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Phylogeny of the Coneflowers and Relatives (Heliantheae: Asteraceae) Based on Nuclear rDNA Internal Transcribed Spacer (ITS) Sequences and Chlorplast DNA Restriction Site Data

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ABSTRACT. The internal transcribed spacer (ITS) region of 18S–26S nuclear ribosomal DNA (rDNA) was sequenced in 65 taxa representing most coneflowers (i.e., species in *Dracopis, Echinacea, Ratibida,* and *Rudbeckia*) and other taxa representing 21 outgroup genera of tribe Heliantheae. Results of parsimony analysis of the rDNA dataset by itself and in combination with the cpDNA dataset uphold the hypothesis from an earlier cpDNA restriction site study that *Echinacea* is not closely related to the other three genera of coneflowers. The data support placement of *Echinacea* in subtribe Zinniinae. The remaining three coneflower genera represent a monophyletic lineage corresponding to subtribe Rudbeckiinae sensu H. Robinson. The rDNA data support two sublineages in *Rudbeckia* congruent with the two traditionally recognized subgenera, subg. *Macrocline* and subg. *Rudbeckia.* In subg. *Macrocline,* two geographic areas of diversification are indicated: southeastern and western United States. The widespread species *R. laciniata* is placed strongly with the western lineage of subg. *Macrocline.* The rDNA data support transfer of *Dracopis* to *Rudbeckia* subg. *Macrocline,* a relationship also supported by multiple morphological characters. The rDNA data do not confidently resolve the sister group of Rudbeckiinae from among the members of Heliantheae investigated. Multiple hypotheses are suggested for the outgroup taxa, such as expanding subtribe Zinniinae to include *Echinacea* and *Trichocoryne,* a genus previously regarded as belonging to subtribe Hymenopappinae (Heleneae or Heliantheae sensu lat.). Our findings further support expansion of subtribe Engelmanniinae to include *Balsamorhiza, Borrichia,* and *Wyethia* even though these taxa lack ray floret complexes and have fertile disc ovaries. We suggest that bioprospectors might usefully search among taxa of Zinniinae for bioactive substances similar to the immune stimulants of *Echinacea.*

Traditionally, the genera *Dracopis* Cass., *Echinacea* Moench, *Ratibida* Raf., and *Rudbeckia* L. have been known as coneflowers. They are among the best known wildflowers in North America. Black-eyed Susan (*Rudbeckia hirta*), brown-eyed Susan (*R. triloba*), prairie coneflowers (*Ratibida* spp.), and purple coneflowers (*Echinacea* spp.) are common in the central prairies of the United States and along woodland margins of the eastern states. In addition, some species in *Rudbeckia* subg. *Macrocline* (Torr. & A. Gray) P. B. Cox & Urbatsch occur in the western United States. With few exceptions, the coneflowers display large, showy capitula characterized by prominently elongated or spherical receptacles and colorful, spreading to reflexed ray corollas. Various species are cultivated for their ornamental qualities and some have become naturalized in areas beyond their native ranges. Other species are rare and their existence is threatened by habitat modifications associated with agricultural practices and urbanization. *Echinacea laevigata* and *E. tennesseensis* are listed as endangered by the Division of Endangered Species (U.S. Fish and Wildlife Service). *Rudbeckia auriculata* and *R. heliopsidis* are considered to be threatened or endangered in the southeast United States (Kral 1983).

A focus by systematists on the prominent morphological similarities shared by the four coneflower genera accounts for the long-standing acceptance of the hypothesis that *Dracopis, Echinacea, Ratibida,* and *Rudbeckia* constitute a natural group (Bentham 1873; Gray 1884; Sharp 1935; Fernald 1950; Stuessy

1977). Cladistic analysis of morphological, chemical, and cytological characters resolved the coneflowers as a single lineage (Cox and Urbatsch 1990; Karis and Ryding 1994). The first challenge to the naturalness of the group was by Robinson (1978, 1981). Robinson's examination of microcharacters led him to restrict the circumscription of subtribe Rudbeckiinae Cass. ex Dumort. to the genera *Dracopis, Ratibida,* and *Rudbeckia.* He placed *Echinacea* in subtribe Ecliptinae Less., close to *Balsamorhiza* Nutt., *Helianthella* Torr. & A. Gray, and *Wyethia* Nutt.

Results from phylogenetic analysis of chloroplast DNA (cpDNA) restriction site data by Urbatsch and Jansen (1995) did not support monophyly of all coneflowers or placement of *Echinacea* in subtribe Ecliptinae but, instead, led to a new hypothesis of relationships. *Echinacea* is placed with members of subtribe Zinniinae Benth. in their cpDNA trees. Subtribe Zinniinae has been variously interpreted by previous workers but has never included *Echinacea* (Stuessy 1977; Robinson 1981; Karis and Ryding 1994).

Another hypothesis tested in the cpDNA restriction site study was the origin of *Ratibida* from within *Rudbeckia. Ratibida* consists of seven, primarily prairie-dwelling species (Richards 1968; Turner 1988). Origin of *Ratibida* from within *Rudbeckia* (in the species group with columnar receptacles later called subg. *Macrocline*) was suggested by Sharp (1935). Should his proposal accurately reflect the evolutionary history of *Ratibida, Rudbeckia* would be paraphyletic. However, cpDNA studies failed to confirm Sharp's idea, instead supporting a sister-group relationship of *Ratibida* and *Rudbeckia* (Urbatsch and Jansen 1995).

Dracopis amplexicaulis has been treated as either a monotypic genus or a species of *Rudbeckia.* Gray (1884) gave it sectional status in *Rudbeckia,* but generic recognition of *Dracopis* has been the tendency of recent workers (e.g., Correll and Johnston 1970; Cronquist 1980). *Dracopis* differs from *Rudbeckia* sensu str. mainly by its lower chromosome number ($n = 16$, as opposed to $x = 18$ or 19 in *Rudbeckia* sensu str.). Unlike most members of *Rudbeckia* sensu str., *Dracopis* also exhibits an annual habit and is epappose. Chloroplast DNA restriction site analysis was inconclusive in resolving the phylogenetic position of *Dracopis* (Urbatsch and Jansen 1995).

Traditionally, *Rudbeckia* has been divided into two well-diagnosed subgenera, subg. *Macrocline* and subg. *Rudbeckia.* Different base chromosome numbers of $x = 18$ (subg. *Macrocline*) and $x = 19$ (subg. *Rudbeckia*) and various morphological characteristics distinguish the two subgenera. Most macroclines are robust perennials with stout, presumably long-lived rhizomes; persistent basal leaves; often clasping, glaucous, cauline leaves; and extremely elongated receptacles. Species in subg. *Macrocline* typically have restricted ranges, being confined to hillside seeps and the margins of bayous, ditches, or streams. The geographic distribution of subg. *Macrocline* is bicentric: six species occur in the western United States and six species are confined primarily to the Gulf coastal plain of the southeast U.S.A. In contrast, most species in subg. *Rudbeckia* are opportunistic annuals or short-lived perennials with an ability to occupy more disturbed habitats than is typical in subg. *Macrocline.* In subg. *Rudbeckia,* basal leaves typically wither by anthesis, cauline leaves are usually petiolate, and receptacles are more spherical than in the macroclines. The native ranges for species in subg. *Rudbeckia* are in the eastern U.S.A.; their occurrence elsewhere has been through human-caused introduction. Attempts at phylogenetic reconstruction within *Rudbeckia* using cpDNA resolved certain species groups but relationships in much of the genus were not robustly reconstructed (Urbatsch and Jansen 1995).

The taxonomic disposition of *Rudbeckia laciniata,* a widespread species common throughout most of its range (the Rocky Mountains, the eastern U.S.A., and southeastern Canada), has been problematic. *Rudbeckia laciniata* has been traditionally assigned to sect. *Macrocline* (e.g., Gray 1884; Perdue 1959). Cox (1991) suggested that the species represents a different lineage because of its unique combination of morphological and cytological characteristics and proposed giving it subgeneric status. Both $x = 18$ and 19 have been reported for *R. laciniata,* as well as various ploidy levels. Perdue (1959, 1960) reported a base chromosome number of $x = 18$ for the species, the base number characteristic of subg. *Macrocline,* and he noted additional numbers of $2n = 36$, 52, 72, and 102+ for the species. Others (Battaglia 1946; Heiser and Smith 1954; Keil and Stuessy 1977; and Pinkava and Keil 1977) report $n = 19$ for *R. laciniata,* which the base number for subg. *Rudbeckia.*

We continue previous investigations to better understand the phylogeny of the coneflowers and their relationships to other Heliantheae Cass. (Cox and Urbatsch 1990; Urbatsch and Jansen 1995). Our major goal is to formulate more precise phylogenetic hypotheses for coneflowers and putatively related genera of Heliantheae. Such information will be useful in devising a more stable classification

system and will provide a basis for evaluating character evolution and for understanding biogeographic distributions. In the present study, sequence data from the internal transcribed spacers (ITS-1 and ITS-2) of 18S–26S rDNA and a portion of the 5.8S nuclear ribosomal gene were analyzed in a phylogenetic context. Investigations reviewed in Baldwin et al. (1995) and others, more recently published (e.g., Bogler and Simpson 1996; Downie and Katz-Downie 1996), amply demonstrate the usefulness of the ITS region for resolving relationships within and among closely related angiosperm genera. In addition, the cpDNA data from Urbatsch and Jansen (1995) for Rudbeckiinae and certain outgroup taxa are re-analyzed by comparing and combining them with the ITS dataset.

Materials and Methods

Taxa. Sixty-five ITS-region sequences spanning ITS-1, ITS-2, and approximately 90 bp of the intervening 5.8S region were analyzed from samples representing 60 species in 25 genera of tribe Heliantheae. Twenty-eight of the 30 species of subtribe Rudbeckiinae sensu Robinson (1981), representing all three genera (*Dracopis, Ratibida,* and *Rudbeckia*) were included. *Echinacea,* a genus historically allied with the three genera of Rudbeckiinae, is represented in this study by six of its nine species. Urbatsch and Jansen (1995) suggested a relationship between *Echinacea* and taxa typically associated with subtribe Zinniinae. In order to test this hypothesis further, seven genera frequently placed in Zinniinae were included in the present study. In addition, representatives of six additional subtribes of Heliantheae were investigated in order to evaluate better the interrelationships among Rudbeckiinae and other members of Heliantheae. *Trichocoryne* S. F. Blake is included in this study because phylogenetic analyses of ITS sequences for Heliantheae sensu lat. by Baldwin and Wessa (in prep.) indicated that the genus is nested within Heliantheae sensu str., close to *Rudbeckia* (other coneflowers and representatives of Zinniinae were not sampled). *Coreopsis* L. was included for rooting purposes based on phylogenetic results of Jansen et al. (1991), Kim et al. (1992), and Kim and Jansen (1995).

For each sample, total genomic DNAs were extracted from approximately one gram of field-collected leaf tissue (kept on ice and subsequently stored at -80^{0}C) or (in a few cases) about 0.25 gram of herbarium-specimen leaves using the 2X CTAB (hexadecyltrimethylammonium bromide) protocol of Doyle and Doyle (1987). Frozen tissue was ground with a mortar and pestle in liquid nitrogen and dry tissue was ground using a small amount of sterile sand. DNAs used earlier for restriction-site analysis (Urbatsch and Jansen 1995) were purified in cesium-chloride gradients as outlined by Sambrook et al. (1989). More recent isolates were subjected to PCR without such purification. Collection and voucher deposition data are given in Table 1.

PCR and DNA Sequencing. Various PCR and sequencing protocols were used as procedures and were gradually optimized. Initially, the asymmetric PCR protocol of Baldwin (1992) and Wojciechowski et al. (1993), employing the primers designed by White et al. (1990), were used to generate single-strand DNA containing the ITS-1 and ITS-2 regions.

Double-strand DNA for direct sequencing was generated in 100 l PCR reactions using 1 unit of *Tfl* polymerase (Epicentre Technologies, Madison, WI), 1.5 mM $MgCl_2$, 0.15 M of each dNTP, 0.3 M of each primer, and approximately 5 ng of DNA. The initial 10 thermal cycles consisted of 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C with a 4 sec per cycle extension. Except for using an annealing temperature of 50°C, the next 20 cycles preceded as before followed by one extension phase of 7 mins.

Because primer ITS5 (White et al. 1990) failed for certain taxa, a replacement designated ''ITS-I'' was designed. It is located approximately 30 bp 5′ to the ITS5 primer and has the following 5′ to 3′ sequence: GTCCACTGAACCTTATCATTTAG. Primer ITS-I was used in equimolar amounts with primer ITS4 (White et al. 1990) to amplify ITS-1 and ITS-2 along with the 5.8S subunit.

The ITS region of certain samples (e.g., most herbarium material in subtribe Zinniinae) proved difficult to amplify in amounts sufficient for sequence analysis. To overcome this difficulty, two sets of nested primers were created to increase PCR product yield. Conserved sequences outside of the ITS region were designed from the published 18S–26S rDNA sequences for *Daucus carota* L. and *Vicia faba* L. (Yokota et al. 1989). The outer primer set was designated 18/350 and the inner set 20/262. Primer 18 is located in the small subunit of 18S–26S rDNA about 220 bp 5′ of ITS-1 and 350 is in the large subunit 350 bp 3′ to ITS-2. Primer 20 is about 200 bp 5′ of ITS-1 and 262 is about 250 bp 3′ ITS-2. Sequences for the primers are as follows (5′ to 3′): 18 (GTAAGCGCGAGTCATCAGCTCG), 350 (CA TCTTTCCCTCGCGGTACTTG), 20 (TCGCGTTG

TABLE 1. DNA sources for coneflowers and other genera of Heliantheae examined in the ITS sequence analyses. Herbarium material from which DNA was extracted is marked with an asterisk. Unmarked samples were extracted from freshly collected leaves.

TAXA	Source localities	Collectors and herbaria	GenBank locus Number ITS 1/2, respectively
Acmella repens (Walter) Rich.	Louisiana: East Baton Rouge Parish	*Urbatsch 7018*, (LSU)	AOU73155/AOU74425
Ambrosia artemisiifolia L.	Louisiana: East Baton Rouge Parish	*Urbatsch 6998*, (LSU)	AAU73794/AAU74437
Ambrosia trifida L.	Louisiana: East Baton Rouge Parish	*Urbatsch 6999*, (LSU)	ATU73795/ATU74438
Berlandiera pumila (Michx.) Nutt.	Florida: Suwannee Co.	*Urbatsch 6649*, (LSU)	BPU73788/BPU74431
Borrichia frutescens (L.) DC.	Louisiana: Cameron Parish	*Wendt & Collins 6671*, (LSU)	BFU73789/BFU74432
Calyptocarpus vialis Less.	Louisiana: East Baton Rouge Parish	*Urbatsch 6996*, (LSU)	CYU73157/CYU74427
Coreopsis tinctoria Nutt.	Louisiana: East Baton Rouge Parish	*Urbatsch 7040*, (LSU)	CTU74393/CTU74442
Dracopis amplexicaulis (Vahl) Cass.	Louisiana: East Baton Rouge Parish	*Lievens 4436*, (LSU)	DAU72805/DAU74401 DAU72806/DAU74402
Echinacea atrorubens (Norton) Cronquist	Texas: Fayette Co.	*Urbatsch 6492*, (LSU)	EAU73149/EAU74419
E. pallida (Nutt.) Nutt.	Louisiana: Bienville Parish	*Urbatsch 7028*, (LSU)	EPU73152/EPU74422
E. paradoxa (Norton) Britton	Oklahoma: Johnson Co.	*Urbatsch 6614*, (LSU)	EPU73153/EPU74423
E. purpurea (L.) Moench	Louisiana: Caldwell Parish	*Urbatsch 5879*, (LSU)	EPU73148/EPU74418
E. simulata McGregor	Missouri: Howell Co.	*Urbatsch 6599*, (LSU)	ESU73150/ESU74420
E. tennesseensis (Beadle) Small	Tennessee: Nursery Stock	*Urbatsch 6442*, (LSU)	ETU73151/ETU74421
Eclipta prostrata (L.) L.	Louisiana: East Baton Rouge Parish	*Urbatsch 6813*, (LSU)	EPU74391/EPU74440
Engelmannia pinnatifida Nutt.	Texas: Washington Co.	*Urbatsch 6482*, (LSU)	EPU73787/EPU74430
Helianthella quinquenervis (Hook. f.) A. Gray	Wyoming: Lincoln Co.	*Hartman 27869*, (RM)	HQU73793/HQU74436
Helianthus simulans E. Wats.	Louisiana: East Baton Rouge Parish	*Urbatsch 7024*, (LSU)	HSU73796/HSU74439
Heliopsis annua Hemsl.	Mexico: Puebla	*Keil 15484*, (TEX)	AF228512/AF228513
Lindheimera texana A. Gray & Engelm.	Texas: Burnet Co.	*Lievens 4378*, (LSU)	LTU73792/LTU74435
Philactis nelsonii (Greenm.) S.F. Blake	Mexico: Chiapas	*Breedlove 28267*, (TEX)*	PNU74396/PNU74452 PNU74445/PNU74453
Ratibida columnaris (Sims) D. Don	Texas: Burnet Co.	*Lievens 4391*, (LSU)	RCU73145/RCU74415
R. latipalearis E. L. Richards	Mexico: Chihuahua	*Tenorio* and *Romero 1730*, (TEX)*	RLU73146/RLU74416
R. mexicana (S. Watson) W.M. Sharp	Mexico: Durango	*Panero 2257*, (TEX)	RMU72816/RMU74412
R. peduncularis (Torr. & A. Gray) Barnhart	Louisiana: Cameron Parish	*Urbatsch 6456*, (LSU)	RPU72817/RPU74413
R. pinnata (Vent.) Barnhart	Iowa: Worth Co.	*Urbatsch 6568*, (LSU)	RPU72818/RPU74414
R. tagetes (James) Barnhart	New Mexico: Doña Ana Co.	*Worthington 14140* (TEX)*	RTU73147/RTU74417
Rudbeckia alpicola Piper	Washington: Chelan Co.	*Urbatsch 6803*, (LSU)	RAU59382/RAU71097

TABLE 1. Continued.

TAXA	Source localities	Collectors and herbaria	GenBank locus Number ITS 1/2, respectively
R. auriculata (Perdue) Kral	Alabama: Geneva Co.	*Cox* 4973 (LSU)	RAU59383/RAU71098
R. californica A. Gray	California: Apline Co.	*Urbatsch 6797*, (LSU)	RCU59384/RCU71099
R. fulgida Aiton var. *fulgida*	Tennessee: Knox Co.	*Urbatsch 6780*, (LSU)	RFU72807/RFU74403
R. glaucescens Eastw.	Oregon: Josephine Co.	*Urbatsch 6720*, (LSU)	RGU71100/RGU72793
R. graminifolia (Torr. & A. Gray) C. L. Boynton & Beadle	Florida: Franklin Co.	*Urbatsch 6720*, (LSU)	RGU72808/RGU74404
R. grandiflora (Sweet) DC.	Louisiana: Beauregard Parish	*Urbatsch 6525* (LSU)	RGU72809/RGU74405
R. heliopsidis Torr. & A. Gray	Alabama: Dekalb Co.	*McInnis s. n.*, (LSU)	RHU72810/RHU74406
R. hirta L.	Missouri: Texas Co.	*Urbatsch 6589*, (LSU)	RHU72811/RHU74407
R. klamathensis P. B. Cox & Urbatsch	California: Trinity Co.	*Urbatsch 6801*, (LSU)	RKU71101/RKU72794
R. laciniata L. var. *laciniata*	Louisiana: West Feliciana Parish	*Cox 4922*, (LSU)	RLU71102/RLU72795 RLU72405/RLU72796 RLU72406/RLU72797
R. maxima Nutt.	Texas: San Jacinto Co.	*Lievens 4435*, (LSU)	RMU72407/RMU72798 RMU72408/RMU72799 RMU72409/RMU72800
R. missouriensis Engelm. ex C. L. Boynton & Beadle	Mississippi: Phelps Co.	*Urbatsch 6581*, (LSU)	RMU72813/RMU74409
R. mohrii A. Gray	Florida: Taylor Co.	*Urbatsch 6701*, (LSU)	RMU72791/RMU72801
R. mollis Elliott	Florida: Suwannee Co.	*Urbatsch 6650*, (LSU)	RMU72812/RMU74408
R. nitida Nutt.	Florida: Clay Co.	*Urbatsch 6659*, (LSU)	RNU72792/RNU72802
R. occidentalis Nutt.	California: Humboldt Co.	*Urbatsch 6799*, (LSU)	ROU59285/ROU74388
R. scabrifolia L. E. Br.	Louisiana: Vernon Parish	*Urbatsch 5974*, (LSU)	RSU72803/RSU74389
R. subtomentosa Pursh	Arkansas: Ashley Co.	*Thomas 97828*, (LSU)	RSU72815/RSU74411
R. texana (Perdue) P. B. Cox & Urbatsch	Texas: Montgomery Co.	*Urbatsch 6495*, (LSU)	RTU72804/RTU74390
R. triloba L.	Virginia: Montgomery Co.	*Urbatsch 6792*, (LSU)	RTU72814/RTU74410
Salmea oligocephala Hemsl.	Mexico: Michoacan	*Escobedo 1861* (TEX)*	SOU73156/SOU74426
Sanvitalia fruticosa Hemsl.	Mexico: Puebla	*Mechano 1446* (TEX)*	SFU74394/SFU74443 SFU74395/SFU74444
Silphium laciniatum L.	Louisiana: Acadia Parish	*Urbatsch 6449*, (LSU)	SLU73790/SLU74433
S. perfoliatum L.	Iowa: Mitchell Co.	*Urbatsch 6547*, (LSU)	SPU73791/SPU74434
Sphagneticola trilobata (L.) Pruski	Louisiana: East Baton Rouge Parish	*Urbatsch 6995*, (LSU)	STU74392/STU74441
Trichocoryne connata S. F. Blake	Mexico: Durango	Baldwin 972	(Baldwin and Wessa, in prep.)
Verbesina occidentalis (L.) Walt.	Tennessee: Roane Co.	*Urbatsch 6788*, (LSU)	VOU73158/VOU74428
Wyethia amplexicaulis (Nutt.) Nutt.	Wyoming: Lincoln Co.	*Hartman 27988*, (RM)	WAU73159/WAU74429
Zinnia angustifolia Kunth	Louisiana: East Baton Rouge Parish, cultivated	*Urbatsch 7036* (LSU)	ZAU74398/ZAU74447
Z. elegans Jacq.	Louisiana: East Baton Rouge Parish, cultivated	*Urbatsch* 7105 (LSU)	ZEU74399/ZEU74448 ZFU74454/ZFU74455
Z. flavicoma (DC.) Olorode & A. M. Torres	Mexico: Oaxaca	*Hartman & Funk 4192*, (TEX)*	ZFU74400/ZFU74449
Z. grandflora Nutt.	Texas: Jeff Davis Co.	*Butterwick & Poole B393*, (TEX)*	ZGU74397/ZGU74446

ACTACGTCCCTGCC), and 262 (ATTCCCAAAC AACCCGACTCG). Primers were obtained either from Oligos etc., Inc., Wilsonville, Oregon, or the GeneLab, School of Veterinary Medicine, Louisiana State University. Sequential PCRs were conducted first using the outer pair of primers with diluted genomic DNA as template. The resulting PCR product was typically diluted 100-fold for use as template with the inner primer pair. For the sequencing reactions the primers of White et al. (1990) were used, with ITS-I often substituting for ITS5.

Multiple methods were used to purify PCR products for sequence analysis. Differential filtration in Millipore Ultrafree-MC tubes (Millipore UDC3THK00) was used for single-strand DNA. Removal of excess primer and dNTP from the amplified double-strand DNA was accomplished by various methods, e.g., Prep-A-Gene, a silica-based purification matrix (Bio-Rad Laboratories), the enzymes Exonuclease I and Shrimp Alkaline Phosphotase (various suppliers), or QIAquick Spin PCR Purification columns. The last method proved to be the easiest to use and provided the most efficient recovery of purified products.

Single-strand, purified DNAs were sequenced by the dideoxy chain termination technique using *Taq* DNA Polymerase (TAQuence, U. S. Biochemical Co. Cleveland, Ohio) as outlined by Baldwin (1992). Double-strand DNA sequencing involved use of either Sequenase (U. S. Biochemical) with ^{35}S-dATP as internal label or cycle sequencing with SequiTherm (Epicentre Technologies, Madison, WI) employing ^{33}P-dATP end-labeled primers. Sequences were resolved on acrylamide gels as described by Baldwin (1992) and Wojciechowski et al. (1993) or on 6% Long Ranger (FMC BioProducts, Rockland, Maine) gels and were visualized by autoradiography. The *Trichocoryne* sequence was supplied by Baldwin and Wessa (in prep.).

For taxa that displayed polymorphic sequences, PCR products were cloned using the TA Cloning System (Invitrogen Version 1.3). Plasmids were purified with the QIAprep Spin Plasmid Miniprep Kit (Qiagen Inc.) or the lysis-by-boiling method (Sambrook et al. 1989). Usually, two or three clones were sequenced for each polymorphic taxon.

Sequence Analysis. Boundaries of the spacers were determined by comparing the sequences to those published for *Daucus carota* L. and *Vicia faba* L. (Yokota et al. 1989) and subtribe Madiinae Benth. of Asteraceae (Baldwin 1992). Cloned PCR sequences were entered into the data matrix as individual OTUs.

Sequence alignments were performed using the CLUSTAL V algorithm (Higgins et al. 1992). Manual adjustments were made when judged necessary. Usually bases of questionable alignment were positioned to minimize their impact on phylogenetic analyses. Also, bases of uncertain alignment where recoded as question marks (unknown or missing data) when their placement in different positions affected tree topologies. Recoding of such bases allowed inclusion of other, unambiguously aligned data from the sequence region in question in the phylogenetic analyses (see Bruns et al. 1992).

The G+C content was determined for each species with the aid of MEGA (Kumar et al. 1992) and MacClade version 3.01 (Maddison and Maddison 1992). MacClade was also used to generate transversion/transition (tv/ti) substitution ratios. Pairwise sequence divergence comparisons were obtained using the distance matrix option in PAUP.

The ITS-1 and ITS-2 sequences have been deposited in GenBank under the locus numbers given in Table 1. The complete data matrix and the trees from this study are deposited in TreeBASE (http://phylogeny.harvard.edu/treebase; SN367–1066).

Phylogenetic Analyses. Phylogenetic analyses of the entire ITS data set of 65 sequences were conducted to test monophyly of subtribe Rudbeckiinae and to examine relationships of the group within Heliantheae. A reduced data matrix consisting of Rudbeckiinae sensu str. and select outgroups was also analyzed to seek maximum resolution of relationships within Rudbeckiinae. Each of seven taxa (*Borrichia frutescens, Coreopsis tinctoria, Echinacea pallida, Eclipta prostrata, Helianthella quinquenervis, Helianthus simulans,* and *Wyethia amplexicaulis*) was used individually as the sole outgroup in separate analyses focused on determining relationships within Rudbeckiinae. These same seven taxa were also used collectively in heuristic searches.

Unweighted parsimony analyses were performed using PAUP 3.1.1 (Swofford 1993) or the beta test version, PAUP* 4.0b1 (Swofford 1998). Heuristic parameters for all searches included using at least 100 or as many as 1000 random sequence additions with TBR branch swapping, MULPARS on, and STEEPEST DESCENT off. Parsimony analyses were performed initially with all potentially informative characters and subsequently by excluding regions of sequence that presented alignment difficulties. A total of 141 potentially informative characters in the entire data matrix presented alignment problems for analysis. Internal branch support was evaluated by bootstrap analysis (Felsenstein 1985) with 100

replicate heuristic analyses using 10 RANDOM addition sequence replicates, MULPARS on, STEEPEST DESCENT off, and TBR branch swapping on. Bootstrap analyses were conducted using all informative characters and, for the large data matrix, with exclusion of the problematic sites.

A separate data matrix was constructed to take advantage of potential phylogenetic information in inferred insertion/deletion (indel) mutations. Inferred indels were recoded as additional binary characters for all sequences. Indels in the same aligned position and of the same length were scored as homologous.

Parsimony analyses were performed to explore the influence of differential weighting of transversion and transition mutations on tree topology in the higher-level analyses. Heuristic search parameters included 100 random taxon-addition sequences with TBR and MULPARS in effect. Gaps were treated as missing data. Weighted parsimony analyses were conducted with transversions weighted 1.2 or 1.5 fold higher than transitions following recommendations of Albert and Mishler (1992).

The ITS trees were compared to those based on cpDNA by (Urbatsch and Jansen 1995). In addition, heuristic and bootstrap analyses were performed as previously described on a combined ITS/cpDNA data set. Both types of data were available for 43 taxa in Heliantheae including 28 taxa of Rudbeckiinae sensu str. The data set contained a total of 1266 characters and *Coreopsis* served as outgroup. The resulting phylogenetic reconstructions were compared with trees generated from the separate data sets. Parsimony and bootstrap analyses were also performed on the cpDNA matrix for the 28 taxa of Rudbeckiinae for which ITS sequences are available. *Echinacea pallida, Heliopsis* Pers., and *Helianthella* were chosen as outgroup taxa for subtribe Rudbeckiinae based on the ITS results, and each was used for that purpose in the searches of the cpDNA data set.

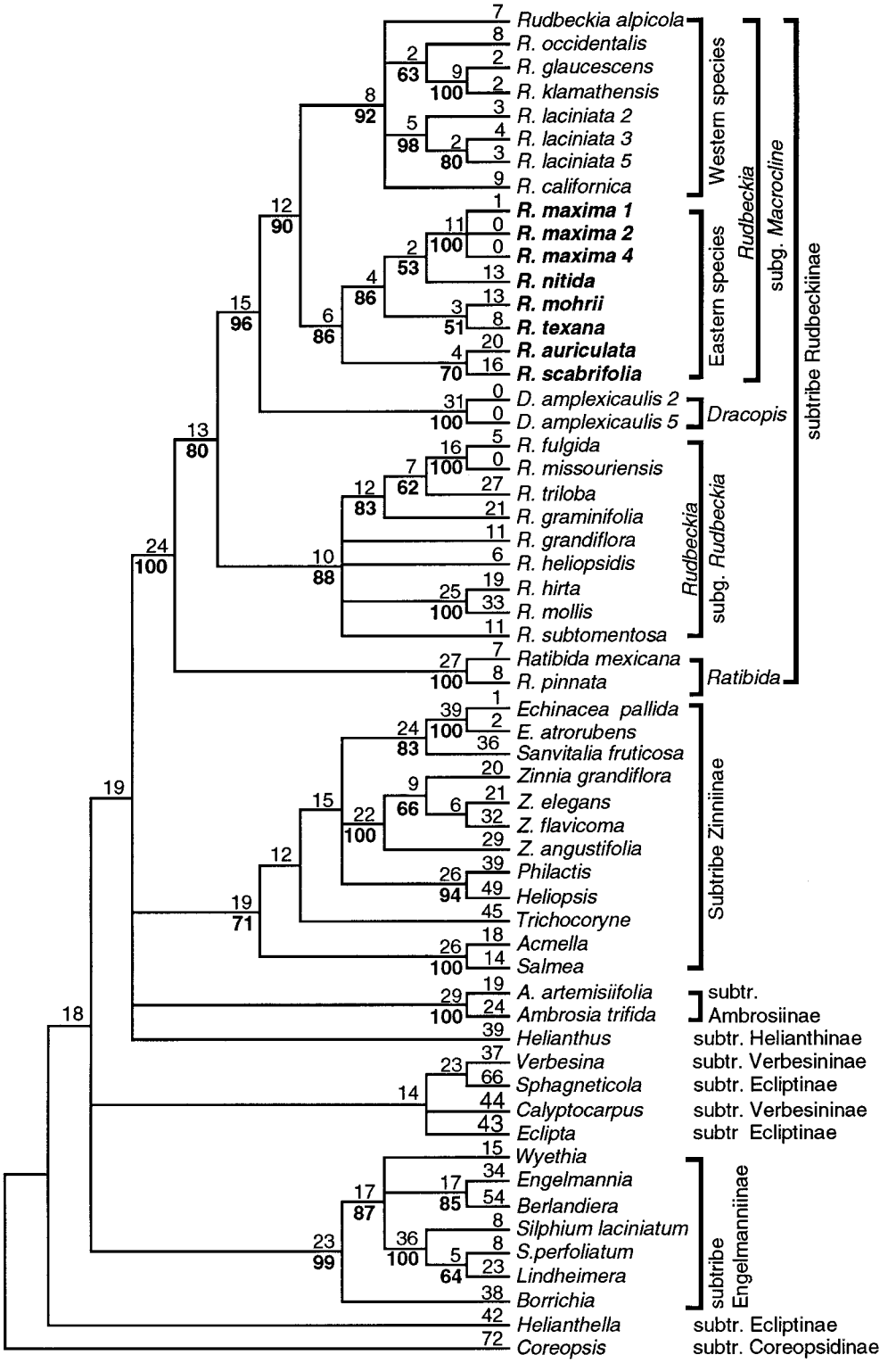
Results

Sequence Characteristics and Variation. Length of the ITS-1 varies from 244 bp in *Lindheimera texana* to 263 bp in *Ambrosia artemisiifolia.* Within Rudbeckiinae, the length of ITS-1 is more uniform, ranging from 246 bp in *Rudbeckia mohrii* to 256 bp in three taxa. ITS-2 varies in length from 209 bp in *R. subtomentosa* to 226 bp in *Silphium laciniatum.* The 218-bp ITS-2 sequences observed in *R. californica* and *R. graminifolia* are the longest for Rudbeckiinae. On average, ITS-1 is about 33 bp longer than ITS-2 in the study taxa. The ranges and means for percentage G + C are very similar for ITS-1 and ITS-2, but somewhat higher for the 5.8S segment investigated. G + C content (excluding indels) ranges in ITS-1 from 41.7% in *Berlandiera pumila* to 56.9% in *Verbesina occidentalis.* G + C content (excluding indels) for ITS-2 ranges from 41.4% in *R. mollis* to 56.6% in *Zinnia flavicoma.* In the 5.8S segment sequenced, G + C content ranges from 52.8% in *Berlandiera pumila* to 60.0% in *Philactis nelsonii.*

Of the gaps inserted to align the sequences, approximately 67.8% involve one base-pair and 20.9% involve two base-pairs. The longest gap necessary to align a sequence (in *Lindheimera* A. Gray & Engelm.) is 12 bp long. The next longest gap, 10 bp, occurs in a single taxon, *Rudbeckia subtomentosa.* Gaps in ITS-1 outnumber gaps in ITS-2 by nearly one third.

Approximately 55% of the characters are potentially informative; 338 of 631 nucleotide positions exhibit at least two states that are each found in at least two taxa. The number of invariant sites in the entire data set is 195; 98 are variable but uninformative. ITS-1 supplies a greater number (171 vs. 147) and higher percentage (32% vs. 27%) of informative sites than does ITS-2. Relative to ITS-1 and ITS-2, the 5.8S subunit contains a much lower proportion of informative sites. In the 5.8S 71% of the sites are invariant compared to 24% in ITS-1 and ITS-2 combined.

In the combined ITS-1, ITS-2, and 5.8S data set, pairwise sequence divergence between species ranges from 0.18% (between *Echinacea paradoxa* and *E. tennesseensis*) to 29.6% (between *Rudbeckia mollis* and *Sphagneticola trilobata*). *Echinacea paradoxa* differs from *E. tennesseensis* by one substitution and six indel events. Mean pairwise distance between species in Rudbeckiinae sensu str. and species in *Zinnia* L. or *Echinacea* exceeds 18.1% in both sets of comparisons. Mean distances between members of Rudbeckiinae and the other taxa of Heliantheae examined are greater than 13.8%. Average mean distance for all sequence pairs in Rudbeckiinae is 11.5%. Interspecific distances in *Rudbeckia* range from 0.7% (*R. glaucescens* and *R. klamathensis*) to 17.8% (*R. mollis* and *R. klamathensis*). The range in distance values is considerably less within *Ratibida* (1.0 to 4.5%) or within *Echinacea* (0.18 to 3.2%) than within *Rudbeckia.* For the ITS-1 alone, interspecific pairwise distances range from 4.0% between *E. pallida* and *E. tennesseensis* to 38.3% between *R. mollis* and *Sphagneticola* O. Hoffmann. Several species pairs


Rudbeckia alpicola
R. occidentalis
R. glaucescens
R. klamathensis
R. laciniata 2
R. laciniata 3
R. laciniata 5
R. californica
R. maxima 1
R. maxima 2
R. maxima 4
R. nitida
R. mohrii
R. texana
R. auriculata
R. scabrifolia
D. amplexicaulis 2
D. amplexicaulis 5
R. fulgida
R. missouriensis
R. triloba
R. graminifolia
R. grandiflora
R. heliopsidis
R. hirta
R. mollis
R. subtomentosa
Ratibida mexicana
R. pinnata
Echinacea pallida
E. atrorubens
Sanvitalia fruticosa
Zinnia grandiflora
Z. elegans
Z. flavicoma
Z. angustifolia
Philactis
Heliopsis
Trichocoryne
Acmella
Salmea
A. artemisiifolia
Ambrosia trifida
Helianthus
Verbesina
Sphagneticola
Calyptocarpus
Eclipta
Wyethia
Engelmannia
Berlandiera
Silphium laciniatum
S.perfoliatum
Lindheimera
Borrichia
Helianthella
Coreopsis
Western species
Eastern species
Rudbeckia
subg. Macrocline
Dracopis
Rudbeckia
subg. Rudbeckia
Ratibida
subtribe Rudbeckiinae
Subtribe Zinniinae
subtr. Ambrosiinae
subtr. Helianthinae
subtr. Verbesininae
subtr. Ecliptinae
subtr. Verbesininae
subtr Ecliptinae
subtribe Engelmanniinae
subtr. Ecliptinae
subtr. Coreopsidinae

within *Echinacea* and within *Ratibida* have identical ITS-2 sequences. The greatest distance value for ITS-2, 33.9%, is for the comparison between *Heliopsis* and *Sphagneticola.*

For certain species, two or three clones of PCR products per sample were chosen at random and sequenced because of polymorphisms evident in the original, direct sequence. The two clones of *Dracopis* sequenced differ by a single indel event in ITS-1. In *Rudbeckia maxima* clones 1 and 2 and clones 1 and 4 differ from each other by two indel events and a transition. Greater variation was observed among the three clones of *R. laciniata*: clone 2 differs from clone 3 by 4 indels, 8 transitions, and 4 transversions; clones 2 and 5 differ by 8 indels, 2 transitions, and 4 transversions. Clones 3 and 5 showed differences at 12 positions, of which 5 are attributable to indel events, 5 to transitions, and 2 to transversions.

Phylogenetic Results. ANALYSES OF THE COMPLETE ITS DATA SET. Parsimony analyses performed on the complete ITS data set for all taxa generated trees in numbers exceeding tree storage capacity. This result is mainly due to the small amount of sequence divergence between congeneric species in *Echinacea* and *Ratibida* and between the intraspecific clones. For this reason all analyses except those for Rudbeckiinae sensu str. excluded all but two exemplar taxa for *Echinacea* and for *Ratibida.* The use of different species pairs for each genus had minimal impact on the resulting tree topologies.

In the first analysis, the data set consisted of equally weighted ITS-1 and ITS-2 sequences along with a portion of the 5.8S region for 57 taxa. Four species each *Echinacea* and *Ratibida* were excluded. Parsimony analysis resulted in 396 minimal length trees of 1842 steps, a consistency index (CI) of 0.396 (uninformative characters excluded), a retention index (RI) of 0.635, and a g_1 statistic of -0.392 for 10,000 random trees generated in PAUP. The CI is somewhat higher than the value of 0.336 for 57 taxa calculated based on data of Sanderson and Donoghue (1989).

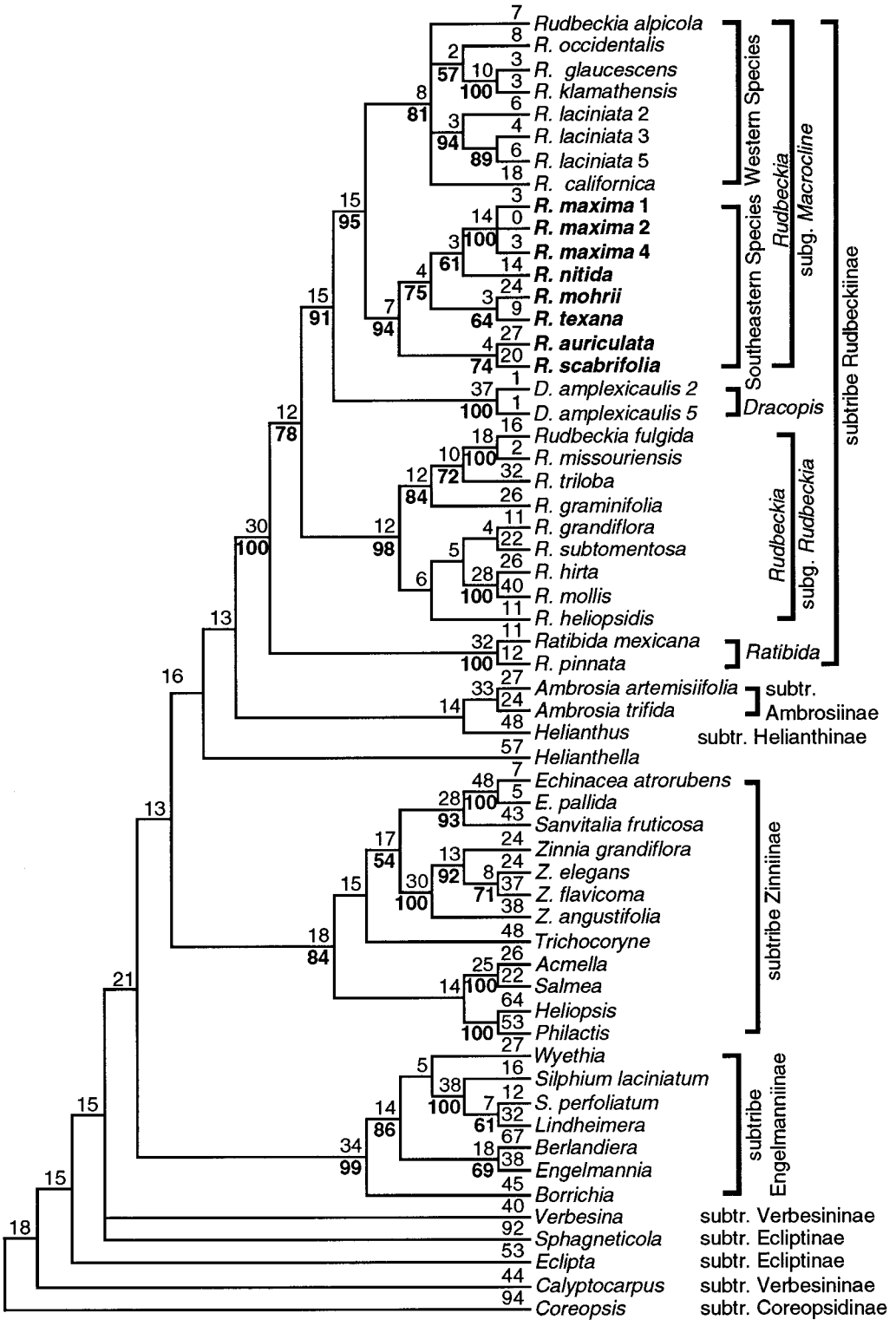
The strict consensus tree from the first analysis shows a high degree of resolution (Fig. 1). Among the major clades receiving moderate to high bootstrap and character support are Engelmanniinae Stuessy, Rudbeckiinae, and Zinniinae. Other deep clades outside of the three subtribes received less than 50% bootstrap support. No sister relationship between Rudbeckiinae and the outgroup taxa is supported.

Because of uncertainties in aligning particular regions of sequence, parsimony analyses were performed on the same set of taxa after removal of problematic basepairs. A total of 147 (79 potentially informative) positions were excluded, 106 bp from ITS-1 and 41 bp from ITS-2. Resolution improved somewhat from the previous heuristic search (trees not shown). The major clades resolved in the previous analysis (i.e., Engelmanniinae, Rudbeckiinae, and Zinniinae) were supported as was the sister group relationship between the *Acmella* Lam./*Salmea* DC. and the *Trichocoryne*/Zinniinae clades. Additional resolution not obtained from the analysis of all ITS characters included (1) *Helianthella, Helianthus,* and *Ambrosia* as a grade basal to Rudbeckiinae and (2) *Sphagneticola*/*Verbesina* L. as a clade sister to Engelmanniinae and the other taxa except for a basal grade of *Calyptocarpus* Less., *Eclipta* L., and *Coreopsis.* The topology of Rudbeckiinae was essentially the same as in the trees from analysis of all ITS characters. In the bootstrap majority-rule consensus tree, Rudbeckiinae and Engelmanniinae received over 92% support. Zinniinae minus *Acmella*/*Salmea, Heliopsis*/*Philactis* Shrad., and *Trichocoryne* received nearly 60% bootstrap support.

Among the ITS sequences of the 65 taxa, 234 indel characters were detected and scored for presence/absence. Parsimony analysis of the indel data set alone for all taxa was terminated due to limited tree storage capacity after 29,000 maximally parsimonious trees (431 steps, CI = 0.504, RI = 0.598) were saved. In the strict consensus tree (not shown), Rudbeckiinae is resolved as monophyletic and *Ratibida* forms a well-supported lineage. Beyond this, minimal resolution was achieved in Rudbeckiinae. Additional monophyletic groups delineated include Zinniinae, with an internal topology

←

FIG. 1. The strict consensus of 48 minimum length trees from parsimony analysis of the ITS-1, ITS-2, and a portion of the 5.8S sequences with equal weighting of all transformations. Each tree has a length of 1732 steps, a consistency index (CI) of 0.438 and a retention index (RI) of 0.561. Branch lengths are given above the branches and bootstrap support values 50% are given in bold print below. Sequences of unique PCR clones are designated with single digit numbers following the specific name.


Rudbeckia alpicola
R. occidentalis
R. glaucescens
R. klamathensis
R. laciniata 2
R. laciniata 3
R. laciniata 5
R. californica
R. maxima 1
R. maxima 2
R. maxima 4
R. nitida
R. mohrii
R. texana
R. auriculata
R. scabrifolia
D. amplexicaulis 2
D. amplexicaulis 5
Rudbeckia fulgida
R. missouriensis
R. triloba
R. graminifolia
R. grandiflora
R. subtomentosa
R. hirta
R. mollis
R. heliopsidis
Ratibida mexicana
R. pinnata
Ambrosia artemisiifolia
Ambrosia trifida
Helianthus
Helianthella
Echinacea atrorubens
E. pallida
Sanvitalia fruticosa
Zinnia grandiflora
Z. elegans
Z. flavicoma
Z. angustifolia
Trichocoryne
Acmella
Salmea
Heliopsis
Philactis
Wyethia
Silphium laciniatum
S. perfoliatum
Lindheimera
Berlandiera
Engelmannia
Borrichia
Verbesina
Sphagneticola
Eclipta
Calyptocarpus
Coreopsis
Western Species
Southeastern Species
Rudbeckia
subg. Macrocline
Dracopis
Rudbeckia
subg. Rudbeckia
Ratibida
subtribe Rudbeckiinae
subtr. Ambrosiinae
subtr. Helianthinae
subtribe Zinniinae
subtribe Engelmanniinae
subtr. Verbesininae
subtr. Ecliptinae
subtr. Ecliptinae
subtr. Verbesininae
subtr. Coreopsidinae

much like that seen in Fig. 1 except that *Heliopsis* and *Philactis* were excluded. The clade of taxa usually associated with Engelmanniinae is also resolved but it is minus *Borrichia* Adans., *Engelmannia* Torr. & A. Gray ex Nutt., and *Lindheimera.*

Parsimony analysis of the combined ITS sequence and indel data sets resulted in six maximally parsimonious trees of 2299 steps (CI = 0.442, RI = 0.619) (Fig. 2). Engelmanniinae, Rudbeckiinae, and Zinniinae clades each received high bootstrap support. The Rudbeckiinae lineage is fully resolved in the strict consensus tree (Fig. 2) except for the western *Macrocline* clade and the lineage of *Rudbeckia maxima* clones. Within the western macroclines, four lineages are supported but their relationships to one another are unresolved. Membership of the Engelmanniinae and Zinniinae clades is as in trees from analysis of ITS sequences without indels recoded (Fig. 1).

Weighted parsimony analysis of ITS sequence data was performed using two different weightings of transversions to transitions, 1.2:1.0 and 1.5:1.0, and resulted in 12 and 40 maximally parsimonious trees, respectively. Tree topologies from both sets of data are identical for the Rudbeckiinae and Zinniinae clades (Fig. 3). In the 1.2:1 analysis, *Helianthus* is sister to Rudbeckiinae, while in the 1.5:1 weighting, *Helianthus* is sister to *Ambrosia,* and the *Ambrosia/Helianthus* clade is sister to Rudbeckiinae. For Engelmanniinae, *Wyethia* is basal to *Berlandiera* DC./*Engelmannia* and *Lindheimera/Silphium* L. in the 1.2:1 reconstruction. In the 1.5:1 strict consensus tree *Wyethia* and the other two clades form a polytomy. *Calyptocarpus, Eclipta, Sphagneticola,* and *Verbesina* constitute a lineage in the 1.2:1 topology that is unresolved in the 1.5:1 analysis. Both sets of bootstrap analyses produced values 94% for Engelmanniinae and Rudbeckiinae. Support for Zinniinae ranges from 65% to 76% in the two different weighted analyses. Bootstrap majority-rule consensus tree topologies are identical for the 1.2:1 weighted analysis, the unweighted ITS data set, and the ITS + indel data set.

Comparison of the unweighted and weighted strict consensus trees reveals some differences in topology. Within subtribe Rudbeckiinae, *R. hirta/R. mollis, R. grandiflora,* and *R. heliopsidis* constitute three branches of a five branch polytomy when equal weights were used (Fig. 1) and a single clade when different weights for transitions and transversions were applied (Fig. 3). In the ITS + indels, subg. *Rudbeckia* is completely resolved (Fig. 2). There is mimimal agreement on the sister group relationship for Rudbeckiinae among results of the various analyses. In the unweighted strict consensus tree for the ITS data set without recoded indels, *Ambrosia, Helianthus,* and Zinniinae are each equally parsimonious sister group candidates. When 1.2:1 weighting is applied to the ITS data set *Helianthus* and Rudbeckiinae are sister. When 1.5:1 weighting is used or when the combined ITS and indel data set is analysed the *Ambrosia/Helianthus* clade is sister to Rudbeckiinae. The composition of Engelmanniinae and Zinniinae is identical in all analyses and the topology of each group differs only in a few details.

Phylogenetic analyses for the combined ITS and cpDNA data matrix resulted in 15 equally parsimonious trees of 1840 steps (CI = 0.6158, RI = 0.7629). The strict consensus of these trees (Fig. 4) is similar in many respects to trees obtained from the ITS data alone. The three major lineages, Engelmanniinae, Rudbeckiinae, and Zinniinae, have bootstrap support values of 100%, 100%, and 56%, respectively. *Helianthella* was placed sister to Rudbeckiinae and *Eclipta* is sister to all ingroup taxa in the strict consensus tree. The *Helianthella*/Rudbeckiinae relationship received less than 50% bootstrap support. Within Rudbeckiinae *Ratibida* is sister to a clade with a basal dichotomy between *Dracopis/Rudbeckia* subg. *Macrocline* and *Rudbeckia* subg. *Rudbeckia.* Bootstrap support for *Rudbeckia* (including *Dracopis*) is 56%. *Dracopis* is basal to *R.* subg. *Macrocline* and the southeastern and western clades receive moderately strong bootstrap support. *Rudbeckia maxima* and *R. texana* are sisters, as are *R. mohrii* and *R. nitida,* which reflect the cpDNA data rather than the ITS pattern of relationship (Fig. 4).

Trees resulting from separate analyses of the ITS and cpDNA data sets (Urbatsch and Jansen 1995) for 43 taxa show considerable agreement. The Engelmanniinae, Rudbeckiinae, and Zinniinae are resolved and strongly supported in trees of both data-sets. *Heliopsis* is placed sister to *Echinacea* in

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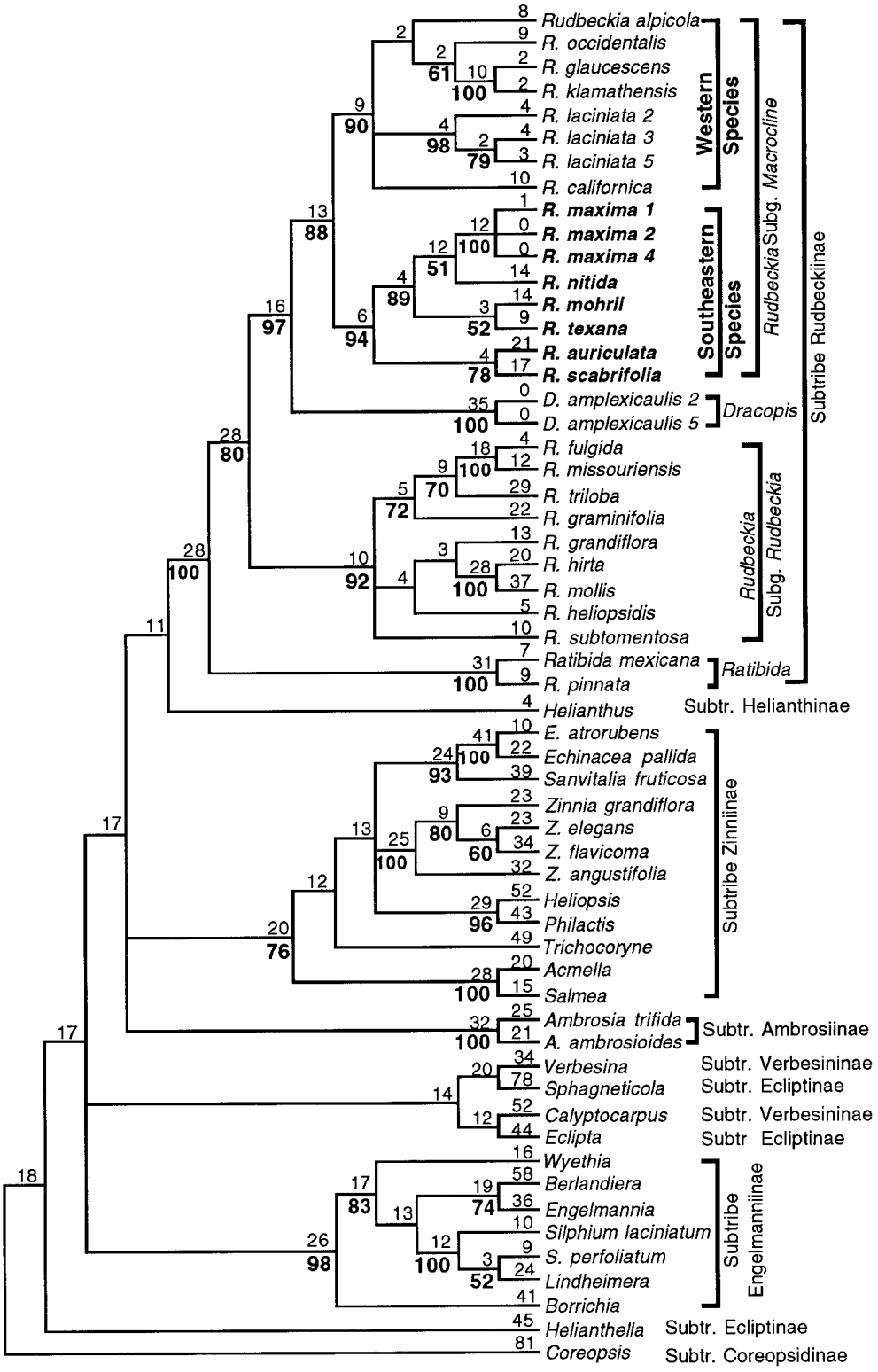
FIG. 2. The strict consensus of 30 minimal length trees from parsimony analysis of the combined ITS-region sequence (including 5.8S data) and indel data sets. Branch lengths and bootstrap values are given above and below the branches, respectively.


Rudbeckia alpicola
R. occidentalis
R. glaucescens
R. klamathensis
R. laciniata 2
R. laciniata 3
R. laciniata 5
R. californica
R. maxima 1
R. maxima 2
R. maxima 4
R. nitida
R. mohrii
R. texana
R. auriculata
R. scabrifolia
D. amplexicaulis 2
D. amplexicaulis 5
R. fulgida
R. missouriensis
R. triloba
R. graminifolia
R. grandiflora
R. hirta
R. mollis
R. heliopsidis
R. subtomentosa
Ratibida mexicana
R. pinnata
Helianthus
E. atrorubens
Echinacea pallida
Sanvitalia fruticosa
Zinnia grandiflora
Z. elegans
Z. flavicoma
Z. angustifolia
Heliopsis
Philactis
Trichocoryne
Acmella
Salmea
Ambrosia trifida
A. ambrosioides
Verbesina
Sphagneticola
Calyptocarpus
Eclipta
Wyethia
Berlandiera
Engelmannia
Silphium laciniatum
S. perfoliatum
Lindheimera
Borrichia
Helianthella
Coreopsis
Western Species
Southeastern Species
Rudbeckia Subg. Macrocline
Dracopis
Rudbeckia Subg. Rudbeckia
Ratibida
Subtribe Rudbeckiinae
Subtr. Helianthinae
Subtribe Zinniinae
Subtr. Ambrosiinae
Subtr. Verbesininae
Subtr. Ecliptinae
Subtr. Verbesininae
Subtr Ecliptinae
Subtribe Engelmanniinae
Subtr. Ecliptinae
Subtr. Coreopsidinae

the cpDNA reconstructions and the clade comprising both groups receives >90% bootstrap support. In the ITS strict consensus tree, *Heliopsis* and *Acmella* constitute a clade that is sister to *Echinacea.* However, bootstrap support for this relationship is less than 50%. *Helianthella* is sister to Zinniinae in the cpDNA trees, a relationship weakly supported based on bootstrap analysis. *Eclipta* is the most basally divergent ingroup clade in the ITS phylogenies.

RELATIONSHIPS IN RUDBECKIINAE S. STR. BASED ON ITS SEQUENCES. In each of the phylogenetic analyses limited to Rudbeckiinae plus individual and collective outgroups, essentially identical major groups were resolved and the tree topologies differ minimally from those obtained from the broad-scale analyses that included other members of Heliantheae. One of the minimal length trees from parsimony analysis with *Echinacea pallida* as outgroup was selected as representative (Fig. 5). *Ratibida, Rudbeckia* subg. *Rudbeckia,* and eastern and western groups of *Rudbeckia* subg. *Macrocline* are each resolved as monophyletic and mostly strongly supported. Three basic topologies that differ from one another in the relative placement of *Ratibida* and *Rudbeckia* subg. *Rudbeckia* are obtained from the use of different outgroups. In the strict consensus trees (not shown) resulting from analyses of the ITS sequences without indels recoded when *Coreopsis, Eclipta, Helianthus* or all seven taxa altogether served as outgroups, *Ratibida* is placed sister to *Dracopis/Rudbeckia,* which in turn contains two major lineages corresponding to *Dracopis*/subg. *Macrocline* and *R.* subg. *Rudbeckia.* When *Echinacea pallida* serves as outgroup, subg. *Rudbeckia* appears as two clades, one composed of *R. fulgida/graminifolia/grandiflora/missouriensis* and the other consisting of the remaining five species. These two lineages and the *Dracopis/R.* subg. *Macrocline* clade form a trichotomy. When *Helianthella* and *Borrichia* are outgroups, relationships among *Dracopis*/subg. *Macrocline,* subg. *Rudbeckia,* and *Ratibida* are unresolved (a trichotomy). *Ratibida* and subg. *Rudbeckia* constitute a clade that is sister to subg. *Macrocline* with *Wyethia* as outgroup. Tree statistics for the seven analyses are as follows: numbers of minimal length trees range from 27 (with *Wyethia* as outgroup) to 135 (with *Echinacea* as outgroup), tree length varies from 588 steps (with *Helianthus* as outgroup) to 624 steps (with *Coreopsis* as outgroup), CI ranges from 0.657 to 0.674, and RI ranges from 0.789 to 0.804.

Similar results were obtained when this set of analyses for Rudbeckiinae was performed on the ITS data with indels coded as a binary character. As in the previous analyses, the sister group relationship between *Ratibida* and *Rudbeckia,* including *Dracopis,* was the most frequently discovered topology (Fig. 5). One result not found with the ITS data without recoded indels when *Wyethia* served as outgroup is that the sister group relationship between *Dracopis* and subg. *Macrocline* received less than 50% bootstrap support. In the analyses of Rudbeckiinae using the ITS and ITS + indel data, *Ratibida* is consistently resolved and receives 100% bootstrap support in all analyses. Bootstrap support for *Rudbeckia* when resolved as a clade in bootstrap majority-rule consensus trees receives 61–84%. In six of 16 analyses, *Rudbeckia* receives < 50% bootstrap support.

Bootstrap support for *Rudbeckia* subg. *Macrocline* sensu str. mostly exceeded 80% in all analyses with different outgroups. Bootstrap values for the clades of subg. *Macrocline* and *Dracopis* range from 80 to 95% depending on the outgroup used. Bootstrap support for the southeastern and the western clades of *R.* subg. *Macrocline* range from 74 to 97% with different outgroups used. *Rudbeckia laciniata,* the only species of *Rudbeckia* that occurs in the Rocky Mountains and is widespread in the eastern United States, is always placed within the western clade.

Phylogenetic analyses of ITS sequence data and the ITS + indel data for the Rudbeckiinae, with various outgroups used individually and collectively, produced a topology wherein *R. alpicola, R californica,* the *R. laciniata* clones, and a clade of the other three taxa (*R. glaucescens, R. klamathensis, R. occidentalis*) form a polytomy. Clades within the western macrocline lineage with > 50% bootstrap support include the group comprising the *R. laciniata* clones and the group comprising the strongly supported clade of *R. glaucescens/R. klamathensis* and its sister, *R. occidentalis.* The greatest resolution within the western clade of *Rudbeckia* subg. *Macrocline* was

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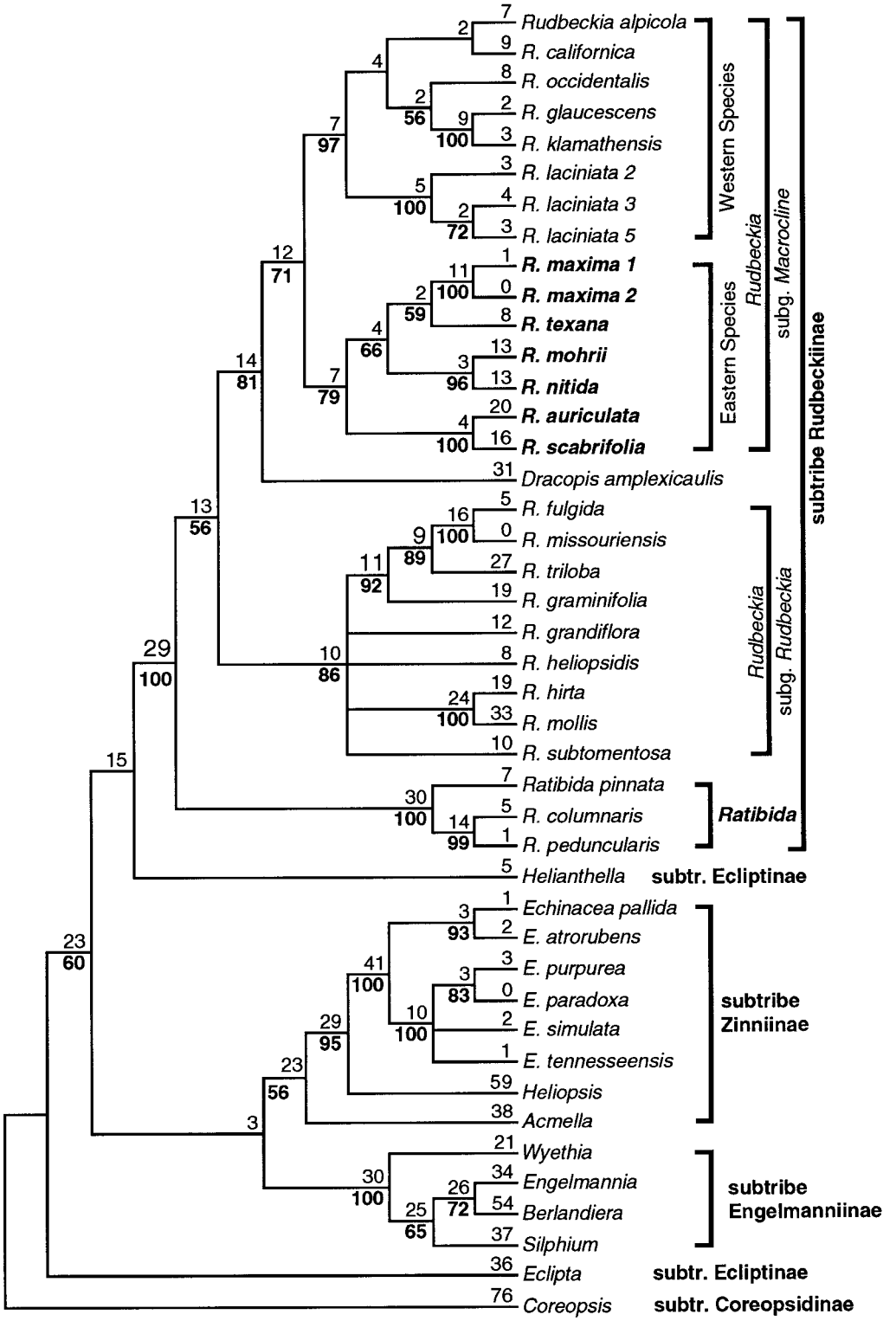
FIG. 3. Strict consensus of six minimum length trees from weighted parsimony analyses with transversions weighted 1.2-fold higher than transitions. Branch lengths are given above the branches and bootstrap support values are given in bold print below.


Rudbeckia alpicola
R. californica
R. occidentalis
R. glaucescens
R. klamathensis
R. laciniata 2
R. laciniata 3
R. laciniata 5
R. maxima 1
R. maxima 2
R. texana
R. mohrii
R. nitida
R. auriculata
R. scabrifolia
Dracopis amplexicaulis
R. fulgida
R. missouriensis
R. triloba
R. graminifolia
R. grandiflora
R. heliopsidis
R. hirta
R. mollis
R. subtomentosa
Ratibida pinnata
R. columnaris
R. peduncularis
Helianthella
Echinacea pallida
E. atrorubens
E. purpurea
E. paradoxa
E. simulata
E. tennesseensis
Heliopsis
Acmella
Wyethia
Engelmannia
Berlandiera
Silphium
Eclipta
Coreopsis
Western Species
Eastern Species
Rudbeckia
subg. Macrocline
subg. Rudbeckia
Ratibida
subtribe Rudbeckiinae
subtr. Ecliptinae
subtribe Zinniinae
subtribe Engelmanniinae
subtr. Ecliptinae
subtr. Coreopsidinae

achieved in analyses of the ITS data-set minus the difficult-to-align regions. In the resulting strict consensus tree, two subclades are resolved–one with *R. californica* sister to *R. laciniata* and the other with *R. alpicola* sister to *R. occidentalis* plus *R. glaucescens*/*R. klamathensis. Rudbeckia alpicola* was placed with the *R. occidentalis* plus *R. glaucescens*/*R. klamathensis* lineage in the results from the weighted analyses (Fig. 3).

Relationships within the southeastern *Rudbeckia* subg. *Macrocline* clade based on ITS data are fully and identically resolved (except for the *R. maxima* clones) and generally well supported (81–94% bootstrap) in trees from most of the analyses conducted in the study (Figs. 1,2,3,5). Three pairs of sister taxa are evident in these trees: *R. auriculata*/*R. scabrifolia* were placed sister to a lineage comprising two pairs of taxa, *R. maxima*/*R. nitida* and *R. mohrii*/*R. texana.* The last four taxa of this lineage forms a polytomy in the ITS + indel analysis for Rudbeckiinae with *Wyethia* as outgroup.

Support for *Rudbeckia* subg. *Rudbeckia* is generally strong, with bootstrap analyses resulting in values ranging from 55–100%. Analyses of the ITS + indel data fully resolved the species into two lineages (Figs. 2,5). The first one, containing *R. fulgida*/*missouriensis*/*triloba*/*graminifolia,* generally received over 70% bootstrap support. Most of the other analyses also resulted in the same topology for this constellation of taxa. The remaining five species were consistently resolved as a clade, with *R. heliopsidis* sister to the clades of *R. hirta*/*R. mollis* and *R. grandiflora*/*R. subtomentosa* in trees of some analyses(Figs. 2, 5). Within this five taxon sublineage only *R. hirta*/*R. mollis* is well supported by bootstrap values. Overall support for the five-taxon sublineage is in the 50–70% range. Analysis of the ITS data minus the recoded indels usually failed to resolve all but the *R. hirta*/*R. mollis* clade.

Although bootstrap support for monophyly of *Ratibida* is 100% in all analyses, resolution of clades within the genus was weakly supported. A pectinate series of clades with *R. pinnata* basally divergent and *R. tagetes*/*R. latipalearis* most apically nested is the most frequently recovered topology from the ITS + indels analyses using different outgroups (Fig. 5). Usually, relationships among *R. columnaris, R. latipalearis,* and *R. tagetes* are unresolved in the trees based on ITS data without the recoded indels. No resolution is achieved in *Ratibida* in the strict consensus tree based on ITS sequences without recoded indels with *Echinacea* as outgroup. Bootstrap support for the lineage exclusive of *R. mexicana* and *R. pinnata* is typically > 90%. Support for the other clades is generally weak ranging from < 50% to 75%.

RESOLUTION OF RELATIONSHIPS IN RUDBECKIINAE S. STR. BASED ON THE ITS AND CPDNA. Figure 6 (right) shows the bootstrap consensus tree for Rudbeckiinae based on cpDNA data with *E. pallida* as the outgroup. In the bootstrap and strict consensus trees, *Dracopis* is sister to a large polytomy that includes *Ratibida* and *Rudbeckia. Ratibida* has 100% bootstrap support and is part of a large polytomy consisting of the species and species groups of *Rudbeckia.* The western species of *Rudbeckia* subg. *Macrocline* are delineated as a clade in the cpDNA reconstruction with just over 50% bootstrap support. Neither the southeastern species of *R.* subg. *Macrocline* nor *R.* subg. *Rudbeckia* are resolved as clades. Strong bootstrap support, however, was obtained for various species groups within *Rudbeckia.* Bootstrap analyses in which *Helianthella* or *Heliopsis* served as outgroup identified the same clades with nearly identical support. In the strict consensus tree from the heuristic search in which *Helianthella* served as outgroup, *Dracopis* is sister to *R. maxima* and *R. texana* of subg. *Macrocline.* Topology of the tree is similar to that of trees generated using other outgroups. The major areas of differences between the two topologies are (1) placements of *Dracopis* and (2) relationships among the southeastern macrocline species *R. maxima, R. mohrii, R. nitida,* and *R. texana.* None of the two sets of conflicting groups is strongly supported in either the cpDNA and ITS trees (Fig. 6).

DISCUSSION

ITS Sequence Characteristics. ITS-1 length variation recorded in this study falls within the range of that reported for other Asteraceae; length variation of the ITS-2 ranges to a few base pairs shorter than previously reported in the family (Baldwin et al. 1995; Susanna et al. 1995). The ITS in the study taxa, as in other angiosperm groups, appears to

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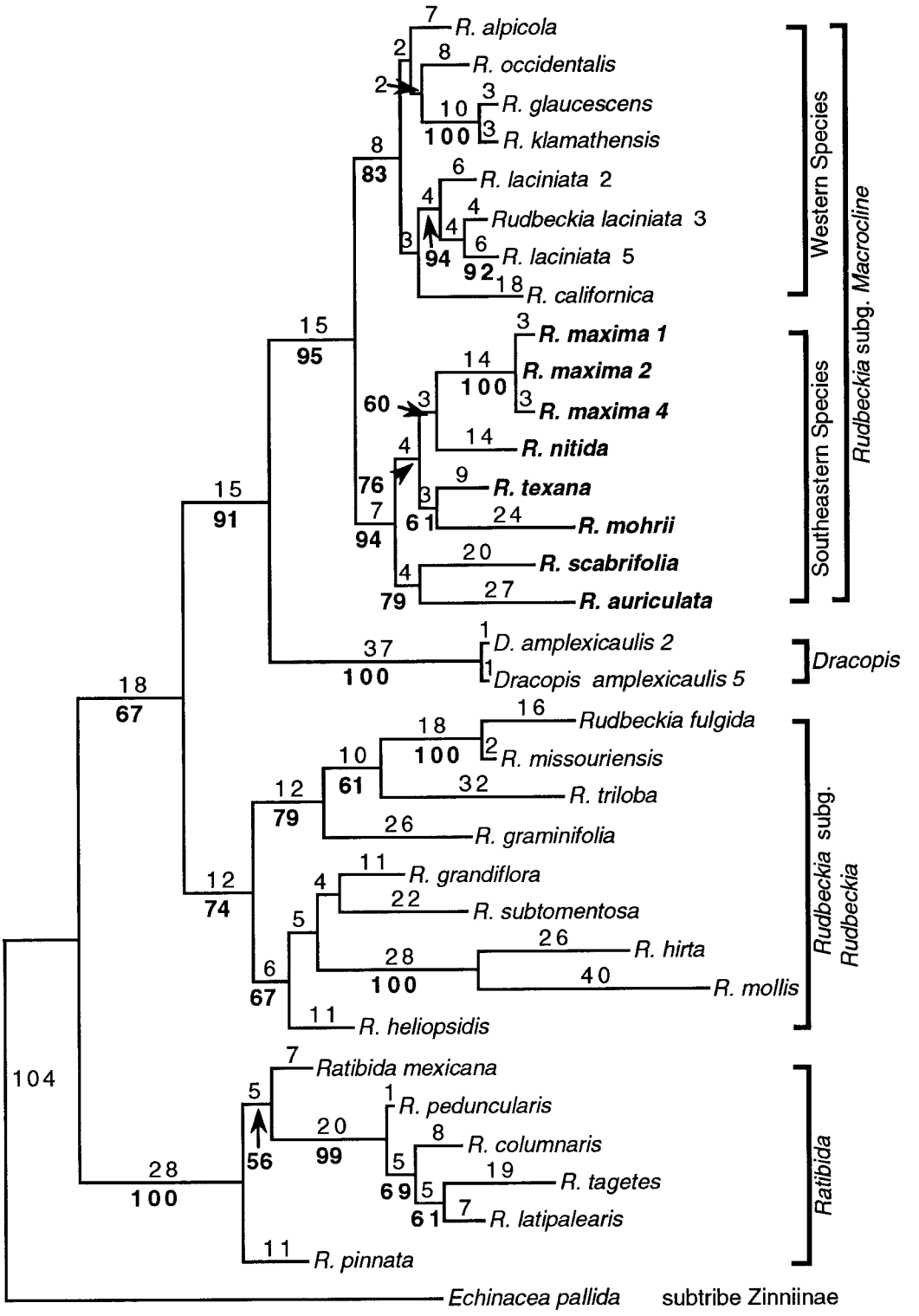
FIG. 4. Strict consensus of 168 minimal-length trees derived from parsimony analyses of the combined ITS and cpDNA data matrices. Branch lengths and bootstrap values are given above and below the branches, respectively.


R. alpicola
R. occidentalis
R. glaucescens
R. klamathensis
R. laciniata 2
Rudbeckia laciniata 3
R. laciniata 5
R. californica
R. maxima 1
R. maxima 2
R. maxima 4
R. nitida
R. texana
R. mohrii
R. scabrifolia
R. auriculata
D. amplexicaulis 2
Dracopis amplexicaulis 5
Rudbeckia fulgida
R. missouriensis
R. triloba
R. graminifolia
R. grandiflora
R. subtomentosa
R. hirta
R. mollis
R. heliopsidis
Ratibida mexicana
R. peduncularis
R. columnaris
R. tagetes
R. latipalearis
R. pinnata
Echinacea pallida
subtribe Zinniinae
Western Species
Southeastern Species
Rudbeckia subg. Macrocline
Dracopis
Rudbeckia subg. Rudbeckia
Ratibida

have evolved mostly by nucleotide substitution and small (one or two base pair) length mutations rather than by large-scale insertions, deletions, or rearrangements.

The motif GGCRYGYGYCAAGGA reported in ITS-1 for Apioideae Seem. and other angiosperms by Downie and Katz-Downie (1996) is also seen in our sequences and in published Asteraceae data sets (Baldwin 1992, 1993). This region is thought to be an essential recognition element for rRNA processing (Liu and Schardl 1994). In other sequence regions, uniform nucleotide composition in ITS-1 is limited to individual sites or a few consecutive sites. For ITS-2, the first five (of six) conservation patterns reported by Hershkovitz and Zimmer (1996) were readily identifiable among the taxa examined. Region six was less evident in Heliantheae sequences. Conservation of the six regions may confer a general secondary structure for ITS-2 (Hershkovitz and Zimmer 1996). These and other conserved sites in Heliantheae served as valuable guides for alignment.

Length and sequence variation found among cloned ITS sequences from the same individuals of three species (*Dracopis amplexicaulis, Rudbeckia laciniata,* and *R. maxima*) demonstrate that homogenization of sequences by concerted evolution is incomplete. In a phylogenetic context, variation among ITS paralogues was not problematical–all cloned sequences from particular species were resolved as clades.

Polyphyly of the Coneflowers. We interpret the phylogenetic analysis of ITS sequences as strongly supporting a subtribe Rudbeckiinae that includes *Dracopis, Ratibida,* and *Rudbeckia* but not *Echinacea* (Figs. 1–4). A similar interpretation was based on findings from cpDNA restriction site analysis (Urbatsch and Jansen 1995). Robinson (1978, 1981) questioned the affiliation of *Echinacea* with the other three coneflower genera. He noted that the nonstriate cypselae and base chromosome number of $x = 11$ in *Echinacea* differ markedly from the striate cypselae and base chromosome numbers of $x = 18$ to 20 in Rudbeckiinae. He also noted differences in endothecial cell walls between *Echinacea* and Rudbeckiinae (Robinson 1981). Such considerations led Robinson (1981) to conclude that Ecliptinae should include *Echinacea* and that *Balsamorhiza, Helianthella,* and *Wyethia* are possible close relatives of the genus.

Relationship of* Echinacea *to* Zinnia *and Relatives. Subtribe Zinniinae as originally circumscribed by Bentham (1873) accommodated six genera, *Aganippea* DC. (now synonymous with *Jaegeria* Kunth), *Heliopsis, Philactis, Sanvitalia* Lam., *Tragoceras* Kunth in Humboldt et al., and *Zinnia.* Stuessy (1977) maintained that concept with the important exception of also including the enigmatic genus *Borrichia* in the subtribe. He offered chromosome number, leaf arrangement, and pappus features as supporting characters for Zinniinae. *Jaegeria* had been removed to Galinsoginae Benth. (Torres and Beaman 1964; Torres 1968). Robinson (1981) included Zinniinae sensu Stuessy (1977) within Ecliptinae, asserting that many characteristics of Zinniinae are also present in Ecliptinae. Karis and Ryding (1994) substantially changed the composition of Zinniinae by including *Acmella, Podachaenium* Benth. ex Oerst., *Spilanthes* Jacq., and *Squamopappus* R. K. Jansen, N. A. Harriman, & Urbatsch, plus the previously mentioned core genera, except for *Borrichia.* They relegated *Borrichia* to Verbesininae Benth. Panero and Villaseñor (1996) published *Tehuana* Panero & Villaseñor and noted its morphological and cytological similarities to *Heliopsis* and *Philactis.* Our attempts to isolate usable DNA from dried leaves of *Tehuana* were unsuccessful.

In the earlier cpDNA restriction site analysis, a clade comprising *Echinacea* and *Heliopsis* constituted one of the most strongly supported lineages, another being Rudbeckiinae (Urbatsch and Jansen 1995). Both clades had relatively high decay indices and bootstrap values. Representatives of *Balsamorhiza, Helianthella,* and *Wyethia,* members of Ecliptinae sensu Robinson (1981), were included in the cpDNA study but did not constitute a clade. Weak support was obtained for a cpDNA lineage including *Helianthella* and the *Echinacea*/*Heliopsis* clade (Urbatsch and Jansen 1995). *Heliopsis* has been regarded as a core member of Zinniinae by previous investigators (Bentham 1873; Hoffmann 1894; Stuessy 1977; Karis and Ryding 1994). The restriction site data were interpreted as support for an hypothesis that *Echinacea* is more closely related to

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FIG. 5. One of 15 minimum length trees from parsimony analysis of ITS-1, ITS-2, and a portion of the 5.8S region plus recoded indel characters for Rudbeckiinae, with Echinacea pallida as outgroup (772 steps, CI = 0.644, RI = 0.756). Branch lengths appear above the branches and bootstrap numbers for 100 replicates appear below.

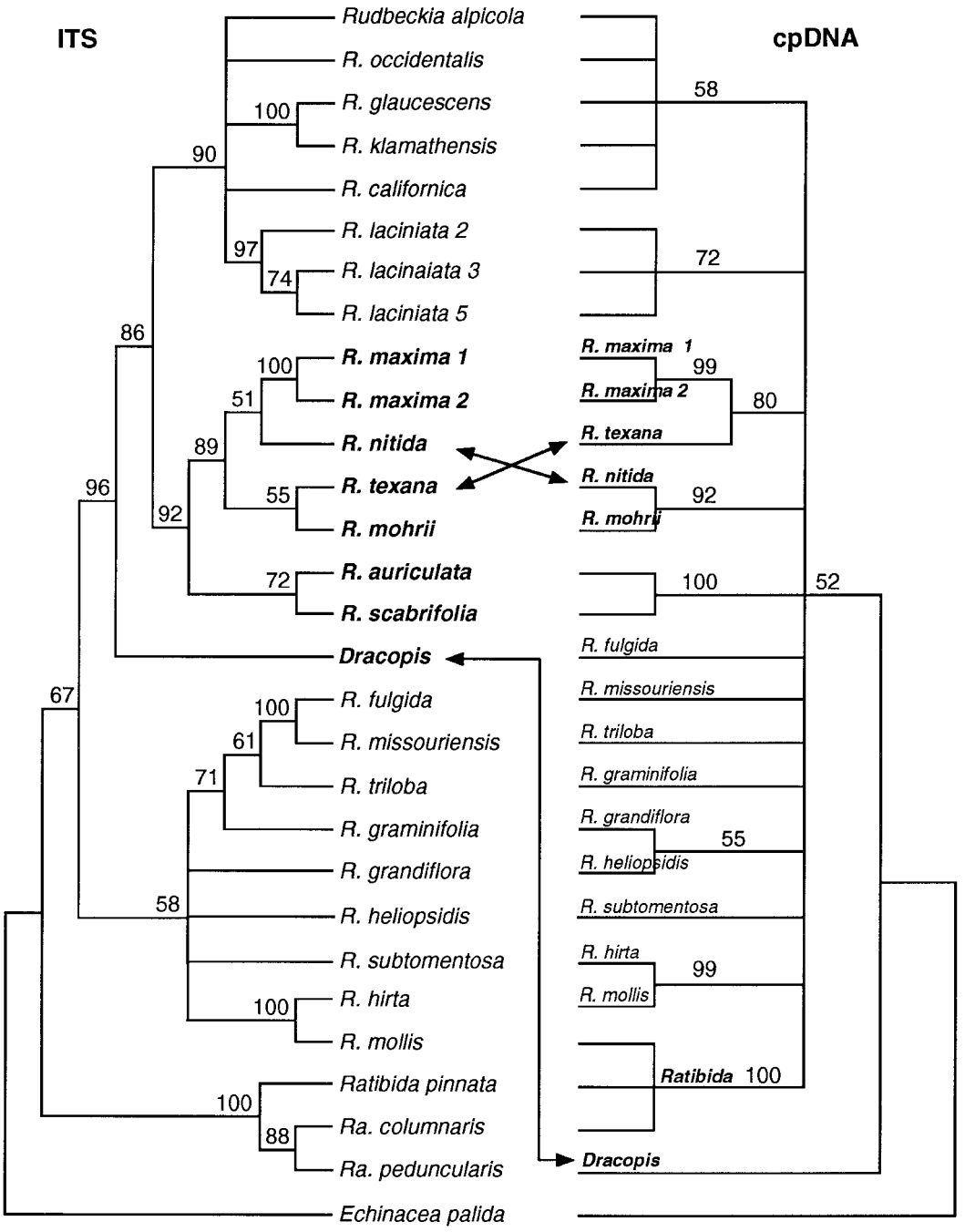

FIG. 6. Comparison of bootstrap consensus trees for the Rudbeckiinae derived from separate analyses of the ITS and cpDNA data sets. Opposing branches of the two trees contain the same terminal taxa unless otherwise indicated. Lines connecting certain terminal taxa indicate resolved relationships that differ between the two trees.

traditional Zinniinae than to other taxa in Ecliptinae sensu Robinson (1981).

Based on our ITS results we affirm a phylogenetic position of *Echinacea* within Zinniinae (Figs. 1–4), which we sampled more thoroughly than in the previous cpDNA investigation (Urbatsch and Jansen 1995). Most analyses of our ITS data gave trees with *Echinacea* and *Sanvitalia* as sister taxa. *Zinnia* is sister to this clade in some of our trees, mostly with low bootstrap support values.

Morphological and cytological data are mostly congruous with placement of *Echinacea* in Zinniinae. Like most other members of Zinniinae, *Echinacea* has solitary capitula, conical receptacles, rigid paleae, and a short, crown-like pappus. As noted by Robinson et al. (1981), the base chromosome number for *Echinacea,* $x = 11$, is similar to the proposed base numbers of $x = 8$–12 for *Zinnia* and *Sanvitalia.* Alternate leaf arrangement in *Echinacea* is the only anomalous characteristic among the otherwise opposite-leaved Zinniinae and possibly the major reason for previous association of *Echinacea* with Rudbeckiinae.

The sister group relationship between *Echinacea* and *Sanvitalia* in the ITS trees (Figs. 1–3) is supported by a potential synapomorphy in fruit anatomy. The outer layer of the cypsela wall in *Echinacea* is composed of a layer of soft, tan tissue several cells thick, that is underlain by a shiny, dark-pigmented layer (Schulthess et al. 1991). Similar characteristics are observed in *Sanvitalia fruticosa* (Urbatsch, unpubl. data).

One surprising result from the present ITS sequence analyses is the inclusion of *Trichocoryne* in Zinniinae (Figs. 1–3). The monospecific genus was assigned by Robinson (1981), with reservations, to Hymenopappinae (Heliantheae sensu lat.). As noted by Robinson (1981), Hymenopappinae and other mostly epaleate subtribes in Heliantheae sensu lat. were regarded as members of Helenieae by earlier synoptical workers (Bentham 1973 and Hoffman 1894), and by Bremer (1994). Results of ITS investigations of helenioid Heliantheae, Baldwin and Wessa (in prep.) concluded that *Trichocoryne* is nested in Heliantheae s. str. and more closely related to *Rudbeckia* than to other sampled taxa (none in traditional Zinniinae). Our subsequent inclusion of *Trichocoryne* of the ITS-region sequence in analysis of the coneflower ITS matrix yielded evidence for placement of the genus in the Zinniinae clade. ITS evidence for placement of *Trichocoryne* in Heliantheae sensu str. in general and Zinniinae in particular is interesting because *Trichocoryne* is epaleate. Presence or absence of paleae traditionally has been a major feature for diagnosing Heliantheae sensu str. and Helenieae, respectively. Some epaleate core Heliantheae genera are known, e.g., the *Geissopappus* group in *Calea* and the distantly related *Tyleropappus* Greenman, and are regarded as isolated descendants of paleate taxa (Robinson 1981). Based on ITS trees of Heliantheae sensu lat. (including Helenieae) Baldwin and Wessa (in prep.) similarly concluded that *Trichocoryne* descended from paleate ancestors, a conclusion reinforced by our findings. Harris (1995) showed that disk flowers and paleae in Heliantheae initially share a common primordium. Suppression of the abaxial portions of such primordia may result in the loss of receptacular bracts. Despite lack of paleae, *Trichocoryne* possesses features characteristic of most Zinniinae, e.g., opposite leaves, highly conical receptacles, and blackened anther thecae. We interpret such morphological characteristics together with ITS sequence data as support for reclassification of *Trichocoryne* within Zinniinae.

Segregation of Zinniinae from Ecliptinae. Based on our results we support recognition of Zinniinae as a taxon apart from Ecliptinae sensu Robinson (1981) and argue for recircumscription of Zinniinae (Figs. 1–3). Renovations of Zinniinae proposed by Karis and Ryding (1994) such as the addition of *Acmella* and *Salmea* are in accord with the ITS data presented here. Other molecular data provide a basis for arguing for removal of *Podachaenium* Benth. and *Squamopappus* Jansen, Harriman, and Urbatsch from Zinniinae (Urbatsch, unpubl. data; Panero et al. 1999). Regardless of the eventual composition of Zinniinae, our DNA data constitute evidence for placement of *Echinacea* and *Trichocoryne* with taxa now included in Zinniinae.

In our analyses (Figs. 1–4) *Helianthella,* a putative relative of *Echinacea, Balsamorhiza,* and *Wyethia* (fide Robinson 1981), was resolved as a basally divergent ingroup taxon sister to a clade of *Helianthus, Ambrosia,* and Rudbeckiinae, or a sister to Rudbeckiinae. Analyses of ITS sequences strongly supports a closer relationship of *Helianthella* to *Encelia* Adans. and *Flourensia* DC. than to various taxa in this study or to *Balsamorhiza* and *Wyethia* (Urbatsch, Baldwin, and Pell, unpubl. data). A subgroup within Ecliptinae composed of *Encelia, Enceliopsis* (A. Gray) A. Nelson, *Flourensia, Geraea* Torr. & A. Gray, and other genera was endorsed by Robinson (1981), who noted fused stigmatic surfaces, pale anther thecae, and sterile rays as supporting evidence of relationship. Karis and Ryding (1994) likewise sup-

ported the concept of an *Encelia, Enceliopsis, Flourensia,* and *Geraea* lineage and designated it the *Encelia* group within their Verbesininae. They included *Helianthella* in Verbesininae without comment on its generic relationship. Restriction site cpDNA studies support the sister status of *Encelia* and *Helianthella* (Panero et al. 1999). The *Encelia/Helianthella* cpDNA lineage is sister to a pair of clades: one branch containing Zinniinae and the other containing *Acmella, Salmea,* and *Spilanthes* (Panero et al. 1999).

Circumscription of Engelmanniinae. Diagnostic characteristics for Engelmanniinae sensu Stuessy (1977) include the apomorphic cypsela-palea-phyllary complex (hereafter, cypsela complex) and such synapomorphic attributes as often alternate and dissected leaves, pistillate ray florets, functionally staminate disk florets, and a base chromosome number of $x = 9$.

The ITS based phylogenies present heretofore unpredicted generic groupings (Clevenger and Panero 1997; Urbatsch et al. 1997). *Borrichia* in the ITS trees is a basally divergent element in a clade of genera traditionally treated as subtribe Engelmanniinae (Figs. 1–3). As mentioned above, *Borrichia* has been placed variously in Zinniinae (Stuessy 1977), Ecliptinae (Robinson 1981), and Verbesininae (Karis and Ryding 1994). Stuessy (1977) noted that the chromosome number of *Borrichia* is consonant with Zinniinae. Robinson (1981) commented on the distinctive qualities of *Borrichia,* but did not suggest possible close relatives within subtribe Ecliptinae. Karis and Ryding (1994) likewise offered no phylogenetic hypothesis concerning *Borrichia.* Expanded ITS investigations (Urbatsch, Baldwin, and Pell, unpubl. data) reaffirm the support presented here for placing *Borrichia* in Engelmanniinae, as do cpDNA investigations (Panero et al. 1999).

Previous hypotheses of relationship for the *Balsamorhiza/Wyethia* clade are not supported based on our ITS data. *Balsamorhiza* and *Wyethia* have been treated as members of Ecliptinae, with *Echinacea* proposed as a close relative (Robinson 1981), or as members of Verbesininae, which contain *Calyptocarpus, Verbesina,* and many other genera in the circumscription of Karis and Ryding (1994). In the phylogeny based on the cpDNA restriction site analysis of Urbatsch and Jansen (1995), *Balsamorhiza* and *Wyethia* are contained in a well-supported clade of core Engelmanniinae. Panero et al. (1999), in a much larger cpDNA study of Ecliptinae, re-affirm this relationship and include additional genera in Engelmanniinae. ITS data offers strong support for aligning *Wyethia* and *Balsamorhiza* with Engelmanniinae (Clevinger and Panero 1997; Urbatsch et al. 1997). *Balsamorhiza, Wyethia,* and *Borrichia* lack apomorphies characteristic of Engelmanniinae sensu str., such as the cypsela complex, and have bisexual disk florets and higher base chromosome numbers ($x = 19, 20$ in *Balsamorhiza* and *Wyethia*) than those members of Engelmanniinae sensu str. Synapomorphic, morphological features for the enlarged Engelmanniinae are pistillate ray florets and large, leaf-like phyllaries in most species.

Stuessy (1977) regarded Ambrosiinae Less. as sister to Engelmanniinae, with *Parthenium* L. and *Parthenice* A. Gray ''transitional'' between the two subtribes. Both genera have cypsela complexes similar to those found in most Engelmanniinae. Stuessy (1973) regarded the cypsela complexes in Ambrosiinae to be pleisiomorphic, with a reversal to the more typical state (palea, phyllaries, and cypselas separate) in ''advanced'' Ambrosiinae, but with continuing specialization in most Engelmanniinae. Karis and Ryding (1994) similarly concluded that the cypsela complexes in Ambrosiinae and Engelmanniinae are symplesiomorphic because the two subtribes and two other groups formed a polytomy in their trees based on morphological data. Bolick (1983) concluded from her cladistic study of Ambrosiinae and Engelmanniinae a sister group relationship between the two subtribes is most likely incorrect. Robinson (1981) concluded similarly that the cypsela complexes originated independently in the two groups, a hypothesis we supported based on our ITS data (Figs. 1–3).

Subtribe Ecliptinae and Relatives. The phylogenetic position of *Eclipta* remains poorly resolved. Along with *Calyptocarpus, Verbesina,* and *Sphagneticola, Eclipta* constitutes a clade in the weighted ITS tree (Figs. 1,3). In most analyses, however, it was at the base of the tree. In results of the various ITS analyses, none of the clades comprising *Eclipta* and a sister group received more than 50% bootstrap support. Robinson (1981) suggested a relationship of *Eclipta* to *Wedelia* Jacq. and a group of approximately 23 other genera including *Calyptocarpus* and *Silphium.* Stuessy (1977) offered a somewhat similar classification for these genera but excluded those of Engelmanniinae. Karis and Ryding (1994) left *Eclipta* without subtribal placement. The ITS data weakly support some of the previously suggested hypotheses of relationship for *Eclipta.* Panero et al. (1999) offered strong support for placement of *Eclipta* basal to a group of genera they call the ''wedelioid complex,'' which contains *Sphagneticola* but not *Verbesina.*

Sister Relationship of Rudbeckiinae. Owing to poor resolution or support for the more basal nodes in ITS trees, relationships among the major clades and, particularly, idendity of the sister group of Rudbeckiinae, remain uncertain. Among the possible candidates for sister group of Rudbeckiinae are two groups that were resolved as such (but with weak support) in the various ITS trees: *Helianthella* and *Helianthus* (also *Helianthus/Ambrosia*). *Helianthella* was resolved as sister to Rudbeckiinae in the ITS data minus difficult-to-align basepairs and in the ITS + cpDNA-based phylogenies. Chloroplast DNA data by itself, however, does not provide evidence for such a relationship (Urbatsch and Jansen 1995). *Helianthus* and *Ambrosia/Helianthus* are sister groups of Rudbeckiinae in the 1.2 and 1.5 weighted analyses, respectively. None of the suggested sister relationships of Rudbeckiinae received more than 50% bootstrap support.

Relationships in Rudbeckiinae. MAJOR CLADES. Our ITS results corroborate previous cpDNA findings on the monophyly of Rudbeckiinae and provide the best resolved phylogeny thus far obtained for this group. Previous phylogenetic investigations of cpDNA identified certain genera and species groups and provided incomplete resolution of relationships among species of Rudbeckiinae (Urbatsch and Jansen 1995). Our ITS analyses identified three major lineages coincident with the traditionally recognized genus *Ratibida* and the two subgenera of *Rudbeckia,* subg. *Macrocline* and subg. *Rudbeckia* (Perdue 1957, 1959). The results of most of our analyses placed the two subgenera of *Rudbeckia* together with *Dracopis,* exclusive of *Ratibida.*

PHYLOGENETIC POSITION OF *RATIBIDA.* The ITS data corroborate previous evidence for monophyly of *Ratibida* (Urbatsch and Jansen 1995). Some synapomorphic features support the *Ratibida* lineage. *Ratibida* has a different base chromosome number (Robinson et al. 1981) and a distinctive chromosomal morphology compared to those of other coneflowers (Perdue 1959). Morphological characteristics uniting species in *Ratibida* include phyllaries in two unequal series, a dark, resinous gland along the adaxial edge of each palea, paleae that enclose the cypselae and are shed with them, and laterally compressed cypselae.

Previous suggestions about phylogenetic relationships in Rudbeckiinae have usually implicated *Dracopis* as the closest relative of *Ratibida.* Based on their ruderal ecology, similar, biseriate involucres, and cylindrical receptacles, Sharp (1935) suggested that *Ratibida* and *Dracopis* arose from a common ancestor within a complex now treated as *Rudbeckia* subg. *Macrocline,* i.e., *Ratibida* would have been nested within *Rudbeckia.* Sharp (1935) regarded the highly dissected leaf blades, laterally compressed cypselae, seed dispersal mechanism, aggressive weediness, and occupation of geologically recent areas seen in *Ratibida* as indications of greater specialization relative to that of *Rudbeckia.* Richards (1968) cited additional similarities shared by *Ratibida* and *Dracopis,* e.g., resin canal position in the stems and similar chromosome numbers. One of the tree topologies obtained in the mostly morphologically-based phylogenetic analysis of Cox and Urbatsch (1990) resolved a sister group relationship of *Ratibida* and *Dracopis.*

The molecular data provide a different picture. In the ITS trees (Figs. 1–3) and in the combined ITS/cpDNA trees (Fig. 4), and the cpDNA trees (Urbatsch and Jansen 1995), *Ratibida* is never nested within *Rudbeckia.* Instead, *Ratibida* is most frequently resolved as sister to *Rudbeckia* plus *Dracopis,* in accord with an hypothesis of divergence of *Ratibida* from a common ancestor with *Rudbeckia* and *Dracopis* prior to the origin of modern species in both groups. Less often, *Ratibida* was placed as one branch of a trichotomy with the two subgenera of *Rudbeckia,* and in some trees *Ratibida* was placed sister to *Rudbeckia* subg. *Rudbeckia,* and this clade in turn was placed sister to *Rudbeckia* subg. *Macrocline* plus *Dracopis.* Based on the weight of evidence from ITS and cpDNA data we continue to recognize *Ratibida* as a lineage distinct from *Rudbeckia.*

RELATIONSHIPS OF *DRACOPIS.* In the ITS trees, *Dracopis* is almost always placed sister to *Rudbeckia* subg. *Macrocline,* usually with high bootstrap support. This result conflicts with the earlier hypothesized sister-group relationship between *Ratibida* and *Dracopis* (Sharp 1935; Richards 1968). Based on the ITS data, the morphological and cytological similarities shared by *Ratibida* and *Dracopis* may be due to convergence or retention of plesiomorphic states.

In cpDNA trees resulting from a broad analysis of Heliantheae and from cpDNA analysis of Rudbeckiinae with most outgroup taxa included, *Dracopis* is placed sister to *Ratibida* and *Rudbeckia* (Urbatsch and Jansen 1995), with weak support. A slightly more parsimonious solution in an analysis including fewer taxa is a cpDNA sister group relationship between *Dracopis* and a clade containing *R. maxima* and *R. texana,* members of *Rudbeckia* subg. *Macrocline.* The cpDNA data thus appear to be congruent (or not significantly incongruent)

with ITS evidence for a close relationship between *Dracopis* and *Rudbeckia* subg. *Macrocline.* Morphology is also congruent with this relationship.

Although *Dracopis amplexicaulis* was originally described as a species of *Rudbeckia,* wherein it was treated as a section by Gray (1884) and Fernald (1950), more recent investigators have emphasized the distinctiveness of *Dracopis* and followed Cassini in assigning it generic status (Gleason 1952; Correll and Johnston 1970; Cronquist 1980). Despite the distinctive characteristics of *Dracopis* (e.g., annual habit, lack of pappus, and low base chromosome number), it shares many characteristics with taxa in *Rudbeckia* subg. *Macrocline,* e.g., rigid, glaucous stems; sessile, clasping, glaucous, cauline leaves; yellow (vs. yellow-orange) ray corollas; and cone-shaped receptacles. Sectional status for *Dracopis* within *R.* subg. *Macrocline* best communicates existing knowledge by emphasizing both phylogenetic relationships and readily diagnosed groups. Maintaining *Dracopis* as a separate genus could only be accommodated by elevating *R.* subg. *Macrocline* and *R.* subg. *Rudbeckia* to generic rank but would necessitate many name changes.

RELATIONSHIPS WITHIN *RATIBIDA.* Phylogenetic resolution within *Ratibida* was hampered by the limited ITS sequence divergence among species. The lineage resolved most frequently (with > 90% bootstrap support) in analyses restricted to Rudbeckiinae comprises *Ratibida columnaris, R. latipalearis, R. peduncularis,* and *R. tagetes* (Fig. 5). Groups resolved within this clade received from less than 50 to 70% bootstrap support. There was no resolution in *Ratibida* in ITS trees with *Echinacea* as outgroup. When the ITS sequence and indel data sets were combined *R. pinnata* was basally divergent to a clade with a pectinate arrangement of the other. Except for the four-taxon clade referred to above most other lineages received weak support from the ITS analyses. Minimal variation in cpDNA among members of *Ratibida* also resulted unstable resoluton of relationships (Urbatsch and Jansen 1995).

SUBGENERA OF *RUDBECKIA.* The ITS data and the combined ITS/cpDNA data, unlike the cpDNA trees (Urbatsch and Jansen 1995), support recognition for the two traditionally defined subgenera of *Rudbeckia,* subg. *Macrocline* and subg. *Rudbeckia.* The two clades were resolved in all analyses except with the ITS indel data alone. Bootstrap support for the subgenera in results of most analyses is above 80%. Support for monophyly of the two subgenera is congruent with different base chromosome numbers and with evidence from some morphological characters. In fact, previous investigators tended to emphasize the distinction between the subgenera more than the characteristics uniting the genus *Rudbeckia* (Perdue 1957, 1959).

On the basis of the ITS data, an argument could be made for elevating the two subgenera of *Rudbeckia* to generic status. Bootstrap support for *Rudbeckia* sensu lat. ranges from 58 to 80% in results of the various analyses. Approximately one-quarter of the ITS analyses failed to resolve *Rudbeckia* as a monophyletic group, and certain analyses, as noted above, provide 50 to over 60% bootstrap support for a sister group relationship between *R.* subg. *Rudbeckia* and *Ratibida.*

The case for retaining *Rudbeckia* is strengthened when morphological and cytological evidence are considered. The two subgenera of *Rudbeckia* are more similar to each other than to *Ratibida* in involucre, cypsela, and palea morphology. The same is true for base chromosome number, with similar numbers of $x = 18$ in *Rudbeckia* subg. *Macrocline* and $x = 19$ in *R.* subg. *Rudbeckia* compared to $x = 13$, 14, and 16 in *Ratibida.* Chromosome morphology is also more similar between the two subgenera of *Rudbeckia* than between either group and *Ratibida.* In *Rudbeckia* sensu lat., the chromosomes are either short or long, with median, submedian, and subterminal primary constrictions. In addition to these chromosome morphologies, *Ratibida* also exhibits medium length chromosomes with subterminal to submedian constrictions, a form not seen in *Rudbeckia* (Perdue 1959).

THE WESTERN LINEAGE OF *RUDBECKIA* SUBG. *MACROCLINE.* For the western North American assemblage of *R.* subg. *Macrocline,* two species groups, each consisting of three species, were recognized by Cox and Urbatsch (1994). Presence or absence of ray flowers readily distinguishes the two groups. Historically, the three radiate species were recognized as separate varieties of *R. californica* and the three eradiate species were treated as distinct varieties of *R. occidentalis* (Cox and Urbatsch 1994). Judging from the degree of divergence in the ITS sequences and results of phylogenetic analysis, recognition of more than two species is warranted. The ITS tree topologies (Figs. 1–5) suggest a different phylogeny than that suggested by the presence or absence of ray flowers. The radiate *R. glaucescens/R. klamathensis* clade based on the results of this study shows affinity to *R. occidentalis,* a widespread eradiate taxon. Relationships of the other western species are not consistently resolved by the

different analyses, but monophyly of radiate and eradiate taxa is not supported.

The relationships of the widespread species *Rudbeckia laciniata* have been controversial largely due to differences in chromosome number determinations. Perdue (1959, 1960) identified the base chromosome number for *R. laciniata* as $x = 18$, which is characteristic of subg. *Macrocline,* where he placed the taxon. Reports for *R. laciniata* of $2n = 38, 76$ (Battaglia 1946) and numerous reports of $n = 19$ (Heiser and Smith 1954; Keil and Stuessy 1977; Pinkava and Keil 1977; and Turner 5684, TEX, in sched.) suggest a base of $x = 19$ which is concordant with the base number of subg. *Rudbeckia.* After considering chromosome numbers and various morphological characteristics, Cox (1991) suggested that *R. laciniata* represents a distinctive lineage that should receive subgeneric status. Chloroplast DNA trees provide weak support for a western subg. *Macrocline* clade containing *R. laciniata* (Urbatsch and Jansen 1995). Our ITS data strongly support placement of *R. laciniata* with the western macroclines. Whether *R. laciniata* originated in western or southeastern North America, areas where it now naturally occurs, remains unknown based on the ITS results.

THE SOUTHEASTERN LINEAGE OF *RUDBECKIA* SUBG. *MACROCLINE.* In the southeastern lineage of *R.* subg. *Macrocline,* the *R. auriculata/R. scabrifolia* clade is strongly supported by ITS and cpDNA data. Both species have relatively restricted distributions in the eastern and western Gulf Coastal plain, respectively. As discussed by Urbatsch and Jansen (1995), some morphological synapomorphies support this lineage.

Relationships among the remaining four southeastern species of *R.* subg. *Macrocline* were resolved differently in ITS trees and cpDNA trees (Fig. 6). The ITS analyses placed the species into two lineages, *R. maxima/R. nitida* and *R. mohrii/R. texana.* Each species pair exhibits a geographical distribution pattern like that of *R. auriculata/R. scabrifolia,* i.e., one species in each pair occurs in the eastern Gulf Coastal plain and the other occurs in the western Gulf Coastal plain. In contrast, the cpDNA analyses resolved an eastern species pair (*R. mohrii/R. nitida*) and a western species pair (*R. maxima/R. texana*) (Fig. 6). One possible reason for the discordance of the cpDNA and ITS trees is the low level of cpDNA variation within *Rudbeckia.* Lineage sorting is another possible explanation for this incongruity, especially if speciation occurred recently and involved large population sizes. The species in this complex are not known to hybridize at present. However, they may have done so in the past resulting in chloroplast capture. For the genus as a whole the species-level cpDNA phylogeny was poorly resolved. The cpDNA trees exhibited numerous polytomies and clades supported by single mutations. The ITS data provide a more robust set of phylogenetic hypotheses for the southeastern macroclines and for other species groups in *Rudbeckia.* Cox and Urbatsch (1989) proposed that *R. texana* and *R. nitida,* recognized as varieties of the same species by Perdue (1962) and Cronquist (1980), be recognized as distinct species. The two molecular data sets are in keeping with this treatment.

RUDBECKIA SUBG. *RUDBECKIA.* Within *R.* subg. *Rudbeckia, R. fulgida* and *R. missouriensis* were once treated as a single species, then as varieties of the same species (Cronquist 1945), and finally as distinct species (Fernald 1950; Perdue 1957, 1959; Cronquist 1980). Among the interspecific crosses attempted with six species of *R.* subg. *Rudbeckia,* the only successful hybridization was between *R. fulgida* and *R. missouriensis* (McCrea 1981). ITS and cpDNA trees support a close relationship between these taxa.

Perdue (1959) noted that *R. triloba* is a ''highly distinct species, not closely related to any other in *Rudbeckia*.'' The ITS and cpDNA data (Figs. 1–5) moderately support a sister-group relationship of *R. triloba* to *R. missouriensis/R. fulgida.* Attempts to hybridize *R. triloba* with other species of *Rudbeckia* were unsuccessful (McCrea 1981), and the results of crossing attempts are equivocal because diploid populations of this species exhibit some degree of self-compatibility and triploid populations are apomictic (McCrea 1981).

Rudbeckia graminifolia, a species known only from five counties in the Florida panhandle, is unusual in the genus because of its small stature and its small, deep maroon ray corollas. The ITS results lead us to conclude that *R. graminifolia* is probably more closely related to *R. fulgida, R. missouriensis,* and *R. triloba* than to other members of *Rudbeckia.* In the best resolved trees, *R. graminifolia* is either sister to *R. triloba* or sister to the other three species.

The *R. hirta/R. mollis* clade received 100% bootstrap support in the ITS and cpDNA analyses. In addition to numerous ITS (Figs. 1–3) and cpDNA mutations (Urbatsch and Jansen 1995), this relationship is also supported by a synapomorphy in life cycle. Both species are annual, biennial, or weakly perennial–features unique for the subgenus. *Rud-*

beckia hirta is somewhat weedy and widespread; *R. mollis* is restricted to dry sandy soils of the eastern Gulf Coastal plain. In ITS trees in which relationships were fully resolved in subg. *Rudbeckia, R. grandiflora* and/or *R. heliopsidis* are sister to the *R. hirta*/*R. mollis* clade. *Rudbeckia grandiflora* is relatively common on prairies from Texas to Georgia northward to Kansas and Missouri; *R. heliopsidis* is rare, occurring in a few scattered localities from southeastern Virginia to northeastern Alabama. Judging from their woody rhizomes, *R. grandiflora* and *R. heliopsidis* apparently are long-lived perennials. *Rudbeckia subtomentosa* lacks what appear to be morphological and ecological apomorphies of the above clades in subg. *Rudbeckia*; *R. subtomentosa* occurs in mesic habitats and has a perennial habit, cauline leaves, and medium-sized capitulae. The ITS trees place *R. subtomentosa* sister to the abovementioned five species or sister to all species in subg. *Rudbeckia,* but neither position has strong support.

HISTORICAL BIOGEOGRAPHY OF RUDBECKIINAE. Sharp's (1935) hypothesis of evolution in the coneflowers appears untenable now that *Echinacea,* his putative primitive taxon, has been shown to fall well outside Rudbeckiinae. Removing *Echinacea* from Rudbeckiinae shifts the center of diversity for the subtribe toward the Appalachians and the Gulf Coastal plain. The center of diversity for a group may or may not include the center of origin. Both the Ozark and Appalachian plateaus have been available for colonization for long periods of time from South America and Mexico, both important centers of diversity for Heliantheae (Cronquist 1977; Bremer 1994). With better knowledge of the broader relationships of Rudbeckiinae, and using methods such as those introduced by Bremer (1995) and Ronquist (1995, 1997), establishment of the probable area of origin for Rudbeckiinae might be possible.

Wood (1970) included *Rudbeckia* among the genera having continuous, transcontinental distributions between the southern Appalachians and western North America. Our refinements of the *Rudbeckia* phylogeny indicate that the distribution of *R. laciniata* is more or less continuous in the northern United States, where it occurs in the Dakotas and then westward into the Rocky Mountains. The other species in subg. *Macrocline* are disjunct between the southeastern and western U.S.A. with a distributional gap ranging from eastern Texas to western Colorado.

The two strongly supported clades in *Rudbeckia* subg. *Macrocline* appear to represent two separate North American radiations, one in the west and one in the southeast. Whether the disjunction is attributable to long-distance dispersal or vicariance involving a widely distributed progenitor is a matter of conjecture, but the overall phylogenetic pattern in Rudbeckiinae leads us to suggest that the subtribe may have diversified to some extent in eastern North America before the evolution of western species of subg. *Macrocline.*

Species in *Rudbeckia* subg. *Macrocline* share a similar biogeographic pattern with Sarraceniaceae. *Rudbeckia glaucescens* co-occurs with *Darlingtonia* Torr. in the western U.S.A., and various macroclines in the southeasterm U.S.A. grow in habitats also occupied by *Sarracenia* L. Among other taxa that share a similar distribution is *Neviusia* A. Gray. *Neviusia alabamaensis* A. Gray is a rare taxon occurring in Alabama, Arkansas, Georgia, and Tennessee; *N. cliftonii* Shevock, Ertter, & Taylor occurs at the southeastern edge of the Klamath–Siskiyou province of northern California. Fossil evidence led Shevock et al. (1992) to suggest that *N. cliftonii* is an old relict species. Other putative Tertiary and pre-Tertiary relictual taxa having a similar California–eastern United States disjunction include *Torreya* Raf., *Dirca* L., and *Calycanthus* L. (Nesom and Mayfield 1995; Sharp 1951; Wood 1970).

As Wood (1970) noted, the Klamath region and the southern Appalachians have served, since the early Cenozoic, as refugia for plants eliminated in other areas by glaciation, coastal plain submergence, climatic desiccation, and other disturbances. Environmental stability in both areas may have allowed long-term persistence (and diversification) of related plants, irrespective of the disjuntion between plants in the two regions being the result of ancient dispersal or break-up of formerly geographically continuous (or nearly so) distributions during wet interglacial periods.

Bayer et al. (1996) suggested that the small seeds of members of Sarraceniaceae are suited for long distance avian dispersal, and that the prehistoric existence of continuous habitat for pitcher plants is unlikely. Most species of macroclines require wet habitats, usually along bayous, seeps, or streams. The cypselas of species of *Rudbeckia* subg. *Macrocline* lack all but rudimentary pappus. Although they are larger than seeds of Sarraceniaceae, they are still quite small. Even without significant pappus, the fruits could easily became lodged in feathers. Although long-distance dispersal by birds may be unlikely, only one such event would have been nec-

essary. Because the western and southeastern species in subg. *Macrocline* appear to represent distinct radiations, dispersal in the group must have been ancient and extremely limited, with no exchange of consequence subsequent to the onset of diversification. Although vicariance can not be ruled out to explain present day distributions in subg. *Macrocline,* the existence of suitable habitats through areas that are not now occupied by these plants must be hypothesized.

Echinacea has had a long, documented history of use as a folk medicine (Kindscher 1989; Moerman 1998). Pharmacological studies of extracts from *Echinacea* have implicated cichoric acid, alkamides, and glycoprotein/polysaccharides as putative active principles possessing immunomodulatory and other activities (Hobbs 1995; Bauer 1996). Our phylogenetic results would suggest that other similar bioactive substances might be sought among members of Zinniinae and related taxa.

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