. semiglobulata (not included in Fig. 5) are sister to the Coespeletia clade. Both Espeletia species grow in sympathy with species of Coespeletia and are restricted to the superpáramos over 4000 m. Espeletia cuniculorum is a rare species, known only from a few collections from the Páramo de los Conejos (Mérida), and it may be a hybrid species. Espeletia semiglobulata grows in the same massif, and its epithet refers to the semiglobular capitula, similar to the capitula of all Coespeletia species. Furthermore, E. semiglobulata shares with C. moritziana, C. spicata and C. timotensis characters such as large pendulous capitula, pluri- or multi-(6-7)-seriate short rays, and reduced length of pollen spines. It was originally assigned to Espeletia because of its thyrsoid synflorescence, but placed in its own section (sect. Badilloa Cuatrec., in Cuatrecasas 2013); the molecular evidence suggests that the delimitation of Coespeletia must be revised.

Coespeletia thyrsiformis (A.C.Sm.) Cuatrec. is at the base of the clade with two Coespeletia species and E. cuniculorum in the AME tree (Fig. 5), but the sequence data reconstruction (Fig. 4) shows it in a clade with other four Ruilopezia species and the two Espeletiopsis with white-purplish flowers and thin leaves. It grows in sympathy with species of
Ruilopezia (e.g. R. leucactina) in the Páramo del Batallón, ca. 100 km southwest from the páramos in Mérida, where the other species of Coespeletia are found. While C. thyrsiformis grows at ca. 3230 m (2500–3510 m) of altitude, in the páramo proper, the other species of the genus grow at ca. 4000 m., in the superpáramos. Because of its characteristic thyrsoid capitulescence, with polycephalous peduncles becoming monocephalous, Cuatrecasas (1986, 2013) suggested that C. thyrsiformis could be more related to the ancestor of the genus that was presumably adapted to lower altitudes. Later migration upward with adaptation to extreme cold habitats would have produced the superpáramo species that are known today. However, an alternative explanation is that the species exhibits introgression with the sympatric Ruilopezia species, explaining its confusing position in the phylogeny.

A fifth species, Coespeletia moritziana, is also sympatric with the superpáramo species of Mérida. However, it appears at the base of a larger clade containing five different genera (Figs. 3–5). This conflicting position was also reported by Rauscher (2000) for ITS. He suggested two possible explanations: parallel evolution or hybridization. Coespeletia moritziana shares numerous morphological and phenological characteristics that place it without doubt in the genus Coespeletia. However, it hybridizes frequently, at least with C. timotensis and E. schultzii, and exhibits a plastic morphology. Coespeletia moritziana forms large well-established populations and could be a species of hybrid origin.

Sister to the Coespeletia clade (minus C. moritziana) is a small clade containing Espeletiopsis angustifolia, E. pannosa and Ruilopezia floccosa. These three species share silvery sericeous pubescence and have common names such as ‘frailejón plateado’ (i.e. silvery frailejón). There is no doubt about the relatedness of two of the Espeletiopsis species, both of which have white-purplish flowers and thin leaves. These two species show no close relationship with other species of Espeletiopsis, and a re-classification could be suggested for them. Ruilopezia floccosa grows in sympathy with E. angustifolia, but no large populations are currently known. It is...
peculiar that whereas most of the *Ruilopezia* species live in subpáramo areas at the limit of the forest, *R. floccosa* is adapted to open grass páramos. Further analyses are needed to determine the origin of this taxon.

The remaining species of *Ruilopezia* are grouped in a clade that includes *Tamania* (Figs. 3–5). Both genera have terminal synflorescences. The life form of *Ruilopezia* was defined as a monocarpic caulirosula, i.e. a rosette of imbricated leaves at the end of a straight stem, with a terminal synflorescence (Cuatrecasas 1933; Cuatrecasas 1934; Cuatrecasas 2013). After flowering, the rosette (or the ramet) dies. *Tamania* is a tree with a monopodial trunk but sympodial (pseudodichotomous) branching, and terminal monochasial synflorescences as well. Cuatrecasas (1986) hypothesized a relationship between these two genera. According to him, these two genera and *Libanothamnus*, which also has terminal synflorescences, share a common ancestor. Interestingly, a clade of most of the *Ruilopezia* species + *Tamania* + *Libanothamnus* is well supported in the tree based on total evidence (Fig. 5).

Three segregated species of *Ruilopezia* (*R. leucactina*, *R. lindenii* and *R. marcescens*) form a separate well-supported clade (Figs. 3–5). *Ruilopezia lindenii* and *R. marcescens* share numerous synapomorphies: large broad herbaceous sterile phyllaries (≥20 mm long), densely glanduliferous floral tube with sparse hairs, and very long disk corollas (6–8 mm long). The three species share creamy-white (sometimes light greenish) ray flowers.

The Colombian clade contains only species of *Espeletia, Espeletiopsis* and *Paramiflos* (Fig. 5). *Espeletiopsis diazii*, a new species recently discovered (Diazgranados & Sanchez 2013) is at the base of this clade. This is a unique species, particularly interesting because it shares with some Venezuelan species of *Espeletia* the oblong shape of the sheaths at the base of linear tomentose leaves, among other characteristics. However, the synflorescence is a typical monochasium that places this species under *Espeletiopsis*. Currently found in a remote páramo in...
Norte de Santander, it may be a descendant of one of the first species that migrated from Venezuela.

A clade containing twelve *Espeletiopsis* species suggests the monophyly of the Colombian *Espeletiopsis*. In addition to *E. diazii*, five species (*E. colombiana* (Cuatrec.) Cuatrec., *E. funkii* (Sch. Bip. ex Wedd.) Cuatrec., *E. guacharaca* (S. Díaz) Cuatrec., *E. petiolata* (Cuatrec.) Cuatrec., and *E. pleiochasia*) and one sample of *E. garciae* (Cuatrec.) Cuatrec. fall outside this clade. The positions of these species, however, are either not supported or represent relationships with sympatric species. Rauscher (2000) found two different ITS haplotypes for *E. garciae* and *E. pleiochasia*, suggesting either hybridization or two possible lineages for ITS.

Sister to the large clade of Colombian *Espeletiopsis*, is a large clade of Colombian *Espeletia*, although support for this clade is very low. Species within this last clade are grouped mainly by their geographic location.

One sample of *Espeletia congestiflora* (Coll. number 3651) seems to be nested with *Espeletiopsis diazii* and related to other *Espeletiopsis* taxa sister to the larger clade containing mainly *Espeletia* species (Figs. 3–4). Diazgranados (2012b) initially treated this collection as a new species to be described (tentatively named *Espeletia multicongestiflora* sp. nov. in his dissertation). However, the species has not been described yet, and can fit in the broad description of *E. congestiflora* sensu lato, which will need to be adjusted. Population of *E. congestiflora* 3651 differs mainly from the typical *E. congestiflora* morphology in having capitulescences with 9–12 congested capitula, rather than 3–7, and alternate sterile bracts along the scape, which is typical of *Espeletiopsis* and not *Espeletia*. This putative new species has yet to be studied, and for the moment the collection was placed within the conservative definition of *E. congestiflora*.
Evolution and geography

Our results support a recent rapid radiation of the Espeletiinae, based on a shallow phylogeny with respect to other sister species, a recent origin (less than 2-4 my BP), and a great number of species. Frequent hybridization is unmistakable, and most species may exhibit incomplete lineage sorting. Trees based on sequence data clearly support an origin of the subtribe in Venezuela, as hypothesized originally by Cuatrecasas (1986).

The apparent mixing of genera in the Venezuelan clade can potentially be explained by a much longer history of introgressive hybridization. The relatively longer branches of the Venezuelan species in comparison with the Colombian species suggest older ages of those taxa. Branch length differences between putatively older Venezuelan and younger Colombian taxa (most pronounced in the combined sequence tree; Fig. 4), could perhaps reflect a progression of species migrations in time and space. Longer branches are more common among the species from the massif of the Venezuelan Mérida páramos, while species at the extremes of the geographic range of the subtribe tend to have shorter branches. Thus, the massif of the Mérida páramos could be hypothesized as a putative center of origin for the subtribe. From there, it followed mainly southward migrations along the Andes cordilleras (Cuatrecasas 1986; Cuatrecasas 2013).

The Colombian clade of *Espeletiopsis* (including *Paramiflos*, Fig. 5) shows numerous relationships between sympatric or parapatric species: *E. guacharaca* and *P. glandulosus* (Cuatrecc.) Cuatrecc. in the páramo de la Rusia (Boyacá); *E. insignis* (Cuatrecc.) Cuatrecc. and *E. sclerophylla* (Cuatrecc.) Cuatrecc. in the páramo de Almorzadero-Chitagá (Norte de Santander); *E. caldasii* (Cuatrecc.) Cuatrecc. and *E. santanderensis* (A. C. Sm.) Cuatrecc. in the páramo de Berlin-Almorzadero (Norte de Santander); *E. corymbosa, E. garciae* (col. 3715) and *E. rabanalensis* S. Diaz & Rodr.-Cabeza in the páramos of Cundinamarca-Boyacá; and *E. sanchezii* S. Díaz & Obando in *E. purpurascens* (Cuatrecc.) Cuatrecc in the páramo complex of Tierranegra–Tamá (Norte de Santander).
The Colombian clade of *Espeletia* species in the AME tree (Fig. 5) does not show resolution at the base; however, numerous small clades reflecting geographic distributions are recovered. Colombian species suggest two important centers of radiation for Espeletiinae: the páramos of Santander and Norte de Santander, close to Venezuela; and the páramos of Boyacá, where the Eastern Cordillera reaches its maximum width and topographic complexity. A smaller center of diversification is the complex of páramos in Cundinamarca, around the ‘Sabana de Bogotá’. The AME phylogeny suggests that Colombian species of *Espeletia* from these areas are more related to each other, in disregard of their altitude or niche specialization, than with other vicariant species. As an example, *E. lopezii* appears to be related to *E. cleefii* Cuatrec. These are two parapatric species that can be found in the Sierra Nevada del Cocuy (Boyacá–Arauca).

*Espeletia lopezii* prefers swampy, very wet meadows, while *E. cleefii* thrives better in scarped ridges of the superpáramo. The former has long robust naked scapes with a simple 3-cephalous cyme, while the latter has scapes with multiple pairs of sterile leaves and 15–27 capitula. There are numerous morphological differences that would classify these two species in totally different groups within *Espeletia*. Similarly, several clades containing morphologically divergent species, found in the same páramo massifs, are recognized as closely related species (i.e. clades for the páramos of Pisba, Frontino, Chingaza-Sumapáz-Tablazo, Tota, Iguáque-La Rusia, Nariño, etc.). It is likely that these clades represent the most recent colonization events that likely occurred after the Last Glacial. Additional glaciations would have enabled secondary crosses between vicariant species, with a diffusion of the geographic structure.

Geographically, the phylogeny suggests that the radiation of frailejones is an ongoing and highly dynamic process. None of the proposed two hypotheses describes entirely this radiation, but rather a combination of the two, with numerous horizontal and vertical migrations, isolations and reconnections, and a strong geographic structure. Frequent hybridization is likely prolonging the radiation momentum.
Based on morphology and his knowledge of the group, Cuatrecasas (2013) proposed a schematic phylogeny of the subtribe (Fig. 4), in which he highlighted two main clades. One contains *Libanothamnus*, *Ruilopezia* and *Tamania*, the group with terminal capitulescences. Our results (Figs. 3–5) support this hypothesis, with *Tamania* clearly nested with *Ruilopezia*. The other clade proposed by Cuatrecasas (2013) includes the rest of the genera, with *Paramiflos* closely related to *Espeletiopsis*, as well as *Coespeletia* with *Espeleta*, and *Carramboa* as sister of the latter clade. Our phylogenetic reconstructions support the position of *Paramiflos* within *Espeletiopsis* (Figs. 3–5). However, it does not support the relationship of *Carramboa* and *Coespeletia* with the large clade of *Espeletia* (with the Colombian species of the genus).

Moreover, the result of two large clades (mostly Venezuelan species and Colombian species) deeply challenges Cuatrecasas’ generic classification, with two possible outcomes: a splitting approach or a lumping approach. The former would imply: 1) preserving the monophyletic *Carramboa*; 2) redefining generic limits of *Coespeletia*, likely including *Espeleta semiglobulata* and *E. cuniculorum*; 3) proposing a new generic combination for *Espeletiopsis pannosa* and *E. angustifolia*; 4) splitting *Ruilopezia* in two genera, one of them including *Tamania*, and the other one the mainly glandulous *Ruilopezia* species; 5) creating a new genus (or two) for the Venezuelan species of *Espeletia* (considered by Cuatrecasas (2013) as section *Weddellia* Cuatrec.); 6) splitting Colombian *Espeletiopsis* in three genera; and 7) keeping Colombian *Espeletia* species as the proper genus *Espeletia*. The lumping approach would imply creating two groups (or three if keeping the monophyletic *Carramboa*): one with the mainly Venezuelan species, in which case should be named *Libanothamnus*; and one group with the Colombian taxa, named *Espeletia*. In any case, it seems premature to address these profound changes in the classification without further analysis (e.g. genomic data using population sampling) to fully understand conflicting inter-specific relationships.
Finally, our conclusions strongly support that relationships between species cannot be established solely in the light of morphology. Behind every species there is an evolutionary hypothesis to test, and frailejones represent a fertile field for studying migration, speciation and hybridization mechanisms.

ACKNOWLEDGMENTS

We wish to thank Gerardo Camilo, Vicki Funk, Jason Knouft, Allison Miller and Peter Raven for enlightening discussions and constructive feedback. We are also grateful for the contributions of many undergraduate and graduate students to the molecular analyses: Christine Abboud, Emily Adamson, Anna Belia de los Santos, Rachel Fauser, Ameera Haider, Hannah King, Shaun Patel, Amith Reddy and Geetha Sridharan. Four students and colleagues deserve our most special acknowledgments for their contributions to the lab work: Talita Carvalho, Maria Pinilla, Carolina Romero and Carolina Sánchez. We thank our local collaborators in Colombia: Camilo Cadena, Santiago Madriñán and Roberto Sánchez; and in Venezuela: Gilberto Morillo and Luis ‘Kike’ Gamez. We especially would like to acknowledge the many friends and colleagues who contributed as field assistants: Camilo Cadena, Monica Carlsen, Andrés Diavanera, Elí Flores, Carlos Gómez, Jennifer Gruhn, Rachel Jabaily, César Alirio Leal, Llizeth Mantilla, Clara Quintero, Diego Rodríguez, Nicolás Rodríguez, Susana Rodríguez, César Sanabria, Roberto Sánchez, Jessica Sarmiento, the park rangers of Pisba, Chingaza, Tamá and Denira, and many others. Without their considerable help, this project could not have been accomplished. Additional plant samples were provided by Fernando Alzate, Rodrigo Camara, Mauricio Castilblanco, Filip Kolar, Betsy V. Rodríguez, Oscar Salazar, Petr Sklenář and Simón Uribe-Convers. Special thanks to the institutions and herbaria that opened to the authors their collections, especially: ANDES, CAS, COL, CUVC, F, FMB, HECASA, HUA, K, MER, MO, NY and US. We thank Carlos Parra, from COL, and Santiago Madriñán, from ANDES, for
providing working and storage facilities at their respective herbaria in Colombia. Special thanks
to Monica Carlsen for her comments and advice on the phylogenetic analyses. We also thank the
Missouri Botanical Garden friends and colleagues for their comments and support.

REFERENCES

Applied Biosystems I. 2007. AFLP Plant Mapping Protocol: Applied Biosystems.
Avise JC. 2004. Molecular Markers, Natural History and Evolution. Sunderland, Massachussetts,
USA: Sinauer Associates, Inc.
Baldwin BG. 2009. Heliantheae alliance. In: Funk VA, Susanna A, Stuessy T, and Bayer RT, eds.
Systematics, Evolution and Biogeography of Compositae. First Edition ed. Vienna,
Austria: International Association for Plant Taxonomy, 689-711.
Baldwin BG, and Markos S. 1998. Phylogenetic utility of the external transcribed spacer (ETS)
of 18S-26S rDNA: congruence of ETS and ITS trees of Calycadenia (Compositae).
Molecular Phylogenetics and Evolution 10:449-463. 10.1006/mpev.1998.0545
Baldwin BG, Wessa BL, and Panero JL. 2002. Nuclear rDNA evidence for major lineages of
helioid Heliantheae (Compositae). Systematic Botany 27:161-198.
Barker FK, and Lutzoni FM. 2002. The Utility of the Incongruence Length Difference Test.
Systematic Biology 51:625-637.
Berry PE, and Calvo RN. 1989. Wind Pollination, Self-Incompatibility, and Altitudinal Shifts in
Pollination Systems in the High Andean genus Espeletia (Asteraceae). American Journal
of Botany 76:1602-1614.
Berry PE, and Calvo RN. 1994. An overview of the reproductive biology of Espeletia
(Asteraceae) in the Venezuelan Andes. In: Philip W. Rundel APSaFCM, Smith AP, and
Meinzer FC, eds. Tropical Alpine Environments Plant Form and Function. Los Angeles:
Cambridge University Press, 229-250.
Blöch MC. 2010. Molecular phylogeny and chromosome evolution of the genus Melampodium
L. (Milleriaceae, Asteraceae) Doktorin der Naturwissenschaften. PhD Thesis, Universität
Wien, Austria.
Brooks TM, Mittermeier Ra, da Fonseca GaB, Gerlach J, Hoffmann M, Lamoreux JF,
Mittermeier CG, Pilgrim JD, and Rodrigues aSL. 2006. Global biodiversity conservation
priorities. Science (New York, NY) 313:58-61. 10.1126/science.1127609
Clevinger JA, and Panero JL. 2000. Phylogenetic analysis of Silphium and subtribe
Engelmanniinae (Asteraceae: Heliantheae) based on ITS and ETS sequence data.
American Journal of Botany 87:565-572.
Cuatrecasas J. 1933. Plantae Colombianaee novae. Trab Mus Nac Ci Nat, Ser Bot 26:1-31.
Cuatrecasas J. 1934. Observaciones geobotánicas en Colombia. Trab Mus Nac Ci Nat, Ser Bot
27:1-144.
Cuatrecasas J. 1976. A New Subtribe in the Heliantheae (Compositae): Espeletiinae. Phytologia
35:43-59.
Cuatrecasas J. 1986. Speciation and radiation of the Espeletiinae in the Andes. In: Vuilleumier F,
and Monasterio M, eds. High Altitude Tropical Biogeography. New York, USA: Oxford
University Press, 267-303.
Cuatrecasas J. 1995. A new genus of the Compositae: Paramiflos (Espeletiinae) from Colombia. 
Proceedings of the Biological Society of Washington 108:748-750.

Cuatrecasas J. 2013. A systematic study of the subtribe Espeletiinae. New York, USA: The New York Botanical Garden.

Cunningham CW. 1997. Can three incongruence tests predict when data should be combined? 
Molecular Biology and Evolution 14:733–740.

Díaz-Piedrahita S, and Rodriguez-Cabeza BV. 2008. Novedades en los géneros Espeletia Mutis ex Humb. & Bonpl. y Espeletiopsis Cuatrec. (Asteraceae, Heliantheae, Espeletiinae). 
Revista Acad Colomb Ci Exact 32:455–464.

Díaz-Piedrahita S, and Rodriguez-Cabeza BV. 2010. Nuevas Especies Colombianas de Espeletiopsis Cuatrec. y de Espeletia Mutis ex Humb. & Bonpl. (Asteraceae, Heliantheae, Espeletiinae). 
Revista Acad Colomb Ci Exact 34:441–454.

Díaz-Piedrahita S, and Rodriguez-Cabeza BV. 2011. Novedades en Asteráceas colombianas - I. 
Revista Acad Colomb Ci Exact 37:411-424.

Díazgranados M. 2012a. A nomenclator for the frailejones (Espeletiinae Cuatrec., Asteraceae). 
Phytokeys 16:1–52. doi: 10.3897/phytokeys.16.3186

Diazgranados M. 2012b. Phylogenetic and biogeographic relationships within the Espeletiinae (family Asteraceae), an endemic subtribe of the South American Páramos (Doctoral Dissertation) Ph.D. Doctoral Dissertation. Saint Louis University.

Diazgranados M. 2013. Aportes a la delimitación de los páramos desde el estudio de los frailejones. In: Cortés J, and Sarmiento C, eds. Visión socioecosistémica de los Páramos y la Alta Montaña Colombiana: Memorias del proceso de definición de criterios para la delimitación de páramos. Bogotá, Colombia: Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, 23–37.

Diazgranados M. 2015. Una mirada biológica a los páramos circundantes a la Sabana de Bogotá. 
In: Guhl E, ed. Los páramos circundantes a la Sabana de Bogotá. Bogota, Colombia: Jardín Botánico de Bogotá, 175–205.

Diazgranados M, and Morillo G. 2013. A new species of Coespeletia (Asteraceae, Millerieae) from Venezuela. Phytokeys:9–18. 10.3897/phytokeys.28.6378

Duckton M, and Austin AD. 2002. Increased Congruence Does Not Necessarily Indicate Increased Phylogenetic Accuracy-The Behavior of the Incongruence Length Difference Test in Mixed-Model Analyses. Systematic biology 51:19-31.

Dolphin K, Belshaw R, Orme CD, and Quicke DL. 2000. Noise and incongruence: interpreting results of the incongruence length difference test. Molecular Phylogenetics and Evolution 17:401-406. 10.1006/mpev.2000.0845

Dolphin K, Belshaw R, Orme CD, and Quicke DL. 2000. Noise and incongruence: interpreting results of the incongruence length difference test. Molecular Phylogenetics and Evolution 17:401-406. 10.1006/mpev.2000.0845

Doyle JJ, and Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19:11-15.

Ekenäs C, Baldwin BG, and Andreasen K. 2007. A molecular phylogenetic study of Arnica (Asteraceae): low chloroplast DNA variation and problematic subgeneric classification. 
Systematic Botany 32:917-928.

Fagua JC, and Gonzalez VH. 2007. Growth rates, reproductive phenology, and pollination ecology of Espeletia grandiflora (Asteraceae), a giant Andean caulescent rosette. Plant biology (Stuttgart, Germany) 9:127-135.
Farris JS, Kallersjo M, Kluge AG, and Bult C. 1995. Constructing a Significance Test for Incongruence. *Systematic Biology* 44:570-572.

Fernández-Alonso PJL. 2002. Algunos patrones de distribución y endemismo en plantas vasculares de los páramos de colombia. Congreso Mundial de Páramos : Memorias: Ministerio de Medio Ambiente. República de Colombia. p 213-229.

Fluxus Technology Ltd. 2012. Network 4.6.1.1. [http://www.fluxus-engineering.com/](http://www.fluxus-engineering.com/).

Friar EA, Prince LM, Cruse-sanders JM, Mcglaughlin ME, Butterworth CA, and Baldwin BG. 2008. Hybrid Origin and Genomic Mosaicism of *Dubautia scabra* (Hawaiian Silversword; Asteraceae, Madiinae). *Society* 33:589-597.

Garcia S, McArthur ED, Pellicer J, Sanderson SC, Vallès J, and Garnatje T. 2011. A molecular phylogenetic approach to western North America endemic *Artemisia* and allies (Asteraceae): Untangling the sagebrushes. *American Journal of Botany* 98:638-653. 10.3732/ajb.1000386

Gruenstaeudl M, Urtubey E, Jansen RK, Samuel R, Barfuss MHJ, and Stuessy TF. 2009. Phylogeny of Barnadesioideae (Asteraceae) inferred from DNA sequence data and morphology. *Molecular Phylogenetics and Evolution* 51:572-587. 10.1016/j.ympev.2009.01.023

Guindon S G. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52:696-704.

Harpke D, and Peterson A. 2008a. 5.8S motifs for the identification of pseudogenic ITS regions. *Botany* 86:300-305. 10.1139/B07-134

Harpke D, and Peterson A. 2008b. Extensive 5.8S nrDNA polymorphism in *Mammillaria* (Cactaceae) with special reference to the identification of pseudogenic internal transcribed spacer regions. *Journal of Plant Research* 121:261-270. 10.1007/s10265-008-0156-x

Hooghiemstra H, and Van der Hammen T. 2004. Quaternary Ice-Age dynamics in the Colombian Andes: developing an understanding of our legacy. *Philosophical transactions of the Royal Society of LondonSeries B, Biological sciences* 359:173-180; discussion 180-171.

Hughes C, and Eastwood R. 2006. Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes. *Proceedings of the National Academy of Sciences of the United States of America* 103:10334-10339.

Huson DH, and Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 23:254–267. 10.1093/molbev/msj030

Jabaily RS. 2009. Systematics and evolution of *Puya* ( Bromeliaceae ). University of Wisconsin.

Jansen RK, and Palmer JD. 1987. A chloroplast DNA inversion marks an ancient evolutionary split in the sunflower family (Asteraceae). *Proc Natl Acad Sci U S A* 84:5818-5822.

Keeley SC, Forsman ZH, and Chan R. 2007. A phylogeny of the "evil tribe" (Vernonieae: Compositae) reveals Old/New World long distance dispersal: support from separate and combined congruent datasets (trnL-F, ndhF, ITS). *Molecular Phylogenetics and Evolution* 44:89-103. 10.1016/j.ympev.2006.12.024

Killeen TJ, Douglas M, Consiglio T, Jørgensen PM, and Mejia J. 2007. Dry spots and wet spots in the Andean hotspot. *Journal of Biogeography* 34:1357-1373. 10.1111/j.1365-2699.2006.01682.x

Knowles L, and Chan Y-H. 2009. Resolving Species Phylogenies of Recent Evolutionary Radiations. *Annals of the Missouri Botanical Garden* 95:224-231. 10.3417/2006102

Knowles LL, and Carstens BC. 2007. Delimiting Species without Monophyletic Gene Trees. *Syst Biol* 56:887-895. 10.1080/10635150701701091

Koopman WJM. 2005. Phylogenetic signal in AFLP data sets. *Systematic Biology* 54:197-217. 10.1080/1063515059024181
Koopman WJM, Wissemann V, De Cock K, Van Huylenbroeck J, De Riek J, Sabatino GJH, 
Visser D, Vosman B, Ritz CM, Maes B, Werlemark G, Nybom H, Debener T, Linde M, 
and Smulders MJM. 2008. AFLP markers as a tool to reconstruct complex relationships: 
A case study in Rosa (Rosaceae). American Journal of Botany 95:353-366.
10.3732/ajb.95.3.353

Koopman WJM, Zevenbergen MJ, and Van den Berg RG. 2001. Species relationships in Lactuca 
s.l. (Lactuceae, Asteraceae) inferred from AFLP fingerprints. American Journal of Botany 
88:1881-1887.

Luteyn JL. 1999. Páramos: a checklist of plant diversity, geographical distribution, and 
botanical literature. Bronx, New York, USA.: New York Botanical Garden Press.

Maddison WP, and Maddison DR. 2011. Mesquite: a modular system for evolutionary analysis. 
Version 2.75. Online at: http://mesquiteproject.org.

Madriñán S, Cortés AJ, and Richardson JE. 2013. Paramo is the world's fastest evolving and 
coolest biodiversity hotspot. Front Genet 4:1–7. 10.3389/fgene.2013.00192

Mallet J. 2007. Hybrid speciation. Nature 446:279-283. 10.1038/nature05706

Masuda Y, Yukawa T, and Kondo K. 2009. Molecular phylogenetic analysis of members of 
Chrysanthemum and its related genera in the tribe Anthemideae, the Asteraceae in East 
Asia on the basis of the internal transcribed spacer (ITS) region and the external 
transcribed spacer (ETS) region of nrDNA. Chromosome Botany 4:25-36.

Mavrodiev EV, Nawchoo I, Soltis PS, and Soltis DE. 2008. Molecular data reveal that the 
tetraploid Tragopogon kashmirianus (Asteraceae: Lactuceae) is distinct from the North 
American T. mirus. Botanical Journal of the Linnean Society 158:391-398.

McKinnon GE, Vaillancourt RE, Steane DA, and Potts BM. 2008. An AFLP marker approach to 
lower-level systematics in Eucalyptus (Myrtaceae). American Journal of Botany 95:368-
380.

Meudt HM, and Clarke AC. 2007. Almost forgotten or latest practice? AFLP applications, 
analyses and advances. Trends in Plant Science 12:106-117. 
10.1016/j.tplants.2007.02.001

Monasterio M, and Sarmiento L. 1991. Adaptive radiation of Espeletia in the cold Andean 
tropics. Trends in Ecology and Evolution 6:387-391.

Moody ML, and Rieseberg LH. 2012. Sorting through the chaff, nDNA gene trees for 
phylogenetic inference and hybrid identification of annual sunflowers (Helianthus sect. 
Helianthus). Molecular Phylogenetics and Evolution 64:145-155.

Moore AJ, Bartoli A, Tortosa RD, and Baldwin BG. 2012. Phylogeny, biogeography, and 
chromosome evolution of the amphitropical genus Grindelia (Asteraceae) inferred from 
nuclear ribosomal and chloroplast sequence data. Taxon 61:211-230.

Morgan DR, Korn R-L, and Mugleston SL. 2009. Insights into reticulate evolution in 
Machaerantherineae (Asteraceae: Astereae): 5S ribosomal RNA spacer variation, 
estimating support for incongruence, and constructing reticulate phylogenies. American 
Journal of Botany 96:920-932. 10.3732/ajb.0800308

Morillo G, and Briceño B. 2007. Estudio sobre Carramboa tachirensis (Aristeg .) Cuatrec. 
(Asteraceae) y sus afines. Rev Fav Agron (LUZ) 24:475-481.

Nolte AW, and Tautz D. 2010. Understanding the onset of hybrid speciation. Trends Genet 26:54– 
58. 10.1016/j.tig.2009.12.001

Panero J. 2007. Tribe Millerieae Lindl. (1829). In: Kubitzki K, ed. The Families and Genera of 
Vascular Plants. Berlin Heidelberg New York: Springer, 477-492.

Parks DH, Porter M, Churcher S, Wang S, Blouin C, Whalley J, Brooks S, and Beiko RG. 2009. 
GenGIS: A geospatial information system for genomic data. Genome Res 19:1896-1904. 
10.1101/gr.095612.109
Planet PJ. 2006. Tree disagreement: measuring and testing incongruence in phylogenies. *Journal of biomedical informatics* 39:86-102. 10.1016/j.jbi.2005.08.008

Posada D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* 25:1253–1256.

QIAGEN. 2006. *DNeasy plant mini kit handbook*.

Quicke DJ, Jones O, and Epstein D. 2007. Correcting the Problem of False Incongruence Due to Noise Imbalance in the Incongruence Length Difference (ILD) Test. *Systematic biology* 56:496.

Rambaut A. 1996. Se-Al: sequence alignment editor. Available online at: [http://tree.bio.ed.ac.uk/software/seal/](http://tree.bio.ed.ac.uk/software/seal/).

Rambaut A. 2009. FigTree v1.3.1. Computer program available from: [http://tree.bio.ed.ac.uk/software/figtree](http://tree.bio.ed.ac.uk/software/figtree). (last accessed April 2012).

Rangel-Ch. O. 2000. *La región de vida paramuna*. Bogotá D.C., Colombia: Instituto de Ciencias Naturales - Instituto Alexander von Humboldt.

Rauscher J. 2002. Molecular phylogenetics of the *Espeletia* complex (Asteraceae): evidence from nrDNA ITS sequences on the closest relatives of an Andean adaptive radiation. *American Journal of Botany* 89:1074-1084.

Rauscher JT. 2000. Molecular systematics of the *Espeletia* complex: evidence from nrITS sequence on the evolution of an Andean adaptive radiation. ProQuest Dissertations and Theses. p 209.

Robinson H. 1981. A revision of the tribal and subtribal limits of the Heliantheae (Asteraceae). p 1-95.

Sánchez A. 2005a. Filogenética molecular de los Espeletiinae, una radiación adaptativa andina MSc. . Universidad de los Andes.

Sánchez A. 2005b. Filogenética molecular de los Espeletiinae, una radiación adaptativa andina M.S. Universidad de los Andes.

Särkinen T, Pennington RT, Lavin M, Simon MF, and Hughes CE. 2012. Evolutionary islands in the Andes: persistence and isolation explain high endemism in Andean dry tropical forests. *Journal of Biogeography* 39:884–900. 10.1111/j.1365-2699.2011.02644.x

Schilling EE, and Panero JL. 2011. A revised classification of subtribe Helianthinae (Asteraceae: Heliantheae) II. Derived lineages. *Botanical Journal of the Linnean Society* 167:311-331. 10.1111/j.1095-8339.2011.01172.x

Schluter D. 2000. *The Ecology of Adaptive Radiation*: Oxford University Press.

Schmidt-Lebuhn AN. 2007. Using amplified fragment length polymorphism (AFLP) to unravel species relationships and delimitations in *Minthostachys* (Labiatae). *Botanical Journal of the Linnean Society* 153:9–19.

Schmidt-Lebuhn AN, Kessler M, and Kumar M. 2009. Promiscuity in the Andes: species relationships in *Polylepis* (Rosaceae, Sanguisorbeae) based on AFLP and morphology. *Systematic Botany* 31:547-559. 10.1043/05-25.1

Seehausen O. 2004. Hybridization and adaptive radiation. *Trends Ecol Evol* 19:198–207. 10.1016/j.tree.2004.01.003

Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, Siripun KC, Winder CT, Schilling EE, and Small RL. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92:142-166.

Shaw J, Lickey EB, Schilling EE, and Small RL. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany* 94:275-288.
Shimodaira H, and Hasegawa M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* 16:1114–1116.

Sklenár P, Luteyn JL, Ulloa C, Jørgensen PM, and Dillon MO. 2005. *Flora Genérica de los Páramos - Guía Ilustrada de las Plantas Vasculares*. New York: The New York Botanical Garden Press.

Small RL, Ryburn JA, Cronn RC, Seelanan T, and Wendel JF. 1998. The tortoise and the hare: choosing between noncoding plastome and nuclear Adh sequences for phylogeny reconstruction in a recently diverged plant group. *American Journal of Botany* 85:1301-1315.

Sobrevila C. 1988. Effects of Distance between Pollen Donor and Pollen Recipient on Fitness Components in *Espeletia schultzii*. *American Journal of Botany* 75:701-724.

Soltis DE, Mavrodiev EV, Doyle JJ, Rauscher J, and Soltis PS. 2008. ITS and ETS Sequence Data and Phylogeny Reconstruction in Allopolyploids and Hybrids. *Systematic Botany* 33:7-20.

Sukumaran J, and Holder MT. 2010. DendroPy: A Python library for phylogenetic computing. *Bioinformatics* 26:1569-1571.

Swofford DL. 2002. *PAUP* : phylogenetic analysis using parsimony (* and other methods), version 4.0b10. Sunderland, Massachussetts, USA: Sinauer.

Thiers B. [continuously updated]. Index Herbariorum: A global directory of public herbaria and associated staff. [http://sweetgum.nybg.org/ih/](http://sweetgum.nybg.org/ih/); New York Botanical Garden's Virtual Herbarium.

Timme RE, Kuehl JV, Boore JL, and Jansen RK. 2007a. A comparative analysis of the *Lactuca* and *Helianthus* (Asteraceae) plastid genomes: identification of divergent regions and categorization of shared repeats. *Am J Bot* 94:302-312. 10.3732/ajb.94.3.302

Timme RE, Simpson BB, and Linder CR. 2007b. High-resolution phylogeny for *Helianthus* (Asteraceae) using the 18S-26S ribosomal DNA external transcribed spacer. *Am J Bot* 94:1837-1852. 10.3732/ajb.94.11.1837

Torres V, Hooghiemstra H, Lourens L, and Tzedakis PC. 2013. Astronomical tuning of long pollen records reveals the dynamic history of montane biomes and lake levels in the tropical high Andes during the Quaternary. *Quaternary Science Reviews* 63:59–72. [http://dx.doi.org/10.1016/j.quascirev.2012.11.004](http://dx.doi.org/10.1016/j.quascirev.2012.11.004)

Trybush S, Hanley S, Cho K-H, Jahodov, rka, Grimmer M, Emelianov I, Bayon C, and Karp A. 2006. Getting the most out of fluorescent amplified fragment length polymorphism. *Canadian Journal of Botany* 84:1347-1354.

Vaezi J, and Brouillet L. 2009. Phylogenetic relationships among diploid species of *Symphyotrichum* (Asteraceae: Astereae) based on two nuclear markers, ITS and GAPDH. *Molecular Phylogenetics and Evolution* 51:540-553. 10.1016/j.ympev.2009.03.003

Van der Hammen T, and Cleef AM. 1986. Development of the high Andean páramo flora and vegetation. In: Vuilleumier F, and Monasterio M, eds. *High Altitude Tropical Biogeography*. New York, USA: Oxford University Press, 153–201.

Vos P, Hogers R, Bleecker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, and Kuiper M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic acids research* 23:4407-4414. 5w0130 [pii]

Wahrmund U, Heklau H, Röser M, Kästner A, Vitek E, Ehrendorfer F, and Hagen KBV. 2010. A molecular phylogeny reveals frequent changes of growth form in *Carlina* (Asteraceae). *Taxon* 59:367-378.

White TJ, Bruns T, Lee S, and Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky J, and White T,
eds. *PCR protocols: a guide to methods and applications*. San Diego, CA: Academic Press, 315-322.

Worley AC, Ghazvini H, and Schemske DW. 2009. A Phylogeny of the Genus *Polemonium* Based on Amplified Fragment Length Polymorphism (AFLP) Markers. *Systematic Botany* 34:149-161.

Young BE, Josse C, Stern M, Vascone S, Olander J, Smyth R, Zador M, Sánchez de Lozada A, Comer PJ, Moull K, Echavarría M, and Hak J. 2015. Hotspot de Biodiversidad de los Andes Tropicales. Resumen técnico del perfil del ecosistema p55.

Zhang J-w, Nie Z-l, Wen J, and Sun H. 2011. Molecular phylogeny and biogeography of three closely related genera, endemic to the Tibetan Plateau, SW China. *Taxon* 60:15-26.

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion Ph.D. dissertation. The University of Texas at Austin.
Table 1 Number of species collected and documented during fieldwork for this project. In addition to these collections, samples from other species were obtained from different sources.

| Genus          | No. of species | No. collected (%) | New species** |
|----------------|----------------|-------------------|---------------|
| Carramboa      | 4              | 3 (75)            | 1             |
| Coespeletia    | 8              | 6 (86)            | 1             |
| Espeletia      | 72             | 56 (79)           | 6             |
| Espeletiopsis  | 23             | 19 (86)           | 4             |
| Libanothamnus  | 11             | 9 (82)            | 1             |
| Paramiflos     | 1              | 1 (100)           | 0             |
| Ruilopezia     | 24             | 15 (63)           | 0             |
| Tamania        | 1              | 1 (100)           | 1             |
| **TOTAL**      | **144**        | **110 (78)**      | **14**        |

* Putative new species not counted in this column.
** New species being described
Table 2 AFLP primer combinations used for genotyping. First triplet for each primer combination corresponds to the selective anchor for EcoRI; second triplet corresponds to the selective anchor for MseI. Mean Y threshold corresponds to the intensity of each band (peak height), in relative fluorescence units (RFU).

| Primer combination | Number of fragments | Minimum size (b) | Maximum size (b) | Mean size (b) | Variance of size (b^2) | Mean Y threshold (RFU) |
|--------------------|---------------------|------------------|------------------|--------------|-------------------------|-----------------------|
| AAC-CAA            | 39                  | 61.7             | 417.5            | 135.5        | 0.0                     | 4603.9                |
| AAC-CAC            | 43                  | 65.9             | 231.5            | 130.0        | 0.1                     | 4296.3                |
| AAC-CAG            | 41                  | 61.7             | 348.5            | 116.2        | 0.0                     | 9722.0                |
| AAC-CAT            | 51                  | 59.8             | 376.6            | 141.9        | 0.0                     | 4027.3                |
| AAG-CAA            | 39                  | 62.5             | 294.0            | 144.6        | 0.0                     | 14775.7               |
| AAG-CAC            | 56                  | 59.6             | 417.8            | 160.3        | 0.1                     | 9138.6                |
| AAG-CAT            | 39                  | 59.8             | 259.3            | 158.6        | 0.1                     | 10322.9               |
| ACA-CAA            | 47                  | 63.1             | 307.0            | 155.7        | 0.0                     | 8447.5                |
| ACA-CAC            | 65                  | 64.9             | 310.4            | 160.3        | 0.1                     | 8102.3                |
| ACA-CAG            | 58                  | 63.8             | 334.5            | 159.8        | 0.1                     | 10157.2               |
| ACA-CAT            | 62                  | 63.5             | 351.1            | 170.6        | 0.0                     | 4192.8                |
| ACG-CAA            | 56                  | 66.7             | 412.5            | 174.5        | 0.0                     | 4644.9                |
| ACG-CAC            | 51                  | 60.4             | 267.0            | 139.4        | 0.0                     | 6245.9                |
| ACG-CAG            | 58                  | 59.5             | 307.2            | 148.4        | 0.0                     | 6970.9                |
| ACG-CAT            | 51                  | 64.9             | 348.0            | 158.3        | 0.1                     | 3253.5                |
| ACT-CAA            | 91                  | 62.6             | 443.8            | 169.9        | 0.0                     | 15551.5               |
| ACT-CAC            | 59                  | 60.7             | 383.9            | 219.0        | 0.1                     | 8792.9                |
| ACT-CAG            | 58                  | 65.9             | 378.5            | 181.1        | 0.1                     | 11644.2               |
| ACT-CAT            | 83                  | 59.0             | 360.8            | 201.9        | 0.1                     | 10579.9               |
| AGC-CAA            | 54                  | 61.4             | 318.2            | 164.8        | 0.0                     | 3303.6                |
| AGC-CAC            | 40                  | 59.2             | 281.8            | 137.8        | 0.1                     | 5745.0                |
| AGC-CAG            | 57                  | 62.9             | 363.1            | 143.6        | 0.0                     | 7534.2                |
| AGC-CAT            | 40                  | 62.0             | 320.3            | 158.4        | 0.0                     | 3997.4                |
| AGG-CAA            | 75                  | 58.2             | 360.5            | 182.6        | 0.0                     | 10933.4               |
| AGG-CAC            | 109                 | 59.6             | 436.4            | 185.5        | 0.2                     | 12275.0               |
| AGG-CAG            | 86                  | 63.8             | 381.6            | 190.8        | 0.1                     | 16182.5               |
| AGG-CAT            | 88                  | 59.7             | 369.7            | 193.8        | 0.1                     | 9577.2                |
| **Total**          | **1665**            | **58.2**         | **443.8**        | **165.5**    | **0.1**                 | **8892.5**            |
**Table 3** Sequence characteristics and tree statistics for all data sets. Tree scores were estimated from the MP consensus tree.

| Statistics                  | rpl16 | ETS   | ITS   | AFLPs  | ETS-ITS | ETS-ITS-rpl16 | All combined |
|-----------------------------|-------|-------|-------|--------|---------|---------------|--------------|
| Aligned length (characters) | 962   | 1,324 | 649   | 1,665  | 1,973   | 2,935         | 4,600        |
| Number of samples           | 144\(^1\) | 150\(^2\) | 142\(^3\) | 134    | 149     | 145           | 111          |
| Number of species           | 127   | 119   | 130   | 115    | 117     | 116           | 95           |
| Variable characters         | 93    | 560   | 245   | 1,665  | 750     | 831           | 2,294        |
| Informative characters      | 35    | 368   | 173   | 1,583  | 507     | 551           | 1,818        |
| Number of MP trees          | 100,000 | 100,000 | 100,000 | 200    | 100,000 | 100,000       | 24           |
| Tree length (best MP tree)  | 84    | 1,155 | 527   | 10,200 | 1,545   | 1,760         | 9,797        |
| CI*                         | 0.464 | 0.113 | 0.454 | 0.159  | 0.338   | 0.405         | 0.200        |
| RI*                         | 0.930 | 0.061 | 0.841 | 0.159  | 0.747   | 0.813         | 0.456        |
| RC*                         | 0.432 | 0.007 | 0.381 | 0.057  | 0.252   | 0.329         | 0.091        |
| HI*                         | 0.536 | 0.887 | 0.546 | 0.841  | 0.662   | 0.595         | 0.800        |
| Model of evolution          | TVM+I+G | TrN+G | TIM2+G | Mkv    | N/A     | N/A           | N/A          |
| -ln L                       | -2288.46 | -9409.64 | -3944.67 | -44743.38 | -14066.11 | -17054.85     | -54659.98    |

\(^1\) Genbank accession numbers KY231675–KY231818  
\(^2\) Genbank accession numbers KY231383–KY231532  
\(^3\) Genbank accession numbers KY231533–KY231674  

*Statistics based on one of the shortest maximum parsimony trees: CI, consistency index; RI, retention index; RC, rescaled consistency index; HI: homoplasy index.
Figure 1 Morphological diversity in the genera of Espeletiinae. (A) Espeletia (72 species); (B) Espeletiopsis (23); (C) Coespeletia (8); (D) Paramilos (1); (E) Ruilopezia (24); (F) Carramboa (4); (G) Tamania (1); and (H) Libanothamnus (11). Illustrations from Cuatrecasas (2013), made by Alice Tangerini and Florence Lambeth (Department of Botany, US National Herbarium, Smithsonian Institution); photographs: Diazgranados.
Figure 2 Minimum evolution (ME) tree for AFLPs. Branch support is indicated in the following order: ME bootstrap value/ML bootstrap value/MP bootstrap value/BI posterior probability (×100); *, value below 50; -, missing value. Support values shown only for nodes with at least one support metric ≥ 50. Genera abbreviations: I., Ichthyothere; Ru., Rumfordia; Po., Polynnia; S., Smallanthus; Ca., Carramboa; C., Coespeletia; E., Espeletia; Es., Espeletiopsis; L., Libanothamnus; P., Paramiflos; R., Ruilopezia; T., Tamananthus; Ta., Tamananthus.
Figure 3 Maximum likelihood tree based on ITS and ETS. Branch support is indicated in the following order: ML bootstrap value/MP bootstrap value/B1 posterior probability (×100); *, value below 50; -, missing value. Support values shown only for nodes with at least one support metric ≥ 50. In green: taxa present in the border between the two countries. Genera abbreviations: S., Smallanthus; Ca., Carramboa; C., Coespeletia; E., Espeletia; Es., Espeletiopsis; L., Libanothamnus; P., Paramiflos; R., Ruilopezia; T., Tamania.
Figure 4 Maximum likelihood tree based on ITS, ETS and rpl16. Branch support is indicated over the branch or close to the angle of the branch in the following order: ML bootstrap value/MP bootstrap value/BI posterior probability (×100); *, value below 50; -, missing value. Support values shown only for nodes with at least one support metric ≥ 50. Genera abbreviations: S., Smallanthus; Ca., Carramboa; C., Coespeletia; E., Espeletia; Es., Espeletiopsis; L., Libanothamnus; P., Paramiflos; R., Ruilopezia; T., Tamania. Schematic phylogeny proposed by Cuatrecasas (2013) displayed with black lines. Geographic distribution of genera (colored dots) shown on map. Background color denotes mountains over 1000 m of altitude.
Figure 5 Bayesian tree estimated with all the available molecular evidence (ETS, ITS, rpl16 and AFLPs). Branch support is indicated over the branch or close to the angle of the branch in the following order: ML bootstrap value/MP bootstrap value/BI posterior probability (×100); *, value below 50; -, missing value. Support values shown only for nodes with at least one support metric ≥ 50. Genera abbreviations: S., Smallanthus; Ca., Carramboa; C., Coespeletia; E., Espeletia; Es., Espeletiopsis; L., Libanothamnus; P., Paramiflos; R., Ruilopezia; T., Tamania. Main páramo massifs indicated in colors.
Figure 6 Cladogram of the Bayesian tree based on the available molecular evidence (ETS, ITS, rpl16 and AFLPs), mapped on the geographic range of the subtribe. (A) top view of the cladogram; (B) side view of the cladogram. Additional line of colored dots in the top view shows the samples arranged by geographic proximity.