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A phylogeny of the Munnoziinae (Asteraceae, Liabeae): circumscription of *Munnozia* and a new placement of *M. perfoliata*

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Abstract. The tribe Liabeae (Compositae, Cichorioideae) comprises three subtribes, Liabinae, Munnoziinae, and Paranepheliinae. For one of these, the Munnoziinae, which contains the genera *Munnozia*, *Chrysactinium*, *Erato*, and *Philoglossa*, the nuclear ITS (internal transcribed spacer) region was sequenced to examine the monophyly of the subtribe and the core genus *Munnozia* within it. Thirty-six samples representing four currently recognized genera of Munnoziinae and two outgroups were included in this study. Molecular phylogenetic analyses confirm the close relationship of *Munnozia* with *Chrysactinium*, and *Erato* with *Philoglossa*. However, the monophyly of the Munnoziinae and *Munnozia* is not supported, in disagreement with the current morphological findings. The discrepancies were attributed to the placements of *Munnozia perfoliata* outside the Munnoziinae and *Munnozia*, and *Chrysactinium* within *Munnozia*. The resulting tree indicates that first, *M. perfoliata* needs to be moved out of the munnoziinae and second, *Chrysactinium* originated from within *Munnozia*. For the first finding, morphological and palynological reevaluation of this species with allegedly related species reveals additional support in agreement with molecular data. Therefore we propose that the genus *Munnozia* be re-delimited to the members having black or dark brown anther theca and sordid or reddish pappus and re-organized.

Key words: Asteraceae, Cichorioideae, Liabeae, Munnoziinae, Liabinae, Paranepheliinae, Phylogeny, internal transcribed spacer (ITS), parsimony and maximum likelihood analyses, monophyly, paraphyly, biogeography.

One of the most successful families in the flowering plants, the Compositae, consists of approximately 20 tribes with distinctive morphologies and molecular markers (Kim and Jansen 1995, Bremer 1996). Only one tribe is neotropical in its origin and distribution, the Liabeae. The Liabeae has approximately 180 species grouped into 15 genera. They are divided into three subtribal groups, Munnoziinae, Paranepheliinae, and Liabinae, based on palynological characters (Robinson 1983).

Results from the studies by Robinson (1983), Bremer (1994), and Funk et al. (1996) demonstrated the monophyly of the first two subtribes but not the Liabinae. These modern findings differ from older treatments as is evidenced by the controversial history of

classification (Cassini 1823, 1825, 1830; Lessing 1832; De Candolle 1836; Weddell 1855–1857; Hoffmann 1890–1984; Rydberg 1927; Blake 1935; Cabrera 1954; Sandwith 1956; D'Arcy 1975; Cronquist 1955; Carlquist 1976; Nash and Williams 1976) and point out the current ambiguity in the placement and relationships of the three subtribes. An example of the blurred areas of tribal and subtribal relationships has been focused on the Liabinae. Since 1983, the circumscriptions of Munnoziinae have not been questioned and all recent studies agree on the monophyly of Munnoziinae. To the contrary, our preliminary result of molecular investigation on Liabeae draws attention to this subtribe, revealing that the currently circumscribed Munnoziinae and *Munnozia* are not monophyletic.

Circumscribed by the synapomorphic character, black or very dark brown anther thecae, the subtribe Munnoziinae contains four genera and about 60 species. The Munnoziinae is readily divided into two groups (Robinson 1983, Bremer 1994, Funk et al. 1996): one lineage includes *Munnozia* and *Chrysactinium*, having spines on pollen grains regularly disposed, subquadrate raphids in the cypsela walls, and the other lineage includes *Erato* and *Philoglossa*, having stiff hairs with bulbous bases and 2–4 angles on the achenes (Robinson 1983). A few traditional and cladistic works have presented the intergeneric relationship of the Munnoziinae. However, the circumscription and relationship to the sister group of Munnoziinae still remains controversial due not only to the lack of congruence among the works but also to the lack of understanding of the core genus. According to the treatment (Robinson 1983), *Munnozia* has ca. 46 species and represents the morphological diversity and biogeographic distribution pattern of the subtribe. *Munnozia* may be used to understand the origin of speciation and pattern of diversification of the subtribe. Therefore, understanding of *Munnozia* can be pivotal for examining the evolution and phylogeny of the Munnoziinae.

Munnozia, the most species-rich genus in the tribe, contributes significantly to morphological and biogeographical diversity in the subtribe and tribe. The characteristic chaffy receptacle was originally used to define *Munnozia* by Cassini and various other authors (1823, 1825, 1830). Later the series of works by Robinson (1974, 1983) modified the diagnostic characters for the genus. Consequently, Robinson transferred several *Liabum* species and related to *Munnozia*. *Munnozia* is taxonomically complex due not only to the large number of species in the genus, but also to ambiguously demarcated generic delimitation from *Liabum*. Nevertheless, the naturalness of the genus has not been examined in the phylogenetic context. As the first step in a comprehensive phylogenetic study of the Liabeae, we have sequenced DNAs of the Munnoziinae using the nuclear ITS (internal transcribed spacer) region. The goals of this study are to (1) clarify the phylogeny of the Munnoziinae, (2) examine the monophyly of *Munnozia* itself, (3) assess the phylogenetic position of *M. perfoliata*, and (4) evaluate the usefulness of the ITS markers for the generic and species level relationships in the tribe.

Materials and methods

Taxon sampling. Thirty-six samples from twenty-six species of the Munnoziinae and nine species from two outgroup genera were used in this study (Table 1). These cover the morphological and biogeographic diversity of both the ingroup, the subtribe Munnoziinae, and the outgroup. Taxon names and voucher information with geographical distribution and their collecting area are listed in Table 1. Most voucher specimens are housed in the US National herbarium. All of the samples used in this study are from personal collections, identified by the primary collector.

Ingroup sampling. The ingroup contains the four genera of the subtribe Munnoziinae. For *Munnozia*, the largest genus in the subtribe with over ca. 40 species, we have included 14 species representing all of the morphologically distinctive clades in the genus. For instance, *M. campii* has white rays and *M. jussieui* has whitish rays

Table 1. A list of taxa used in this study with voucher information and geographic location

Genus	Species	Author	DNA source/Voucher	Distribution	Accession No.
Chrysactinium	*acaule*	(Kunth) Weddell	Funk#11425A (USA)	Ecuador	AF539940
Chrysactinium	*acaule*	(Kunth) Weddell	Funk#11457 (USA)	Ecuador	AF539939
Erato	*polymnioides*	De Candolle	Dillon#8015 (USA)	Ecuador, Peru, Bolivia	AF539946
Erato	*polymnioides*	De Candolle	Funk#11455 (USA)	Ecuador, Peru, Bolivia	AF539949
Erato	*vulcanica*	(Klatt) Robinson	Funk#4810 (USA)	Ecuador, Costa Rica, Venezuela, Columbia	AF539947
Erato	*vulcanica*	(Klatt) Robinson	Funk#4814 (USA)	Ecuador, Costa Rica, Venezuela, Columbia	AF539948
Liabum	*barahonense*	Urban	Funk#11464 (USA)	endemic to Dominican Republic	AF539952
Liabum	*bourgeaui*	Hieronymus in Ule	Funk#4803 (USA)	Mexico and central America	AF539922
Liabum	*bourgeaui*	Hieronymus in Ule	Funk#4811 (USA)	Mexico and central America	AF539924
Liabum	*igniarium*	(Kunth) Lessing	Funk#11459 (USA)	Columbia and Ecuador	AF539923
Munnozia	*campii*	Robinson	Funk#11456 (USA)	Ecuador	AF539927
Munnozia	*foliosa*	Rusby	Beck #1804* (USA)	Peru, Bolivia	AF539935
Munnozia	*foliosa*	Rusby	Solomon & Daly#8020* (USA)	Peru, Bolivia	AF539936
Munnozia	*fosbergii*	Robinson	Funk#11454 (USA)	Colombia	AF539929
Munnozia	*gigantea*	(Rusby) Rusby	Dillon#8032 (USA)	Peru, Bolivia	AF539945
Munnozia	*hastifolia*	Robinson	Funk#12087 (USA)	Colombia, Venezuela, Ecuador, Peru, Bolivia, Argentina	AF539926
Munnozia	*jussieui*	Robinson & Brettell	Panero#3029* (USA)	Colombia, Ecuador	AF539925
Munnozia	*lanceolata*	Ruiz & Pavon	Hutchinson&Wright#5928* (USA)	Peru	AF539944
Munnozia	*lyrata*	(A. Gray) Robinson	Panero #1201 (USA)	Peru	AF539933
Munnozia	*nivea*	(Hieronymus) Robinson	Harling & Wright#23691 (USA)	Columbia, Ecuador, Peru	AF539942
Munnozia	*perfoliata*1	(Blake) Robinson	Panero & Clarke#3038 (USA)	Colombia	AF539937
Munnozia	*perfoliata*2	(Blake) Robinson	Panero & Clarke#3038* (USA)	Colombia	AF539938
Munnozia	*pinnatipartita*	(Hieronymus) Robinson	Panero & Clarke #2995 (USA)	Ecuador	AF539941
Munnozia	*senecionidis*1	Bentham	Funk#11321 (USA)	Costa Rica, Colombia, Panama, Venezuela, Ecuador, Peru, Bolivia,	AF539932
Munnozia	*senecionidis*2	Bentham	Funk#11343 (USA)	Costa Rica, Colombia, Panama, Venezuela, Ecuador, Peru, Bolivia,	AF539934

Table 1 (continued)

Genus	Species	Author	DNA source/Voucher	Distribution	Accession No.
Munnozia	*Senecionidis*3	Bentham	Sanchez & Dillon #8018 (USA)	Costa Rica, Colombia, Panama, Venezuela, Ecuador, Peru, Bolivia,	AF539931
Munnozia	*pinnulosa*	(Kuntze) Robinson	Funk#11316 (USA)	Bolivia	AF539928
Munnozia	*pinnulosa*	(Kuntze) Robinson	Funk#11344 (USA)	Bolivia	AF539930
Munnozia	*wilburii*	Robinson	Funk#4802 (USA)	endemic to Costa Rica	AF539933
Philoglossa	*mimuloides*	(Hieronymus) Robinson	Funk#11453 (USA)	Colombia, Ecuador, Peru, Bolivia	AF539950
Philoglossa	*minuloides*	(Hieronymus) Robinson	Dillon#8029 (USA)	Colombia, Ecuador, Peru, Bolivia	AF539951
Sinclairia	*angustissima*	(Gray) Turner	Soule#2693 (TEX)*	Mexico	AF539953
Sinclairia	*liebmannii*	Schultz-Bipontinus ex Rydberg	McVaugh & Koelz#1642* (TEX)	Mexico	AF539954
Sinclairia	*moorei*	Robinson & Brettell	Panero#5301 (USA)*	Mexico	AF539957
Sinclairia	*polyantha*	(Klatt) Rydberg	Funk#4813 (USA)	Central America	AF539956
Sinclairia	*vagans*	Robinson & Brettell	Smith#31848 (TEX)*	Guatemala	AF539955

*DNA was extracted from herbarium material. Taxa are listed alphabetically by genus name and specific name

(sometimes turning lavender) that are rarely yellow. *Munnozia nivea* and *M. pinnatipartita*, which belong to a small separate subgenus, have the leaves pinnate or pinnatifid while all other members of the other subgenus have simple leaves.

Munnozia perfoliata is a small creeping annual while most of the members of this genus are perennials, having either a shruby or subshruby habit. Eventually, two different individuals of the same collection of *M. perfoliata* were included because of the unusual position within the analysis. Although they were from the same collection numbers, they were from separate extractions of herbarium and fresh material and the habit of the plant made it likely that they were sampled from two different individuals. The other *Munnozia* species sampled were from throughout the range of the genus from an endemic in Costa Rica, *M. wilburii*, to a widespread variable taxon from the Andes, *M. senecionidis*. Three species that have particular morphological problems are represented by two collections each for a total of 17 collections sampled from *Munnozia*. The other genus, *Chrysactinium*, includes six species and we sampled two collections of the largest and most variable species *C. acaule* from different sites in Ecuador. The other two genera are represented by two distinct samples of a single variable species. The genus *Philoglossa* includes five species, but the widest ranging and most variable is *P. mimuloides*. One accession each of *P. mimuloides* was sampled from Ecuador and Peru. Two of the four species of *Erato* were represented in the study, *Erato polymnioides* from both Ecuador and Peru, and *E. vulcanica* from Costa Rica and Ecuador.

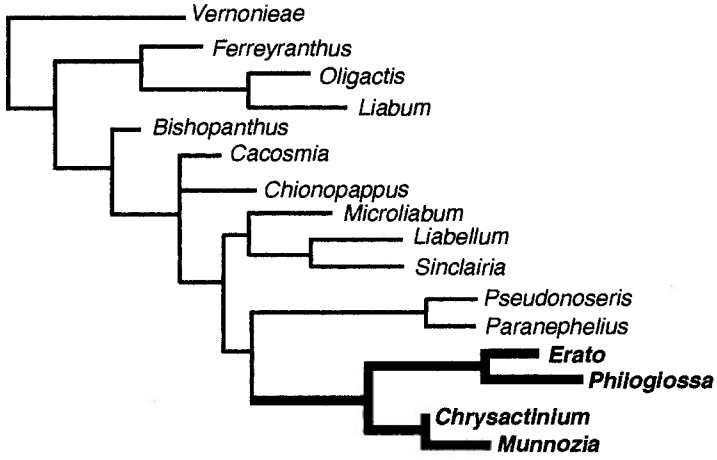
Outgroup sampling. *Sinclairia* and *Liabum* were used as outgroups in the molecular analysis based on their biogeographic diversity and on the results of the previous morphological analysis (Funk et al. 1996). The genus *Liabum* is represented by three taxa. One variable taxon, *L. bourgeaui*, represented by two collections, is native to Mexico and Central America, and was collected from Costa Rica. The second species *Liabum igniarium* is from Colombia and Ecuador, and the final species, *L. baharonense*, is an endemic from the Dominican Republic. The second outgroup, *Sinclairia*, is represented by four species, *S. angustissimum* from Mexico, *S. liebmannii* and *S. vagans* from Guatemala, and *S. polyantha* from Costa Rica. Thus, a total of seven species were used to define the outgroup.

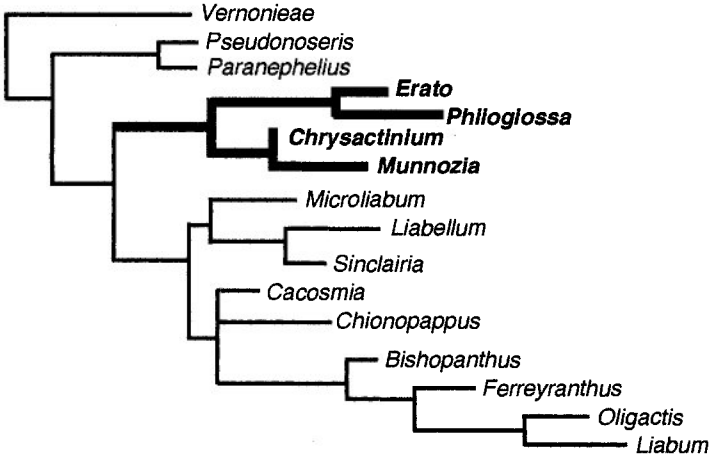
DNA extraction and PCR amplification. Most of the leaf material used was collected in the field and stored in silica gel. In a few cases, either frozen tissue or herbarium specimens from US or TEX served as source material (Table 1). Total genomic DNA was isolated from leaves using a modified 2X CTAB procedure (Doyle and Doyle 1987). Some DNAs contaminated with high concentrations of polysaccharides were purified using a Geneclean II^{R} kit (Bio 101 Inc.). The concentration of DNA samples was checked on 1% agarose gel/1X TBE buffer run with HindIII-digested lambda and HaeIII-digested DNA standards.

Amplifications were performed in 50 ul reactions with 1 ul of 10–40 ng genomic DNA, 1 ul of 10 uM primers (ITS5HP and ITS4), 5 µl of 10X Tfl polymerase buffer, 2.5 mM of each dNTP and 0.5 µl of 5 units/µl Taq polymerase (Promega). DNA was initially denatured at 94 °C for 5 min, followed by 30 amplification cycles consisting of 1 min denaturation at 94 °C, 1 min annealing of primer at 50 °C and 1 min 30 sec extension at 72 °C. Amplification was terminated by a final extension cycle of 72 °C for 7 min and a 4 °C soaking file. The PCR product was precipitated using a 20% Polyethylene Glycol solution (PEG 8000/2.5 M NaCl). When necessary, additional purification of the amplified ITS region was accomplished by gel purification followed by beta-agarase (New England Biolab) treatment. The two amplification primers, ITS 5HP (Suh et al. 1993) and ITS 4 as well as two internal primers (ITS 2 and ITS 3) were used for sequencing (White et al. 1990).

DNA sequencing and alignment. The purified templates were labeled by cycle sequencing using FS chemistry dye terminators according to conditions recommended by the supplier (Applied Biosystems). Excess dye terminators were removed with Sephadex (G-50) spin columns. Sequences were obtained on Applied Biosystems model 373 and and 377 automated fluorescent DNA sequencers. Data collection was carried out with Applied Biosystem Sequence analysis^{TM} 3.1, implemented on Macintosh G3 computers, followed by contig assembly using Sequencer^{TM} from Gene Codes.

All sequences were manually realigned using Se-Al version 1.0a1 (Rambaut 1996). There were no ambiguous regions in the alignment. The ITS region boundaries were defined by comparison with previously published sequences of Asteraceae (Kim and





Figs. 1 and 2. Redrawn from Funk et al. (1996). The two equally parsimonious trees of the Liabeae generated from 42 morphological characters, Tree length (L) = 93, Consistency Index (CI) = 0.71, Retention Index (RI) = 0.44

Jansen 1994, Kim et al. 1998). All sequences are submitted to GenBank (See Table 1 for accession numbers).

Phylogenetic analyses

Parsimony analyses. Phylogenetic analyses were conducted using PAUP* 4.0 (Swofford 1998). All characters were unordered and equally weighted. Gaps were coded as hyphens (-) in the PAUP* analyses. Several ambiguous sites were encountered in the ITS1 regions of *Munnozia lyrata*, *M. nivea* and *M. gigantea*. Those sites were coded using IUPAC (International Union of Pure and Applied Chemistry) ambiguity codes.

To find the most parsimonious trees, heuristic searches were conducted using random addition with TBR, MULPARS, and STEEPEST DESCENT on. Starting trees were constructed using 100 replicates with random addition sequence. Assuming an unequal substitution rate in the ITS region, the data were analyzed as follows: 1) ITS1 and ITS2 sequences were considered separately; 2) ITS1 and ITS2 data sets were combined; and 3) the entire ITS region as a whole was analyzed (ITS1, 5.8 rDNA and ITS2 (ITS region)). In order to assess node support, bootstrap analyses (Felsenstein 1985, Hillis and Bull 1993) and decay analyses (Bremer 1988, Donoghue et al. 1992) were performed. In the bootstrap runs, PAUP was set for 100 bootstrap replicates with TBR and MULPARS options. Two outgroup genera, *Liabum* and *Sinclairia*, were selected based on the morphological grounds cited above. To compare the length of the

shortest trees, a Branch-and-Bound search was performed with MULPARS ON. The tree length and its branching order were identical to the tree generated by the heuristic search. For this study, we present the tree obtained by the Branch-and-Bound search.

Maximum likelihood. To compare and to assess whether the short internal branch length in several clades affects the placement of *M. perfoliata* under different evolutionary criteria, additional analyses were performed. First the original data set of thirty-six was reduced to fifteen taxa (Table 1). A maximum parsimony heuristic search was performed with the same options as the ones for the original data sets, using PAUP 4.0* (Swofford 1998). Using the maximum parsimony tree, the program Modeltest 3.0 (Posada and Crandall 1998) was utilized to determine the model that fits best for the data tested by the hierarchical likelihood ratio (LR) test ($\alpha = 0.01$) under the nested requirement. When the competing models were nested, the LR test statistic (δ) is distributed as χ^2 distribution with degree of freedom equal to the difference in number of free parameters between two models (Huelsenbeck and Crandall 1997). For ITS data, the model selected is the Tamura-Nei model (Tamura and Nei 1993) with gamma-distributed site-to-site rate variation. With the model determined, the maximum likelihood tree search was done using PAUP 4.0*.

Results

Phylogenetic analyses. Of the 641 aligned ITS nucleotide positions, 210 appeared to be potentially informative (33%), and 357 were constant (56%). Initially, seventy-four characters including gaps, were treated as ambiguous or missing (11%). We performed both maximum parsimony (MP) and maximum likelihood (ML) analyses using the aligned ITS data sets. With MP, the combined data sets, as well as alignments for each of the two separate regions of the spacer were analyzed. Since many trees with equal lengths were generated from each data set, strict consensus trees were also utilized to compare the branching orders (Fig. 4). The trees of the complete ITS regions (Fig. 3) are congruent with one of the trees randomly chosen with one exception, the placement of *Munnozia fosbergii*. This taxon varies from being within Clade H in the MP tree but is placed with *M. gigantea* and Clade E in the consensus tree for ITS 1. Despite the instability of the Munnoziinae (Clade D), all of the strict consensus trees from the four data sets are congruent with respect to (1) recognition of the currently circumscribed Munnoziinae as paraphyletic, (2) paraphyly of *Munnozia*, and (3) placement of *M. perfoliata* close to the outgroup. However, the branching order within Clade D (Fig. 3), which includes subclades, E, F, G, H, I, *Munnozia lanceolata*, *M. foliosa* and *M. fosbergii*, and within the *Sinclairia* clade are incongruent among the data sets. The poor resolution may be attributed to using a short and conservative region for this recently derived group.

To confirm the result of the parsimonious tree search, the maximum likelihood tree search was conducted. Modeltest 3.0 version identified TrNef + G with log likelihood score of 3069.2844 as the best model of the DNA evolution for the ITS data set. With the best model TrNef + G chosen, a heuristic tree search was performed. Focusing on our interest in the placement of *M. perfoliata*, the parsimonious tree search was performed with the same data set to compare the topologies. In both topologies (Figs. 3 and 5), *M. perfoliata* appeared outside the Munnoziinae clade with strong support (bootstrap 100%, DI > 10).

Proposed relationships based on ITS data. The ITS trees generated by parsimony and likelihood (Figs. 3 and 5) identified three major clades: Clade A and the Munnoziinae clade consisting of Clades C + D.

Clade A. Clade A, under both analytical methods, consists of two accessions of *Munnozia perfoliata*. Both maximum parsimony and likelihood are consistent in the placement of *M. perfoliata* near the base of the ITS cladogram, and below *Sinclairia* which was used as an outgroup (Clade B, Figs. 3 and 4). Clade A is strongly supported (bootstrap 100%, DI > 10, Figs. 3–5). This placement is also suggested by

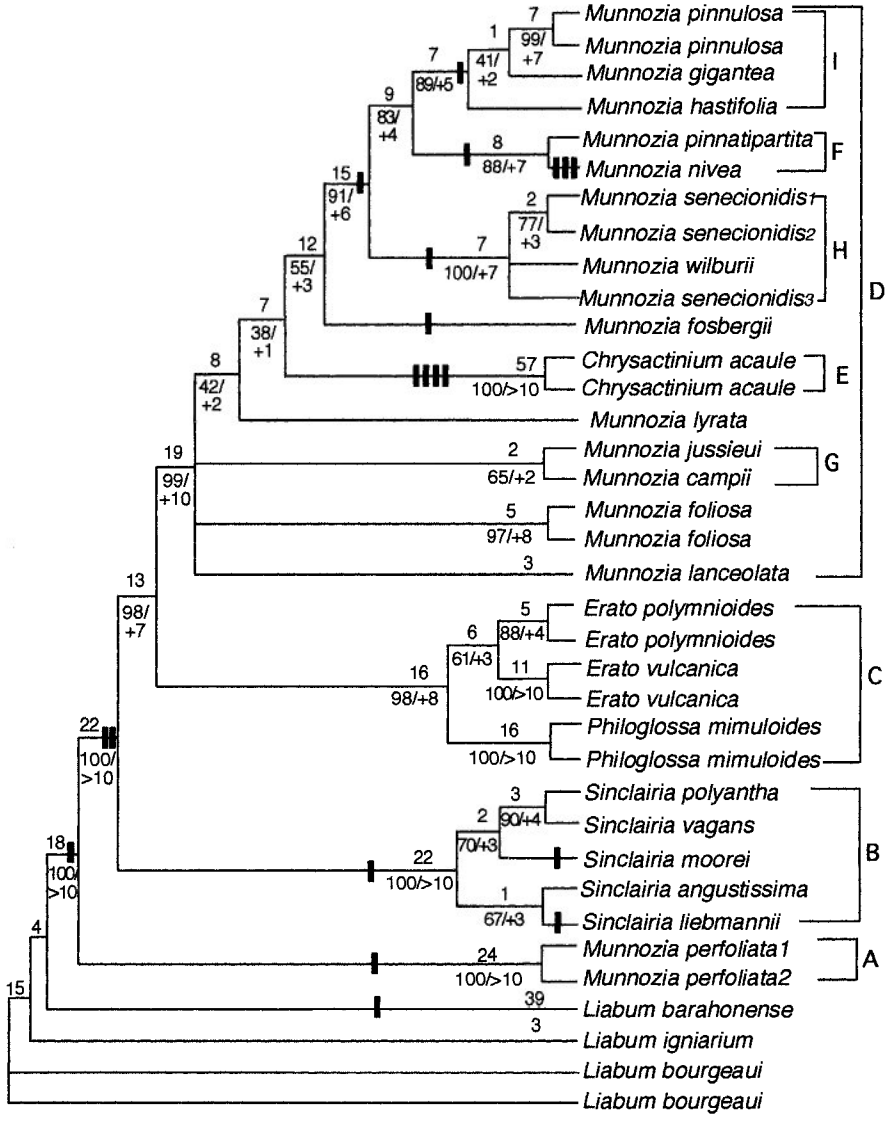


Fig. 3. One of the 22 most equally parsimonious trees obtained from analyses of ITS sequence data matrix (L = 530, CI = 0.704, RI = 0.863). The numbers above the branch indicate the number of characters changed under ACCTRAN optimization using PAUP 4.0. The numbers below the branches indicate bootstrap value and decay index, respectively. The black bar mapped on the node indicates the non-homoplastic informative gaps

the sequence divergence estimate for *M. perfoliata* relative to the majority of *Munnozia* examined, ranging from 11.75%–18.75%, making it the most distant species from core *Munnozia*. To force *M. perfoliata* into the *Munnozia* (Clade D) would required 22 extra steps over the most parsimonious arrangement. Thus, it appears that *M. perfoliata* is an outgroup for Munnoziinae as well as *Sinclairia*.

Clade B. The five species of *Sinclairia* used as part of the outgroup form a monophyletic group with strong support (bootstrap 100%, DI > 10) in the ITS tree. This clade appears to be sister to the subtribe Munnoziinae.

Subtribe Munnoziinae. The subtribe Munnoziinae clade consists of two lineages (Fig. 3): one (Clade C) containing *Philoglossa* and *Erato*, and the other (Clade D) with *Munnozia* and *Chrysactinium*. The parsimony tree supports their close relationships with 13 synapomorphies (bootstrap 98%, DI = 7) and their sister relationship is also supported in the strict consensus tree (Fig. 4). Phylogenetic analyses of ITS sequence data have shown that as currently circumscribed the subtribe is not monophyletic (Figs. 3–5) because of the placement of *M. perfoliata* outside the Munoziinae clade. In addition, *Chrysactinium* is nested

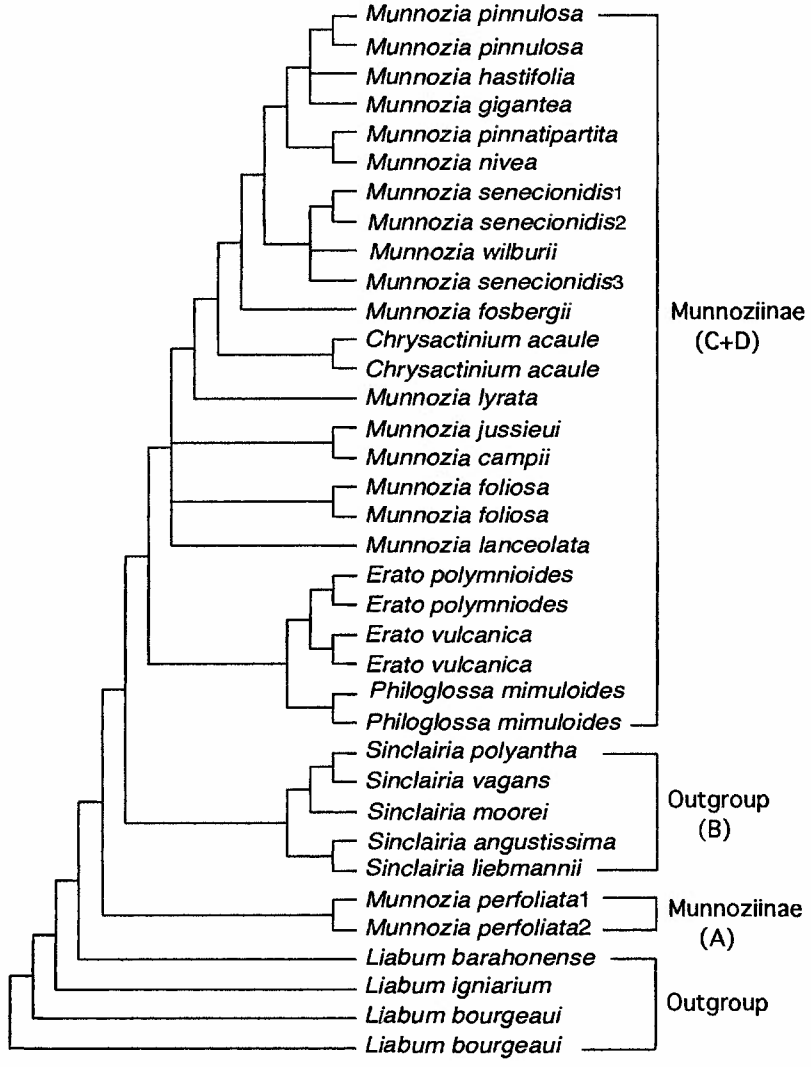


Fig. 4. The strict consensus tree of 22 most equally parsimonious trees, generated using ITS sequence data

inside *Munnozia*. If both *Chrysactinium* and *M. perfoliata* are forced to meet the monophyly of Munnoziinae, 26 extra steps are required relative to the shortest tree for the ITS sequence data.

Clade C. The ITS phylogeny (Figs. 3 and 4) supports the strong sister relationships between *Philoglossa* and *Erato*, and identifies these two genera as a monophyletic group. The clade is stable with strong support (bootstrap 98%, DI = 8). The ITS tree is consistent with the result from morphological cladistic analyses (Robinson 1983, Funk et al. 1996).

Clade D: Clade D (Fig. 3) contains the genera *Munnozia* and *Chrysactiniun*. These two genera have traditionally been placed together as closely related taxa. As mentioned above, the ITS tree shows *Munnozia* to be paraphyletic. The branch leading to *Chrysactinium* is very long, with 57 autapomorphies, in fact it is the longest on the cladogram. To disrupt the integrated relationship of *Chrysactinium* within *Munnozia*, four extra parsimonious steps are required.

Within *Munnozia* (Clade D, Fig. 3), the ITS tree identifies a core *Munnozia* group consisting of the clades F, H, I and *M. forsbergii* with weak support (bootstrap 55%, DI = 3). The rest of the clades within Clade D are weakly resolved, forming a polytomy among *M. foliosa*, *M. lanceolata*, *M. lyrata* and Clade G. With respect to floral and leaf features, there are two

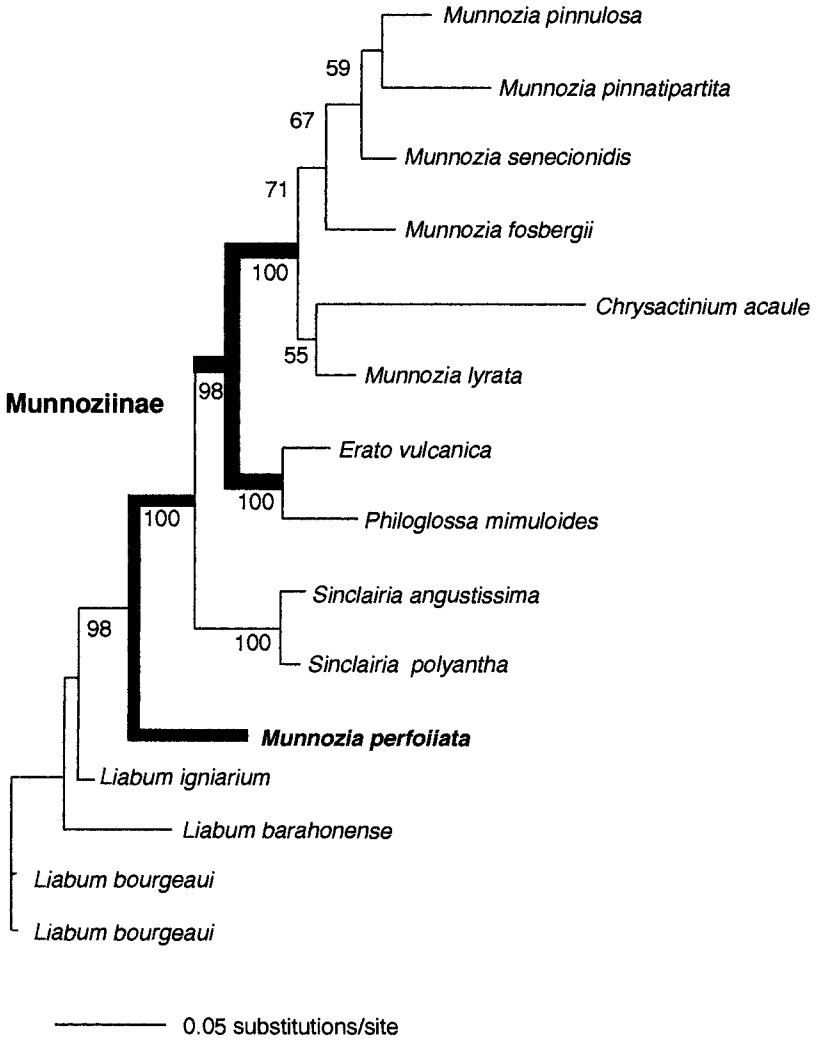


Fig. 5. The maximum likelihood tree of 15 ITS sequences generated using the best model Trnef+G. The data set for maximum likelihood reduced from the original data set of thirty-six. The numbers below the branches indicate bootstrap value. The scale bar corresponds to 0.05 substitutions per site

clades of interest identified in the ITS tree (Fig. 3). One is Clade F (*M. pinnatipartita* and *M. nivea*) and the second is Clade G (*M. jussieui* and *M. campii*). The strongly supported, Clade F, belongs to the subgenus *Kastnera* sensu Robinson and Marticorena (1986) with bootstrap value of 88% and decay index of 7. Clade G is a morphologically distinct group by whitish flower, and is identified in the ITS tree. However, Clade G is not strongly supported (bootstrap 65%, DI = 2) and its relationship to sister group is not resolved in the ITS tree (Figs. 3 and 4). Clade I, sister to Clade F, consists of *M. pinnulosa*, *M. gigantea* and *M. hastifolia*. This clade is fairly stable (bootstrap 89%, DI = 5). Of all the species in *Munnozia*, the most variable in morphology and the most frequently collected species is *M. senecionidis*. Three species are sequenced and *M. senecionidis*1 and 2 are placed on the ITS tree close to *M. wilburii* and *M. senecionidis*3 (Fig. 3), forming a polytomy. Even though these taxa are unresolved relative to each other, this clade is strongly supported (bootstrap 100%, DI = 7).

Variability of the ITS region. The results of phylogenetic analyses of all four separate data sets are summarized in Table 2. The ITS + 5.8S region within *Munnozia* varied from 624 to 629 bp, with *M. lyrata* having the longest length. *Erato polymnioide*s was the shortest (617 bp). The 5.8S region of all taxa examined was 169 base pairs long, 4–5 base

Table 2. A summary of the results of phylogenetic analyses using parsimony for four separate data sets (ITS1, ITS2, ITS1 + 2, and ITS1 + 5.8S + ITS2). The asterisk (*) indicates the sequence divergence in pairwise comparisons among all taxa examined

	ITS 1	ITS 2	ITS 1& 2	ITS1/2/5.8S
Total length (bps)	256	216	472	641
Length variation without gaps	244–255	209–211	454–464	617–629
#s of the most parsimonious trees	14	52	52	22
Tree length	257	211	487	530
Consistency Index (CI)	0.743	0.706	0.698	0.704
Retention Index (RI)	0.896	0.865	0.866	0.863
#s informative characters (%)	117 (46%)	83 (38%)	200 (42%)	210 (33%)
#s variable chr, but uninformative	24 (9%)	30 (14%)	54 (11%)	74 (12%)
% of G + C content	0.48	0.53	0.50	0.51
*(%) seq. divergence	0.3–28.6%	0.4–20.8%	0.4–27.4%	0.3–21.5%

pairs longer than those of other angiosperms published (Baldwin et al. 1995, Wen and Zimmer 1996, Karol et al. 2000). Both ITS1 and ITS2 contributed to the spacer length variation, with the degree of length variation's being similar between the two regions (Table 2). However, the ITS1 contained more informative characters (46%) with a lower GC content (53%). The entire sequence alignment contained 45 gaps (7.2%). Of those, 27 were informative (60%). The non-homoplastic informative gaps are mapped on Fig. 3. At the interspecific level the ITS sequence divergence ranged from 4.42% to 18.74% within the ingroup, and from 7.82% to 21.5% between ingroup and outgroup. *Chrysactinium acaule* is the most divergent (20.65%) among ITS sequences examined.

Discussion

Monophyly and sister relationship. All of the parsimony analyses agree with one another on four points. First, of the taxa examined in this analysis *Sinclairia* (Clade B) is the sister group to the Munnoziinae clade (Clade C and D); second, the subtribe Munnoziinae as currently circumscribed is not monophyletic; third, the currently circumscribed genus *Munnozia* may not be monophyletic; and fourth, *M. perfoliata* (Clade A) needs new taxonomic placement within the Liabeae.

The results of our DNA study are consistent with Bremer's (1994) and one of the scenarios suggested by Funk et al. (1996; Fig. 2). Considering the traditional delimitation of Munnoziinae sensu Robinson (1983), the ITS tree identifies two separate groups, one including *M. perfoliata* and the other consisting of the remainder of the subtribe (Clade C and D). However, because of the close placement of *Sinclairia*, the outgroup, to the members of the Munnoziinae clade the final conclusions on circumscription of Munnoziinae and its sister relationship need to wait until an on-going study on the tribe is completed (in preparation). In particular, the large genus *Liabum* needs comprehensive study, first because it is known to be morphologically and biogeographically diverse, and second because the ambiguity of its generic delimitation from *Munnozia* has been documented.

The Munnoziinae clade now consists of two major lineages. One includes *Erato* and *Philoglossa*, which have been recognized previously as a group supported by several morphological synapomorphies: pollen grains with regularly dispersed spines, and leaves and stems with tomentum (Robinson 1983, Funk et al. 1996). The ITS phylogeny corroborates the morphological study showing a strong support (bootstrap 100%, DI = 8) for this clade. The other, consisting of the *Munnozia* clade and *Chrysactinium*, has been

considered to be a congeneric group based on the reduced acaulescent habit, long scapose heads, black anther theca and regularly disposed spines on the pollen wall (Robinson 1983, 1986). Their close relationship has been emphasized by previous workers (Robinson 1983, Bremer 1994, Funk 1996, Figs. 1 and 2). Our ITS tree (Figs. 3 and 4) places *Chrysactinium* within *Munnozia* with moderate support. This result supports the previous morphological findings. The long branch length of *Chrysactinium* may result from rapid evolution after having diverged (Figs. 3 and 4) and may be confounding the topology. Therefore, the phylogenetic relationship of *Chrysactinium* to the members of *Munnozia* is not completely certain (bootstrap 38%, DI = 1, Figs. 3 and 4).

Munnozia, the most diverse genus, both in terms of biogeography and morphology, has been divided into two subgenera based on morphological characters, *Munnozia* and *Kastnera* (Robinson 1983). The palynological study contradicted the morphological finding, revealing that the internal structure of pollen wall represents several types in *Munnozia* (Robinson 1986). On the ITS tree, the genus appears to split off into several clades: the subgenus *Munnozia* which is core *Munnozia* with typical distinctive morphological elements (Clades F, H, I), the subgenus *Kastnera* (Clade F), and the last four consisting of four monotypic or small clades (*M. foliosa*, *M. lyrata*, *M. lanceolata* and Clade G). Despite weak support within the Munnoziinae clade (D), the ITS tree identifies several morphological subclades recognized by a previous worker (Robinson 1983): 1) the subgenus *Kastnera* (Clade F) defined by the lack of projections on the receptacle, 2) Clade G having the only white-rayed species, 3) Clade H endemic to Costa Rica, *M. wilburii*, and three accessions to a widespread variable taxon, *M. senecionidis*, and 4) Clade I containing *M. pinnulosa*, *M. gigantea* and *M. hastifolia*.

In our study, portions of the traditional subgeneric classification are not supported by the ITS study. Given that the ITS tree is correct for phylogenetic inference, we propose that the classification of *Munnozia* be reorganized in the hope of clarifying the generic delimitation and interspecific relationship. More extensive sampling of both *Munnozia* and *Chrysactinium* will help clarify the phylogeny and relationship of this genus within Munnoziinae.

Phylogenetic position of *M. perfoliata*. *Munnozia perfoliata* is an annual herbaceous species originally described as *Liabum perfoliatum* by Blake (1927) and transferred to the currently recognized status by Robinson and Brettell (1974). Subsequently the palynological data collected by Robinson and Marticorena (1986) confirmed its placement in *Munnozia* (Robinson and Marticorena 1986). The authors noted that the species is very distinctive in size of columellae cluster. Our molecular study (Clade A of Figs. 3–5), however, shows that *M. perfoliata* is outside the major clade of *Munnozia*, and lies next to the outgroup *Sinclairia*. The phylogenetic trees (Figs. 3-5) show that ITS sequence divergence of *M. perfoliata* from other *Munnozia* species ranges between 11.86% to 17.19%. However, the sequence divergence of *Munnozia* to the sister group *Sinclairia* is significantly less, with ranges between 8.52% and 12.78%, and thus is more closely related to *Sinclairia* taxa than to those in *Munnozia*. This is in disagreement with the current morphological understanding. Following the results of this molecular study, morphological and additional SEM investigations have been conducted on *M. perfoliata*, another closely related species, *M. chachapoyensis*, and a new species. Those detailed examinations corroborate the result of our molecular study with characters including the presence of bullate leaf surfaces, pale yellow anther thecae, and irregularly dispersed spines on the pollen. Considering their distinctiveness in morphological and palynological features, and the relative divergence in DNA sequence, these taxa have been moved to a new genus *Dillandia* (Funk and Robinson 2001).

Utility of ITS sequence data for developing phylogeny of closely related genera of Asteraceae. In many angiosperm groups that have been studied over the last decade, ITS sequences have proven the most valuable for examining relationships within genera and among the more closely related genera within a tribe (Downie et al. 2000) and family (Baldwin 1992, Baldwin et al. 1995). However, across the tribes of Asteraceae, divergence among ITS sequences is so high that problems with alignment and homoplasy are sufficient to make family-wide phylogenetic studies impossible with this molecular marker (Baldwin1992, Kim and Jansen 1994). Our primary interest in using ITS sequence data was to evaluate the possibility of utilizing the region to infer the phylogeny of the subtribe, Munnoziinae and later for the tribe Liabeae. Numerous studies have shown the ITS region to be sufficiently variable and to be useful in comparisons at the generic level and below in Asteraceae. It has a divergence level of 0.2 to 15% for species within the Madiinae (Baldwin 1992), 0 to 8.6% for *Calycadenia* (Baldwin 1993), 0.8 to 10.6% for species in *Krigia* (Kim and Jansen 1994), 0 to 4.1% for the aureoid complex of *Senecio* (Bain and Jansen 1995), 0.9 in *Cheirolophus* to 5.9% in *Centaurea* within genera, and intergeneric divergence of 3.3% to 20.6% within Centaureinae (Susanna et al. 1995), 0–1.11% in *Argyranthemum* and 0.2–16.5% among its closely related genera (Francisco-Ortega et al. 1997), and 1–10% among *Eupatorium* and 8 to 27% within Eupatorieae (Schmidt and Schilling 2000). Within the family Asteraceae, the ITS region has changed to the extent that it is possible to use ITS sequences to infer the phylogeny even among distinct allopatric populations in *Calycadenia*; the range of ITS sequence divergence is up to 3.7% with this molecular marker (Baldwin 1993). Our ITS sequence data show a sequence divergence from 0.3% to 20.65% at both interspecific and intergeneric levels. For example, congenera of *Sinclairia* (Clade B, Fig. 3) show pair-wise sequence divergences from 0.8% to 2.8%, while those for the genus *Munnozia*, which is considered to be the most variable of the tribe in both morphology and biogeographic distribution, vary from 0.16% to 17.07%. Among all of the genera and species of Munnoziinae examined, the pair-wise sequence divergence ranged from 0.3% to 20.65%. Considering a phylogenetic tree with reasonably high CI (0.704) and RI (0.861), ITS sequence data contained substantial phylogenetic signal. Several deep nodes are highly supported (bootstrap > 75%) although a few nodes of Clade D within the Munnoziinae are very weakly supported (bootstrap > 50%) and collapsed in the strict consensus tree (Figs. 3 and 4). The poor resolution at the base of the Munnoziinae clade may be attributed to low sequence divergence in ITS data. Ultimately the combining of data from various sources can lead to a better resolution (Donoghue and Sanderson 1992, Olmstead and Sweere 1994). In this study, nevertheless, ITS data provides a useful measure of phylogenetic relationships at the generic and specific level within Munnoziinae.

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