

# MOLECULAR PHYLOGENETICS OF THE *ESPELETIA* COMPLEX (ASTERACEAE): EVIDENCE FROM nrDNA ITS SEQUENCES ON THE CLOSEST RELATIVES OF AN ANDEAN ADAPTIVE RADIATION<sup>1</sup>

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The subtribe Espeletiinae (Asteraceae, Heliantheae) comprises morphologically and ecologically diverse plants endemic to the tropical montane paramos of the Andes of Venezuela, Colombia, and Ecuador. Though the ecophysiology and ecology of this adaptive radiation have been well studied, relationships among taxa in the subtribe and between the subtribe and other taxa in the Heliantheae are poorly known. In this study, sequences from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA are used to test previous hypotheses about the phylogenetic position of the Espeletiinae within the Heliantheae and to determine which taxa are the subtribe's closest relatives. Gene phylogenies based on maximum parsimony analyses reveal that the Espeletiinae clade is nested well within the subtribe Melampodiinae and thus should be considered a monophyletic complex of species, not a separate subtribe. The most parsimonious gene trees suggest that the genus *Ichthyothere* may be the sister taxon to the *Espeletia* complex and that the genus *Smallanthus* and a species of *Rumfordia* are likely among the complex's other closest living relatives. These data offer preliminary insights into the origins of this adaptive radiation and the broader phylogenetic context in which it occurred.

**Key words:** adaptive radiation; Asteraceae; *Espeletia*; Espeletiinae; *Ichthyothere*; ITS; Melampodiinae; paramo.

**Adaptive radiation in the Espeletiinae**—The high Andes of northwestern South America contain a system of islandlike tropical montane habitat known as paramo. Although paramo has existed only since the final uplift of the Andes 2–4 million years ago (Van der Hammen and Cleef, 1986), they are considered the most floristically diverse and endemic-species-rich high montane ecosystems in the world (Smith and Cleef, 1988; Luteyn, 1999). While many of the current paramo taxa are derived from ancestors in both northern and southern temperate mountains, others originated in the diverse surrounding tropical forests (Smith and Cleef, 1988). Among the taxa thought to have evolved from ancestors in the tropical montane forests is the subtribe Espeletiinae (Asteraceae: Heliantheae). This group comprises eight genera and more than 100 described species endemic to Venezuela, Colombia, and Ecuador. The species are noted for their unusual giant rosette growth form and densely pubescent leaves, and many have come to dominate and characterize these tropical alpine landscapes.

Several characteristics of the Espeletiinae make the group ideal not only for gaining insight into the origin of paramo diversity, but also for understanding better the mechanisms of

speciation and adaptive evolution in plants. Since their relatively recent origin in the late Pliocene or early Pleistocene, members of the Espeletiinae have shown a remarkable diversity of morphology, growth form, and ecological specialization (Cuatrecasas, 1986). In addition to the giant polycarpic rosettes most characteristic of the group, there are tiny, mat-forming rosettes, sessile and caulescent monocarpic rosettes, branched shrubby trees, and even broad-leaved trees more than 10 m tall. Espeletiinae species can be found from upper montane cloud forests at about 2000-m elevation to the edge of glaciers at over 4600 m, having adapted to a wide range of conditions from gaps in the montane forest to wet paramo bogs to xeric periglacial talus slopes. Many have considered this rapid diversification a classic example of adaptive radiation in plants (Carlquist, 1974; Monasterio and Sarmiento, 1991).

**Taxonomy and hypotheses of closest relatives**—Despite much interest in the evolution, ecology, and ecophysiology of the Espeletiinae, phylogenetic relationships within the subtribe and between it and the rest of the Heliantheae are still unclear. The species assemblage was first described by Bonpland (von Humboldt and Bonpland, 1809) as a single genus, *Espeletia*, with two species. Over the next two centuries, exploration of remote Andean mountaintops revealed dozens of new species. The morphological diversity and distinctiveness of these species convinced Cuatrecasas (1976) that the creation of a new subtribe, Espeletiinae, was justified. Initially seven genera were recognized: *Espeletia*, *Espeletiopsis*, *Coespeletia*, *Ruilopezia*, *Libanothamnus*, *Carramboia*, and the monotypic genus *Tamania*. Later, an eighth genus, *Paramiflos* (also monotypic), was recognized (Cuatrecasas, 1995).

While Bonpland initially believed that *Espeletia* sensu lato (s.l.) is closely related to the genus *Silphium* (von Humboldt and Bonpland, 1809), Kunth (1820) grouped it with genera such as *Polymnia* and *Unxia*. In one of the early monographs on *Espeletia*, Smith and Koch (1935) identified *Polymnia* as likely the closest relative, although they gave few details sup-

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porting their hypothesis. Robinson (1981), however, determined that *Polymnia*, previously monographed by Wells (1965), is polyphyletic, comprising two phylogenetically distinct groups of species. Therefore, all but two species of *Polymnia* were removed from the genus and given the reestablished generic name *Smallanthus*, originally described by MacKenzie in 1933. Robinson (1981) later commented on the similarities between the Espeletiinae and *Smallanthus*, thus implicitly favoring this genus as the closest relative to the Espeletiinae, rather than the revised *Polymnia*.

Additional candidates for the closest relatives of the Espeletiinae can be inferred from several tribal and subtribal taxonomic treatments. In the first publication that considered the Espeletiinae within a subtribal classification of the Heliantheae (Bentham and Hooker, 1873), *Espeletia* s.l. was placed within the subtribe Melampodiinae, a system that continued until recently (for a detailed taxonomic history see Stuessy, 1973, 1977). In a more modern treatment of the Melampodiinae, Stuessy (1973) created groups of generic affinity in which *Espeletia* s.l. was linked with both *Polymnia* (i.e., *Smallanthus*) and *Unxia*. Later, in his broader study of the Heliantheae, Stuessy (1977) placed *Espeletia* s.l. in "group 2" of the Melampodiinae, which included *Polymnia* (*Smallanthus*), *Rumfordia*, *Sigesbeckia*, *Trigonospermum*, and *Unxia*; other genera of the subtribe were *Acanthospermum*, *Lecocarpus*, and *Melampodium*. While the Espeletiinae were recognized as a distinct subtribe by Robinson in his 1981 revision of tribal and subtribal limits in the Heliantheae, he thought that at least two other subtribes, the Melampodiinae and the Milleriinae, were closely allied. In this revision, the Melampodiinae were reduced from previous concepts of the subtribe to just five genera—*Smallanthus*, *Acanthospermum*, *Ichthyothere*, *Lecocarpus*, and *Melampodium*. The Milleriinae include several of the "group 2" Melampodiinae genera identified by Stuessy, such as *Axiniphyllum*, *Rumfordia*, and *Sigesbeckia*, along with *Milneria* and *Guizotia*.

More recently, Karis (1993) applied modern cladistic techniques to large sets of morphological characters across the entire tribe Heliantheae. In the results of this study, the Espeletiinae grouped not with taxa of the Melampodiinae, but rather in the subtribe Verbesiniinae, with genera such as *Zaluzania*, *Encelia*, *Flourensia*, *Verbesina*, and *Perymenium*. Both *Smallanthus* and *Polymnia* grouped with taxa traditionally associated with the Melampodiinae, such as *Rumfordia*, *Melampodium*, and *Milneria*. The most recent subtribal circumscriptions based on these data have therefore concluded that the Espeletiinae are neither deserving of subtribal status nor associated with the Melampodiinae (Karis and Ryding, 1994).

Other research, however, has shown little support for this new systematic treatment. The first molecular study that included some of these taxa did not support the results of the morphological cladistic analysis. Using chloroplast restriction site data, Panero, Jansen, and Clevinger (1999) found that species of the Espeletiinae were most closely related to the genera *Smallanthus* and *Rumfordia* in a majority of the most parsimonious trees. However, this study focused on the Ecliptiinae and therefore included only a few of the taxa in the Melampodiinae. Additional arguments for the Espeletiinae's association with the Melampodiinae are based on findings that at least some species of *Espeletia* contain melampolide sesquiterpene lactones (Torrenegra and Tellez A., 1995), phytochemicals thought to be associated (though not exclusively) with taxa in the Melampodiinae.

Additional data are needed to resolve the phylogenetic position of the Espeletiinae within the Heliantheae and to elucidate the origins of this adaptive radiation. The internal transcribed spacer (ITS) region of nuclear ribosomal DNA has been an invaluable tool in the reconstruction of plant phylogenies (Baldwin et al., 1995), especially at lower taxonomic levels, and has been useful in the identification of the closest relatives of other important adaptive radiations (e.g., Baldwin, 1992). In this paper, I present the results of a molecular phylogenetic study of DNA sequence data from the nuclear ribosomal ITS region to address the question of which taxa are most closely related to the Espeletiinae.

## MATERIALS AND METHODS

**Taxon sampling and collecting**—All Espeletiinae accessions and some *Smallanthus* were collected in the field and dried in silica gel for preservation of DNA. Vouchers were deposited in the Missouri Botanical Garden (MO) as well as national and local herbaria in Venezuela (VEN, MER, MERF, PORT) and Colombia (COL, FMB) (see Index Herbariorum for abbreviations). All other material was sampled from herbarium sheets from the Missouri Botanical Garden and the Field Museum of Natural History, Chicago (F). The taxa and accessions are listed in the Appendix (<http://ajbsupp.botany.org/v89/>). Taxa were chosen that represented a broad sampling of the potential close relatives of the Espeletiinae, on the basis of the taxonomic work of Robinson (1981) and Stuessy (1977); in particular, taxa from the subtribes Melampodiinae and Milleriinae (sensu Robinson) were selected. Additional taxa (e.g., *Zaluzania*, *Encelia*) were included to test the alternative hypothesis suggested by the results of the morphological cladistic analysis (Karis, 1993; Karis and Ryding, 1994). After a preliminary analysis, several of the taxa that seemed closely related to the Espeletiinae were sampled more thoroughly to test for monophyly and to increase resolution on long branches. Within the Espeletiinae, taxa were chosen from all eight genera and included a broad sample of the morphological and geographical variation found in the subtribe.

**DNA extraction, amplification, and sequencing**—Total DNA was extracted from both silica-dried and herbarium material using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987; Hillis et al., 1996), with a 4% (mass per volume) CTAB solution. Three separate 20- $\mu$ L polymerase chain reactions (PCRs) were set up for each individual in order to decrease the risk that observed polymorphisms would result from PCR error. Each reaction contained a final concentration of 2.5 mmol/L  $MgCl_2$ , 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 0.2 mmol/L of each deoxynucleotide, 0.1 mg/mL bovine serum albumine (BSA), 0.2 mmol/L of each primer, and 0.5 units/ $\mu$ L Taq polymerase. The cycling program included 30 s of denaturation at 94°C, followed by 35 cycles of amplification (94°C [20 s], 54°–56°C [35 s], 72°C [45 s]), and finally a single extension for 3 min at 72°C. Silica-dried samples were amplified as a single fragment using the primers ITS-1 (White et al., 1990) (TCC GTA GGT GAA CCT GCG G) and ITS-4 (White et al., 1990) (TCC TCC GCT TAT TGA TAT GC) at an annealing temperature of 54°C. ITS-1 and ITS-2 regions were amplified separately for herbarium samples using the following pairs of primers—(1) ITS-1 region: ITS-1 (White et al., 1990) and ITS-1 5.8S (O'Kane, 1993) (ACT CGA TGG TTC ACG GGA TT) with an annealing temperature of 54°C; (2) ITS-2 region: either ITS-3 (White et al., 1990; as modified by O'Kane, 1993) (GCA TCG ATG AAG AAC GTA GC) and ITS-4 (White et al., 1990) (TCC TCC GCT TAT TGA TAT GC) with an annealing temperature of 54°C, or ITS-5.8F (AAG CCA YTA GGC CGA G) and ITS2-26S.4 (CGC CTG ACC TGG GTG CG) with an annealing temperature of 56°C.

The three reactions were combined into a single tube and visualized on a 1.8% agarose gel. Reactions that resulted in multiple bands (often representing amplification of fungal DNA associated with the leaves; J. T. Rauscher, unpublished data) were purified on a 1.8% agarose gel, and the bands of the appropriate size were excised. Excised bands and PCR solutions from single-band amplifications were purified using either a glass milk purification method (Hillis et al., 1996) or Wizard PCR Preps (Promega, Madison, Wisconsin,

USA). Most sequencing was done manually using the *fmol* DNA cycle sequencing system (Promega) labeled with [<sup>35</sup>S]α-dATP, run on a 6% Long Ranger (FMC BioProducts, Rockland, Maine, USA) gel, and exposed to x-ray film; other sequences were obtained using an automated sequencer (ABI 377; at the sequencing facility maintained by the International Center for Tropical Agriculture, Cali, Colombia) and ABI Prism BigDye Terminator Cycle Sequencing kits with Amplitaq DNA polymerase (Applied Biosystems, Foster City, California, USA). Both 5' and 3' strands were sequenced for each PCR product, and careful attention was paid to the potential for signal from more than one sequence (double bands or peaks), as might be expected in a multicopy gene such as ITS. GenBank accession numbers are listed in the Appendix (<http://ajbsupp.botany.org/v89/>).

**Analysis**—All sequences (including ITS-1, the 5.8S gene, and ITS-2) were aligned by eye using Se-Al, version 1.d1 (Rambaut, 1995). Gaps were considered as missing data and either ignored or coded separately in a presence/absence matrix. Phylogenetic analysis was done using PAUP\* 4.0b4 (Swofford, 2000). Nucleotide sites with more than one band or peak were coded as degenerate sequence and treated as “polymorphic” under the parsimony options of PAUP\*, because this variation most likely resulted from divergent repeat copies. Trees were constructed using the maximum parsimony criterion and an heuristic search with 1000 random additions (tree bisection-reconnection [TBR] branch swapping, MULPARS, ACCTRAN). From the resulting most parsimonious trees, a strict consensus tree was constructed. Clade support was estimated using both bootstrap values (500 searches with 10 random additions, MAXTREES = 100, implemented in PAUP\*) and decay-index values (Bremer, 1988; Donoghue et al., 1992). In addition, pairwise sequence divergences were calculated for all taxa.

To examine alternative, less parsimonious topologies, I constructed constraint trees in MacClade 4.0 (Maddison and Maddison, 1992) that forced the Espeletiinae to form sister-taxon relationships with clades not suggested by the most parsimonious trees. Heuristic searches, with 500 random additions, were performed in PAUP\* to find the shortest trees consistent with these alternative topologies. To test the statistical significance of the difference in length between the topologically constrained trees and the optimal trees, I used PAUP\* to perform a nonparametric Wilcoxon's signed-ranks test (Templeton, 1987; also see Mason-Gamer and Kellogg, 1996). A single tree from the set of most parsimonious trees was compared with one of the trees found during each constrained search, and the *P* value calculated. If the *P* value was close to significant, at either the one-tailed or two-tailed level, several other randomly chosen trees were tested to estimate the range of possible *P* values that might result from comparing different equally parsimonious trees within each of the sets of trees.

## RESULTS

The final aligned data matrix for the 65 sampled taxa consisted of 688 nucleotide positions; unaligned sequences ranged from 635 to 654 base pairs (bp) in length, while the matrix of insertions and deletions included 72 characters. Both the maximum within-clade sequence divergence of several major clades and the range of divergence between taxa in the Espeletiinae and taxa in these other clades are shown in Table 1. In the first phylogenetic analysis, in which gaps were treated as missing and the insertion/deletion matrix excluded, 379 of the 688 characters were variable, 302 of which were parsimony informative. The search resulted in 30 shortest trees of 1350 steps (1264 if polymorphisms were scored as ambiguous) with a consistency index of 0.51, a retention index of 0.75, and a rescaled consistency index of 0.38. Including the insertion/deletion character matrix added an additional 44 parsimony-informative characters and resulted in 120 most parsimonious trees of 1449 steps (1363 with polymorphisms ambiguous) with a consistency index of 0.52, a retention index of 0.76, and a rescaled consistency index of 0.40. The differ-

TABLE 1. Within- and between-group percent nucleotide sequence divergence for the *Espeletia* complex and closely related clades of taxa from the ITS gene tree.

Taxa	Approximate maximum within-group divergence (%)	Approximate divergence from Espeletiinae (%)
<i>Espeletia</i> complex	3.9	—
<i>Ichthyothere</i>	12.3	8.7–13.6
<i>Smallanthus</i>	7.3	6.9–11.2
<i>Rumfordia</i> I	1.6	5.7–8.7
<i>Ichthy.-Small.-Rumf.</i> I	15.9	5.7–13.6
<i>Trig.-Sig.-Axini.-Mil.-Rumf.</i> II	10.4	8.5–13.5
<i>Melampod.-Acanth.-Leco.</i>	15.2	9.8–15.8

*Note:* The second column shows the approximate maximum percentage sequence divergence calculated within the taxon or group of taxa indicated. The third column indicates the range of divergence values obtained through pairwise comparisons of all sequences in that taxon or clade to all sequences in the *Espeletia* complex. Ranges should be considered approximate owing to limited sampling of some taxa. See text for names of abbreviated genera.

ence in the number of most parsimonious trees found in the two analyses is explained by two insertions: one supporting an alternative topology for the clade containing *Rumfordia floribunda* DC., *R. revealii* H. Rob., and *R. penninervis* S.F. Blake, and another supporting the monophyly of *Sigesbeckia* over the paraphyletic arrangement suggested by the data with insertion/deletions excluded. All subsequent analyses included the insertion/deletion matrix.

The strict consensus of the 120 most parsimonious trees is shown in Fig. 1. One of the most parsimonious trees is represented as a phylogram in Fig. 2, upon which insertions and deletions have been mapped and labeled to indicate homoplasies. Although both these trees have been rooted so that *Guardiola* appears to be the sister taxon to a large clade of Melampodiinae taxa (Fig. 1, clades I–III), the true root is unknown owing to the wide taxonomic sampling and lack of an appropriate outgroup. Midpoint rooting groups *Guardiola* with the non-Melampodiinae outgroups. Despite the lack of a reliable root, clades I–III (Fig. 1) are assumed to be a monophyletic group for the purposes of the subsequent results and discussion. This assumption is justified by previous taxonomic work that has identified most of the taxa contained in this clade as belonging to a closely related group of genera (Stuessy, 1977; Robinson, 1981). To identify the true root of the tree requires a much broader analysis of the entire tribe Heliantheae.

The consensus of the most parsimonious trees supports the placement of the Espeletiinae within a group of genera considered members of the subtribe Melampodiinae (sensu Stuessy, 1977 and Robinson, 1981) or Milleriinae (sensu Robinson, 1981) (Fig. 1, clades I–III). Of these taxa, the genus *Ichthyothere* is most closely related to the Espeletiinae, supported by a minimum of six unambiguous nucleotide changes, a decay-index value of two, and a bootstrap value of 73%. Other close relatives include the genus *Smallanthus* (clearly phylogenetically distant from the three species of *Polymnia* sampled in this study) and one species of *Rumfordia*. The sister taxon to this grouping (Fig. 1, clade I) is a clade including *Trigonospermum*, *Milleria*, *Sigesbeckia*, *Axiniphyllum*, and the other three sampled species of *Rumfordia* (Fig. 1, clade II). Somewhat more distantly related is a group including *Melampodium*, *Acanthospermum*, and *Lecocarpus* (Fig. 1, clade III).

In order to estimate the support of these data for grouping

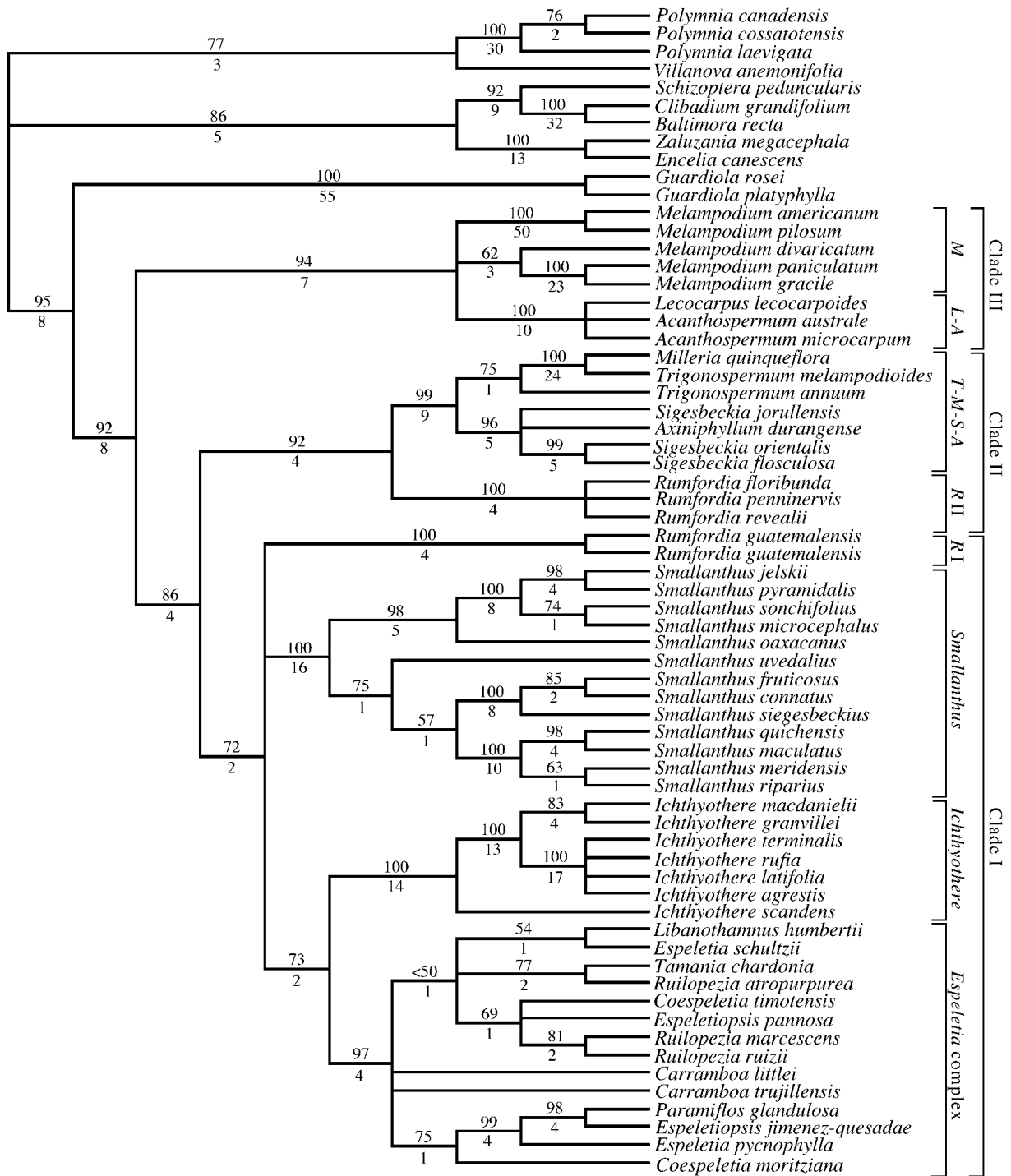


Fig. 1. Strict consensus of the 120 maximally parsimonious trees for ITS sequences from the *Espeletia* complex and potential close relatives in the Heliantheae. Numbers above the branches are percentage bootstrap values based on 1000 replications; numbers below are decay-index values.

the Espeletiinae with clades other than just *Ichthyothere*, several constraint trees were created. The results for each of the alternative topologies tested, including the length of the most parsimonious constrained trees, the number of additional steps required, and the *P* value resulting from Templeton's (1987) application of Wilcoxon's signed-ranks test are shown in Table 2. Among the suboptimal alternatives analyzed, the next most parsimonious reconstruction of the ITS data was a tree 1451

steps long (two steps less parsimonious than the optimal tree) in which *Ichthyothere*, *Smallanthus*, and *Rumfordia guatemalensis* (J.M. Coult) S.F. Blake form a clade that is the sister taxon to the Espeletiinae (Table 2b). A tree with four additional steps makes a clade containing just *Ichthyothere* and *Smallanthus* the sister taxon (Table 2c). Trees that force *Smallanthus* to be the closest relative of the Espeletiinae require five extra steps (Table 2d), as do three other topologies (Table

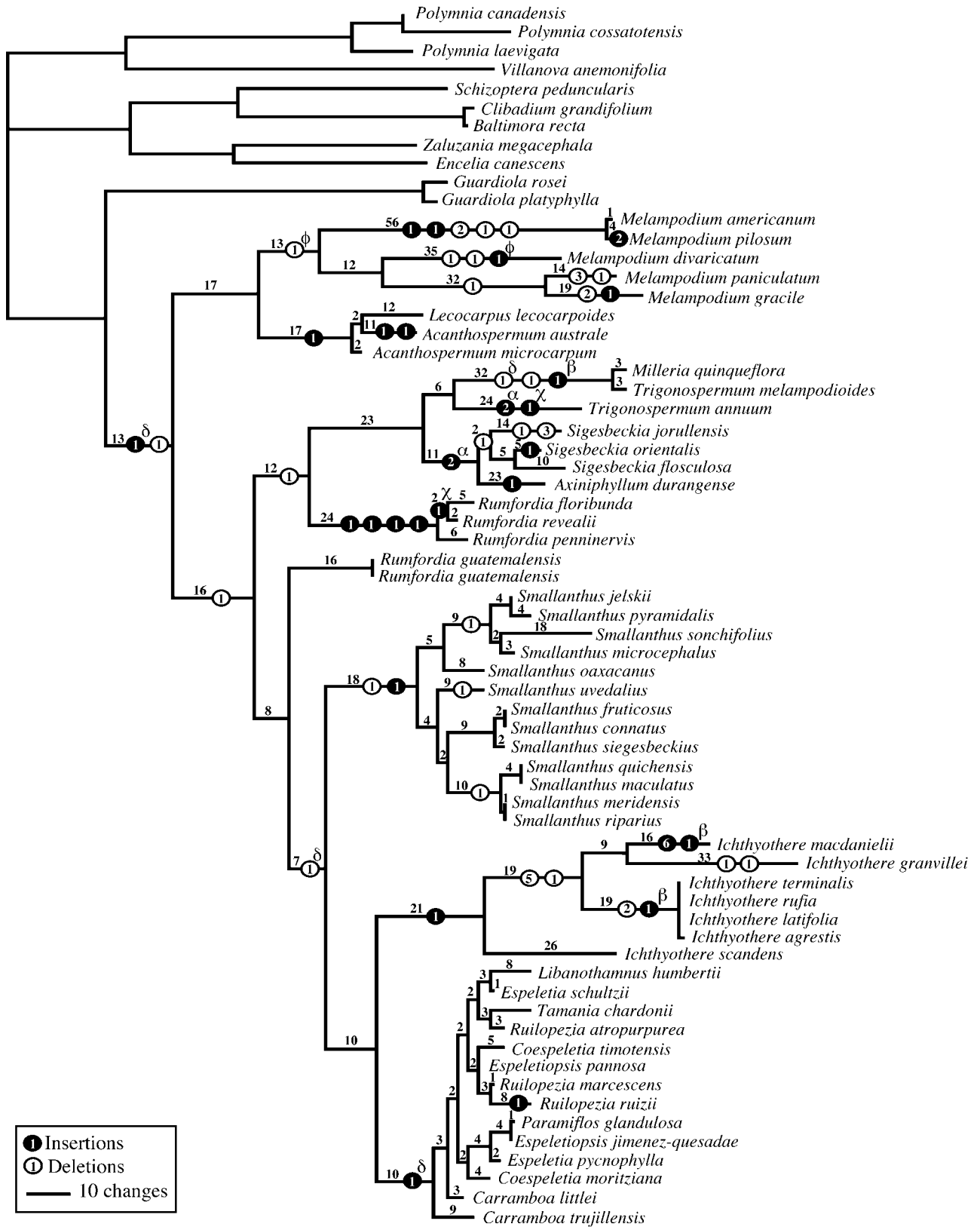


Fig. 2. Phylogram of one of the 120 maximally parsimonious trees for ITS sequences from the *Espeletia* complex and potential close relatives in the Heliantheae. Numbers above branches are branch lengths. Insertions (black circles) and deletions (white circles) are mapped on the branches for taxa most closely related to the *Espeletia* complex; numbers in each circle indicate the length in nucleotides of the insertion or deletion; symbols identify homoplasious changes.

TABLE 2. Tree lengths and results of Wilcoxon's signed-ranks test for maximally parsimonious trees found when different taxa or groups of taxa are forced to be the closest relatives of the *Espeletia* complex.

Sister taxon to the <i>Espeletia</i> complex	Shortest tree length	Added steps	Wilcoxon <i>P</i> values
a) <i>Ichthyothere</i>	1449	—	—
b) <i>Ichthy.-Small.-Rumfordia</i> I	1451	+2	0.70
c) <i>Ichthyothere-Smallanthus</i>	1453	+4	0.42
d) <i>Smallanthus</i>	1454	+5	0.31
e) <i>Ichthyothere-Rumfordia</i> I	1454	+5	0.24
f) <i>Rumfordia</i> I	1454	+5	0.32
g) <i>Rumfordia</i> I- <i>Smallanthus</i>	1454	+5	0.31
h) <i>Ichthy.-Small.-R I-R II-Trigon.-Mill.-Sigesbeck.-Axini.-Melampod.-Acanth.-Leco.</i>	1461	+12	0.04–0.13**

Notes: *P* values are calculated for Wilcoxon's signed-ranks test (as implemented in the Templeton [1987] test) by comparing the most parsimonious tree to the constrained tree. Values are approximate since in most cases only one of the multiple constrained trees has been compared to one of the 120 most parsimonious unconstrained trees. The range of *P* values in the last row is based upon multiple comparisons of constrained and unconstrained trees. See text for full names of abbreviated genera. \*\* *P* < 0.05 (two-tailed).

2e, f, g). Most of the alternative topologies listed in Table 2 are not significantly different from the most parsimonious tree under the Templeton test (*P* < 0.05, two-tailed). Topologies in which the Espeletiinae clade was forced outside of the entire clade of Melampodiinae and Milleriinae resulted in significant *P* values (Table 2h).

The ITS data provided strong support for the monophyly of the Espeletiinae, *Ichthyothere* and *Smallanthus*, but not for the genus *Rumfordia* (Fig. 1). Three of the species, *R. floribunda*, *R. revealii*, and *R. penninervis* (*Rumfordia* II, Fig. 1) form a well-supported clade (bootstrap 100%, decay-index 18), which is the sister taxon to the clade containing *Milleria*, *Trigonospermum*, *Sigesbeckia*, and *Axiniphyllum*. The remaining species, *R. guatemalensis*, is either the sister taxon to an Espeletiinae-*Ichthyothere-Smallanthus* clade (Fig. 3a) or to an Espeletiinae-*Ichthyothere* clade (Fig. 3b) in the set of most parsimonious trees. Alternative topologies were also tested with respect to the phylogeny of *Rumfordia*. Forcing *R. guatemalensis* to group with clade II (Fig. 1) instead of clade I (Fig. 1) results in a tree two steps longer (Fig. 3c); forcing the Rum-

fordia II clade to group with clade I (Fig. 1) instead of clade II (Fig. 1) requires four additional steps (Fig. 3d). The shortest trees consistent with a monophyletic *Rumfordia* were 1456 steps longer than the most parsimonious tree (Fig. 3e), though still not quite significant under the Templeton test. The final rearrangement tested, in which *Rumfordia* was forced to group with other taxa possessing functionally hermaphroditic disc florets (*Axiniphyllum* and *Sigesbeckia*), was 21 steps less parsimonious and highly significant under the Templeton test (Fig. 3f).

DISCUSSION

**Subtribal affinities**—The results of this analysis strongly suggest that the subtribe Espeletiinae is most closely related to taxa traditionally considered members of the subtribes Melampodiinae and Milleriinae. As previous chloroplast studies have shown (Panero, Jansen, and Clevinger, 1999), there is no evidence for the relationship between the Espeletiinae and Verbesininae suggested by morphological cladistic analyses (Kar-

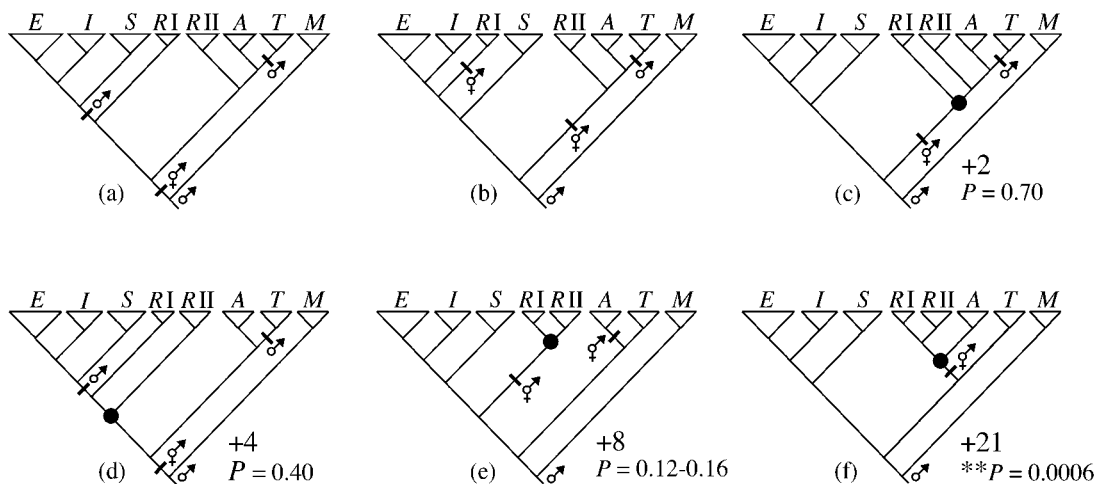


Fig. 3. Alternative tree topologies with implications for disc-floret evolution in taxa closely related to the *Espeletia* complex. The first two trees (a) and (b) represent different topologies within the set of 120 maximally parsimonious unconstrained trees. Other trees (c–f) represent the most parsimonious topologies required when branches are constrained at the point on the tree indicated by black circles. Next to each constrained tree is the minimum number of additional steps required and the approximate *P* value of the Templeton (1987) test when the topology is compared to one of the most parsimonious unconstrained trees. *P* values should be considered approximate (as explained in Table 2). Symbols for hermaphroditic and functionally male disc florets on each tree indicate the minimum number of evolutionary changes in this character required for each alternative topology.

Figure abbreviations: E = *Espeletia* complex; I = *Ichthyothere*; S = *Smallanthus*; R I = *Rumfordia* I; R II = *Rumfordia* II; A = *Axiniphyllum*, *Sigesbeckia*; T = *Trigonospermum*, *Milleria*; M = *Melampodium*, *Acanthospermum*, *Lecocarpus*.

is, 1993); in fact, the Templeton test statistically rejects the possibility that the Espeletinae fall outside the larger group of Melampodiinae and Milleriinae (Table 2h). These results make it difficult to justify subtribal status for the eight genera of the Espeletinae. Although clearly a monophyletic group (see below), this clade is phylogenetically nested within the sampled taxa, and, as a result, formal taxonomic recognition of the Espeletinae will make any broad concept of the Melampodiinae necessarily paraphyletic. Instead, it may be best to recognize these genera as a closely related complex within the Melampodiinae. For the remainder of this discussion, these genera will be collectively called the “*Espeletia* complex.”

In general, the relationships suggested by the ITS phylogeny of these taxa support previous noncladistic circumscriptions of the Melampodiinae (or sections within it) based on morphological data. Two of the major clades resulting from this study (Fig. 1, clades I and II; clade III) correspond remarkably well to the “group 1” (*Melampodium*, *Acanthospermum*, and *Lecocarpus*) and “group 2” (*Espeletia*, *Polymnia* [*Smallanthus*], *Rumfordia*, *Sigesbeckia*, *Trigonospermum*, and *Unxia* [not sampled in this study]) Melampodiinae suggested by Stuessy (1977). While Robinson’s concept of the subtribe Milleriinae seems to be an artificial subset of a broader Melampodiinae based on these data, his work (Robinson, 1981) recognizes several taxa that Stuessy did not consider part of this group, such as *Ichthyothere*, *Axiniphyllum*, and *Milleria*.

***Ichthyothere*: sister taxon to the *Espeletia* complex?**—This study is the first to suggest that species of the genus *Ichthyothere* are very closely related to and perhaps the closest extant relatives of the *Espeletia* complex. Although Robinson’s previous taxonomic work suggested that *Ichthyothere* might be closely related (at least in a closely allied subtribe), it was never proposed as the closest relative. This is not surprising given the significant morphological evolution both groups have undergone since their divergence. The *Espeletia* complex’s radiation of morphology and ecology is well known (Cuatrecasas, 1976; Monasterio and Sarmiento, 1991). *Ichthyothere* is also morphologically distinct compared with its closest relatives. Its characteristically small capitulum with few (usually one or two) fertile ray florets has contributed to its uncertain taxonomic position within the Heliantheae. In the most recent treatments, *Ichthyothere* has been placed in the Melampodiinae (Robinson, 1981), the Milleriinae (Stuessy, 1977), and a group of taxa unclassified to subtribe (Karis and Rytting, 1994).

Despite this morphological divergence, there may be unique characters that support the relationship between the *Espeletia* complex and *Ichthyothere*. One such character is the lack of an expanded outer series of herbaceous involucre bracts. Most taxa within the Melampodiinae and Milleriinae (sensu Robinson) possess a distinct, expanded outer series of involucre bracts, or phyllaries. *Smallanthus* and *Rumfordia*, in particular, are noted for their broad, leafy phyllaries. In *Ichthyothere*, however, the phyllaries tend to be highly reduced. Similarly, the *Espeletia* complex lacks this distinct outer series, although the phyllaries are not reduced to the extent they are in *Ichthyothere*. A single exception in the *Espeletia* complex is the species *Paramiflos glandulosa* Cuatrec., which does possess a distinct outer series of glandular phyllaries (Cuatrecasas, 1995); *Paramiflos*, however, is almost certainly nested within the *Espeletia* complex (Fig. 1) so this character is probably not plesiomorphic. Alone, the lack of expanded phyllaries is,

at best, weak morphological evidence for the relationship between these two groups. More detailed study of the development and anatomy of the phyllaries and other morphological characters in these taxa is needed.

**Alternative hypotheses**—The ITS data alone provide only moderate support for the relationship between the *Espeletia* complex and *Ichthyothere*. Although the branch uniting these taxa includes six unambiguous nucleotide synapomorphies, homoplasy in these characters results in only moderate bootstrap (73%) and decay-index (2) values. Given the moderate support for this relationship, alternative phylogenetic hypotheses must be considered. The approach used in this study was to create an explicit hierarchy of likely alternative hypotheses and estimate their relative support given this data set. Relative support for each alternative can be demonstrated by both the number of additional steps that would be required on the tree and the approximate *P* value resulting from a Templeton test comparing the alternative trees with the most parsimonious trees.

This hierarchy of likely alternatives is shown in Table 2 by the number of additional steps required to force different sister taxon relationships. The next most parsimonious topology supported by the ITS data makes the *Espeletia* complex the sister taxon to a diverse clade containing *Ichthyothere*, *Smallanthus*, and *Rumfordia guatemalensis*; at a cost of only two additional steps (Table 2c), this should be considered an important alternative hypothesis. Though somewhat less likely given the additional five steps required, *Smallanthus* or *R. guatemalensis* may also be candidates for being closest relatives of the *Espeletia* complex. Because alternative topologies less than 11 or 12 steps longer than the most parsimonious tree cannot be rejected statistically using the Templeton test (Table 2), these less parsimonious alternatives should be considered until additional phylogenetic data are available.

**Monophyly of the *Espeletia* complex**—The ITS sequence data strongly support the monophyly of the *Espeletia* complex. This result is not surprising, given the number of cytological and morphological synapomorphies shared by members of this group. All species examined to date are exclusively diploid with a base chromosome number of  $n = 19$  (Powell and King, 1969; Powell and Cuatrecasas, 1970, 1975; Powell and Powell, 1978; Spooner et al., 1995; Carr et al., 1999). Cuatrecasas (1986) identified several morphological features that distinguish the *Espeletia* complex, such as spirally arranged xeromorphic or coriaceous leaves as well as aspects of achene structure and other floral characteristics. Molecular evidence supporting the monophyly of the complex has also been presented in chloroplast restriction studies (Panero, Jansen, and Clevinger, 1999).

Because of the small number of taxa included in this analysis, a detailed discussion of relationships within the *Espeletia* complex will be reserved for forthcoming results. However, these preliminary analyses suggest a taxonomically relevant pattern worth mentioning briefly. Several of the genera proposed by Cuatrecasas (1976) seem to be polyphyletic and/or paraphyletic on the ITS gene tree, including *Espeletia*, *Espeletopsis*, *Coespeletia*, and *Ruilopezia*. Additional sampling within the complex, however, is required before the phylogenetic status of these genera can be reliably assessed.

***Ichthyothere***—The genus *Ichthyothere* is a group of about 25 species distributed in South and Central America. The monophyly of the genus was strongly supported by both bootstrap and decay-index values (Fig. 1) as well as by a single base-pair deletion (Fig. 2). Although sampling was limited in this study to only seven species (and only a single representative from each species), the resolution detected within *Ichthyothere* permits some preliminary discussion of evolution within the genus. Most notable is the well-supported basal split between *I. scandens* S.F. Blake and all other species sampled in this study. *Ichthyothere scandens*, found in Central and northwestern South America, differs from the rest of the genus by 14 unambiguous characters, including two deletions, one of which is a rare 5-bp deletion on the 5' end of the ITS-1 sequence. In the original publication of this species, Blake (1921) described *I. scandens* as morphologically distinct from all other species of *Ichthyothere* on the basis of its scandent habit and its capitula grouped in loose panicles. Robinson (1980) also recognized the distinctiveness of this species and suggested that it and another species from Colombia, *I. garciabarrigae* H. Rob. (not sampled in this study), belong to a unique subgenus within *Ichthyothere*. Both are typically found at elevations above 1000 m.

The other species of *Ichthyothere* sampled here are small, erect herbs characterized by tightly clustered or glomerulate groups of capitula and are generally found below 1000 m elevation in or near the Amazon basin. The first of two clades found within this group of species includes *I. granvillei* H. Rob. and *I. macdanielii* H. Rob. Robinson's work (1983) suggests that species such as *I. davidsei* H. Rob. and perhaps *I. petiolata* H. Rob. may be closely related to this clade as well. The second clade includes *I. rufia* Gardn., *I. agrestis* Baker, *I. terminalis* (Spreng.) S.F. Blake, and *I. latifolia* Baker and shows little to no interspecific sequence variation.

***Smallanthus***—The genus *Smallanthus*, though perhaps not the sister taxon to the *Espeletia* complex, is clearly a close relative. Like the *Espeletia* complex and *Ichthyothere*, the monophyly of the genus, for the taxa sampled, is highly supported by both bootstrap and decay-index analyses (Fig. 1). Robinson's decision to separate *Smallanthus* from the genus *Polymnia* (Robinson, 1978) is well justified; the three sampled species of *Polymnia* form a distinct clade, which is phylogenetically distant from *Smallanthus* and from all taxa traditionally included in or considered close to the Melampodiinae.

Among the 13 species of *Smallanthus* sampled in this study (from about 20 known species), the ITS tree contains at least four distinct lineages. The first includes five taxa—four South American species, *S. jelskii* (Hieron.) H. Rob., *S. pyramidalis* (Triana) H. Rob., *S. sonchifolius* (Poepp. & Endl.) H. Rob., and *S. microcephalus* (Hieron.) H. Rob., and one Mexican–Central American species, *S. oaxacanus* (Sch. Bip. ex Klatt) H. Rob., the sister taxon to the South American clade. This split between South American and Central American species is also found in two of the other major lineages. In the first, the South American species *S. siegesbeckius* (DC.) H. Rob. groups with the Central American species *S. fruticosus* (Benth.) H. Rob. and *S. connatus* (Spreng.) H. Rob.; in the second, *S. meridensis* (Steyerm.) H. Rob. and *S. riparius* (Kunth) H. Rob. (northwestern South America) group with *S. maculatus* (Cav.) H. Rob. and *S. quichensis* (Coul.) H. Rob. (Central America). That three of the major clades of *Smallanthus* contain both South American and Central or North Amer-

ican species suggests that dispersal across the Isthmus of Panama has occurred multiple times in the history of the genus. The fourth major clade contains a single species, *S. uvedalius* (L.) Mackenzie, the only species distributed as far north as the United States.

***Rumfordia***—On the basis of the results of this study, the genus *Rumfordia* is probably not monophyletic. Although monophyly cannot be rejected unequivocally by the Templeton test, the additional eight steps required to make *Rumfordia* monophyletic (Fig. 3e) suggest that it is unlikely. *Rumfordia* is either paraphyletic (both with respect to the *Sigesbeckia*–*Axiniphyllum*–*Trigonospermum*–*Milleria* clade and the *Espeletia* complex–*Ichthyothere*–*Smallanthus* clade) or polyphyletic in the most parsimonious trees (Fig. 3a, b); the specific topology is poorly resolved owing to a lack of characters that identify the proper position of the *R. guatemalensis* (*Rumfordia* I) clade. The suboptimal tree, in which *Rumfordia* is still paraphyletic but *R. guatemalensis* is basal with respect to the *Trigonospermum*–*Milleria*–*Sigesbeckia*–*Axiniphyllum* clade (Fig. 3c), is only two steps less parsimonious and thus a strong alternative hypothesis.

The molecular distinction between the *R. guatemalensis* and *R. floribunda-revealii-penninervis* (*Rumfordia* II) clades is not unexpected given previous morphological work. Both Greenman (1903) and Sanders have noted that *R. guatemalensis* is more similar to species of *Smallanthus* based on its “habit, leaf form, texture and venation, inflorescence pubescence, phyllary shape, ray- and disk-corolla structure and number, and anther structure” (Sanders, 1977, p. 302). Its placement in the genus *Rumfordia*, however, was justified mainly by its possession of perfect disc florets, thought to be a synapomorphy of the genus or a larger clade; other closely related taxa (e.g., the *Espeletia* complex, *Smallanthus*, *Ichthyothere*) have male-sterile disc florets. Additional evidence for the distinction between these two clades comes from chromosome numbers; *R. floribunda* has a base number of  $n = 24$  (Sanders, 1977) while *R. guatemalensis* is  $n = 13$  (Strother, 1983).

**Other taxa and implications for disc-floret evolution**—Closely related to the *Rumfordia* II clade is a well-supported (98% bootstrap, decay-index 10) clade of genera: *Trigonospermum*, *Milleria*, *Sigesbeckia*, and *Axiniphyllum*. This clade is also supported by chromosome counts, as all taxa counted to date have a base number of  $n = 15$  or are polyploids from this base number (Turner and King, 1964; Powell and King, 1969; Solbrig et al., 1972; Keil and Stuessy, 1975, 1977; Jansen et al., 1984; Lane and Li, 1993; Hu and Gu, 1996). Both clades of *Rumfordia* as well as *Sigesbeckia* and *Axiniphyllum* are unusual with respect to the rest of the Melampodiinae because of their perfect disc florets; all other taxa have functionally male disc florets. Although variation in this character originally led Bentham and Hooker (1873) to place taxa with perfect disc florets, such as *Rumfordia* and *Sigesbeckia*, outside the subtribe Melampodiinae, the close relationship between these species was presaged by several authors, including McVaugh and Anderson (1972), McVaugh and Laskowski (1972), Stuessy (1977), Turner (1978a, b), and Robinson (1981).

If having functionally male disc florets is plesiomorphic for the taxa of interest in this study (as is suggested by the fact that all the closest outgroups, including *Melampodium*, *Lecocarpus*, *Acanthospermum*, and *Guardiola*, have this character

state), then both of the two most parsimonious trees require a minimum of three changes: either a change to hermaphroditic disc florets and two reversals (Fig. 3a) or two changes to hermaphroditic discs and a single reversal to functionally male discs (Fig. 3b). In an alternative tree, two steps less parsimonious, in which *Rumfordia* is paraphyletic (Fig. 3c), only two changes are required, one change to perfect discs and a single reversal. While it is unclear whether the true history of these taxa involved two or three changes in disc-floret system of mating, it is certain that more than a single change is required, since a single change to hermaphroditic disc florets requires a tree 21 steps less parsimonious and statistically rejected under the Templeton test (Fig. 3f). It is not surprising that this character can be quite evolutionarily labile in these taxa and other Heliantheae. Stuessy (1973) has previously discussed the artificiality of grouping genera by the sterility of disc florets while Turner (1978a) has argued that frequent changes in disc-floret function between closely related species of *Chrysanthellum* resulted from a simple underlying genetic system.

As mentioned previously, the well-supported clade that contains *Melampodium*, *Acanthospermum*, and *Lecocarpus* (Fig. 1, clade III) agrees with the informal "group 1" Melampodiinae identified by Stuessy (1973) on the basis of shared possession of inner involucral bracts that enclose and fuse with the ray achenes. While the ITS data in this study provide no information with respect to the monophyly of *Melampodium*, there is strong support for the relationship between continental species of *Acanthospermum* and the Galapagos Islands endemic genus *Lecocarpus*.

**Chromosome evolution**—The likely base chromosome number of several of the major lineages sampled here is well established; for others it is less clear. The *Espeletia* complex is  $n = 19$ , with no known polyploidy. The chromosomes of several species of *Smallanthus* have been counted (e.g., Turner, Powell, and King, 1962; Solbrig et al., 1972; Robinson et al., 1981; Carr et al., 1999), and although there is variation in the genus, including polyploid counts, the lowest and by far the most common count is  $n = 16$ , suggesting that it may be the base number. Chromosome number for *Ichthyothere* is known for only two species, one of which is  $n = 16$  (Turner et al., 1979), the other, a likely polyploid, with a number of ca. 33 (Coleman, 1970). Therefore, estimating the base number is speculative at best. However, given that *Smallanthus* is probably a close relative and also has a count of  $n = 16$ , this base number may be accurate. As stated previously, the base number for the *Rumfordia* I clade is  $n = 13$  while that for the *Rumfordia* II clade is  $n = 24$  (possibly a result of polyploidy from an ancestor with a count of  $n = 12$ ). A base number of  $n = 15$  is likely for the clade including *Axiniphyllum*, *Sigesbeckia*, *Trigonospermum*, and *Milleria*. Finally, the base chromosome number for the clade containing *Melampodium*, *Lecocarpus*, and *Acanthospermum* is difficult to estimate at this time; species of *Melampodium* are quite variable, with low numbers ranging from 8 to 12 (e.g., Stuessy, 1972; Keil and Stuessy, 1975; Robinson et al., 1981; Jansen et al., 1984), while *Lecocarpus* and *Acanthospermum* both are  $n = 11$  (Eliasson, 1970; Jansen et al., 1984; Husaini and Iwo, 1990; Jose and Mathew, 1995).

Figure 4 shows the estimated base chromosome numbers for the taxa of interest and a possible reconstruction of chromosome evolution inferred from the ITS gene tree. A hypothesis that can be reliably inferred from this tree is that the com-

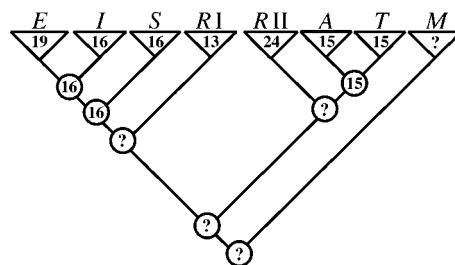


Fig. 4. Evolution of chromosome number mapped on one of the most parsimonious ITS trees for taxa closely related to the *Espeletia* complex. Base chromosome numbers are shown for each taxon or clade and hypothesized ancestral base chromosome numbers are shown in circles at each node. Nodes marked with "?" indicate ambiguous ancestral reconstructions. Figure abbreviations: E = *Espeletia* complex; I = *Ichthyothere*; S = *Smallanthus*; R I = *Rumfordia* I; R II = *Rumfordia* II; A = *Axiniphyllum*, *Sigesbeckia*; T = *Trigonospermum*, *Milleria*; M = *Melampodium*, *Acanthospermum*, *Lecocarpus*.

mon ancestor of the *Espeletia*-*Ichthyothere*-*Smallanthus* clade had a base number of  $n = 16$ . The origin of the *Espeletia* complex may, therefore, be associated with an aneuploid increase in chromosome number to  $n = 19$ .

**Implications for the origin of the *Espeletia* complex**—This first estimate of relationships among the taxa most closely related to the *Espeletia* complex can provide some preliminary insights into the origins of this adaptive radiation. Cuatrecasas (1986) suggested that the ancestors of the complex were woody, a trait reflecting the supposed ancestral habit of the Heliantheae and Asteraceae. While the most recent common ancestor of the extant species of *Espeletia* may indeed have been a woody tree, there is no evidence that this is plesiomorphic. In fact, most species of *Ichthyothere*, *Smallanthus*, *Rumfordia*, *Sigesbeckia*, *Axiniphyllum*, *Trigonospermum*, and *Milleria* are herbs or erect herbs. Although some species of *Smallanthus* are woody shrubs and small trees, there is no reason to assume that this habit is ancestral in this genus or any of the other closely related genera. Therefore, if the most recent common ancestor of the *Espeletia* complex was a woody tree, this habit was likely the result of relatively recent evolution from more herbaceous ancestors.

The time of origin of the *Espeletia* complex is not discussed in detail in this paper, but it is worth noting that levels of sequence divergence may be consistent with previously proposed hypotheses. Because the northern Andes uplifted to their present elevations only during the late Pliocene or early Pleistocene, the upper montane forests and paramos of this area are thought to have existed only for the last 2–4 million years (my; Van der Hammen and Cleef, 1986). This approximate time range has been used as an estimate of when the early ancestors of the *Espeletia* complex arose. Similarly, in the silversword radiation of Hawaii, another adaptive radiation in the Heliantheae, the age of the most recent common ancestor has been estimated at about 5 my BP (Baldwin and Sanderson, 1998). When the levels of sequence divergence between these two radiations are compared, they are quite similar. As shown in Table 1, the maximum nucleotide divergence within the *Espeletia* complex found in this study was approximately 3.9%; ITS studies in the silverswords reported a maximum within-group divergence of about 4.5%. In addition, the ca. 6–14% range of divergence between *Espeletia* and its purported closest relatives (*Ichthyothere*, *Smallanthus* and *Rumfordia guatemalensis*) is

similar to that reported between the silverswords and their closest relatives in California, ca. 5.6–10.5%. Until we better understand the phylogeny of the Heliantheae, we cannot know how comparable the rates of sequence evolution between these two groups are. Nevertheless, assuming that these rates are not significantly dissimilar, the observed levels of sequence divergence may reflect the similar time scale in which evolution in these taxa is thought to have occurred.

**Conclusions**—From the consensus ITS gene tree presented in this analysis and its approximate concordance with previous taxonomic work, it is clear that the *Espeletia* complex is a member of the subtribe Melampodiinae and closely related to such genera as *Ichthyothere*, *Smallanthus*, and *Rumfordia*. While the precise intergeneric and interspecific relationships of these taxa and whether *Ichthyothere* is the closest extant relative of the complex are still not known, analysis of the most parsimonious and several less parsimonious trees has provided a more specific set of hypotheses of phylogeny, which can now be tested. Future work, including analysis of additional taxa and other nuclear and chloroplast loci, should strengthen our phylogenetic inferences and broaden our insight into both evolution of the Melampodiinae and the origin and adaptive radiation of the *Espeletia* complex.

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