INTERFAMILIAL RELATIONSHIPS OF THE ASTERACEAE: INSIGHTS FROM *rbc*L SEQUENCE VARIATION[1]

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ABSTRACT

Nucleotide sequences of the chloroplast gene *rbc*L were analyzed to examine relationships among the large, distinctive family Asteraceae and eight putatively closely related families. Phylogenetic analysis of a total of 24 sequences of *rbc*L identified a lineage consisting of two families, the Goodeniaceae and Calyceraceae, as the sister group to the Asteraceae. In addition, a strongly supported major monophyletic clade consisting of Asteraceae, Goodeniaceae, Calyceraceae, *Corokia* (Cornaceae sensu Cronquist), Menyanthaceae, Lobeliaceae, and Campanulaceae was found. These results clearly distance from the Asteraceae certain groups previously considered closely related; moreover, the results support alternative hypotheses of affinity that were based upon floral and inflorescence morphology, biogeography, pollen morphology, chemistry, and pollen-presentation mechanisms.

The angiosperm family Asteraceae has long been recognized as one of the large, "natural" families with well-established limits defined by several specialized floral characteristics and distinctive secondary chemistry. Many recent studies have illuminated numerous phylogenetic controversies within the family (Bremer, 1987; Bremer et al., 1992; Jansen et al., 1990, 1991a, b; Jansen & Palmer, 1987a, b, 1988; Karis et al., 1992; Keeley & Jansen, 1991; Kim et al., 1992; Watson et al., 1991). However, relationships among the Asteraceae and other families have remained obscure, due to considerable parallel and convergent evolution of conventional characters used to infer affinities, lack of recent studies employing modern methods of phylogenetic analysis, and substantial confusion as to relationships among the various families within the subclass Asteridae itself (but see Olmstead et al., 1992).

At least 12 families have been proposed as closest relatives of the Asteraceae based on a variety of traditional taxonomic characters. Although Hutchinson (1969) noted the superficial similarity of the inflorescence of the Dipsacales (Caprifoliaceae, Valerianaceae, and Dipsacaceae) to that of the Asteraceae, he suggested convergence as the basis for this and identified the Campanulales (Campanulaceae and Lobeliaceae) as the closest relative. Aspects of the distinctive chemistry of the Asteraceae (e.g., alkaloids, polyacetylenes, terpenes, inulin for carbohydrate storage) have been noted in several members of the Campanulales (Campanulaceae, Lobeliaceae, Goodeniaceae, Stylidiaceae), while other chemical evidence has pointed to the Apiaceae and Araliaceae (Hegnauer, 1964, 1977). Cronquist (1955) advocated the Rubiales as closest relatives of the Asteraceae (grouped with the Gentianales in the system of Takhtajan, 1980), but also acknowledged strong similarities in floral and inflorescence morphology between the Asteraceae and the Calyceraceae (as did Takhtajan, 1980). An association with the Calyceraceae is also supported by biogeography and capitular structure (Turner, 1977) and pollen morphology (Skvarla et al., 1977). Others (reviewed in Skvarla et al., 1977) have noted a palynological resemblance of Valerianaceae, Goodeniaceae and Brunoniaceae to Asteraceae. In a morphological cladistic study of tribal relationships within the Asteraceae, stylar morphology, chemical characters, and pollen-presen-

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tation mechanism were identified by Bremer (1987) as potential synapomorphies linking the Lobeliaceae and Asteraceae. Finally, strong similarities to the highly specialized secondary pollen-presentation mechanism of the Asteraceae have been documented in Goodeniaceae, Brunoniaceae, Campanulaceae, and Lobeliaceae (Leins & Erbar, 1990). As noted in many of the above attempts to resolve this controversy, most of the morphological or chemical characters that support a particular hypothesis of ancestry are often also found in several related groups, and some must certainly be the product of parallel evolution.

Several of the recent molecular advances in elucidating phylogenetic relationships within the family have employed restriction site analysis of chloroplast DNA. However, this approach is generally unsuitable at the interfamilial level because the homology of site changes becomes doubtful due to increased levels of both nucleotide sequence divergence (causing multiple "hits" within restriction sites) and length variation causing problems in alignment of sites (Downie & Palmer, 1992b; Palmer et al., 1988). At higher taxonomic levels, restriction site analysis of only the more conserved inverted repeat region of chloroplast DNA may be used to circumvent these problems (e.g., Downie & Palmer, 1992a), but fewer characters are generated than in whole genome surveys. DNA sequence analysis of the slowly evolving chloroplast gene *rbc*L and nuclear rRNA genes has proven highly effective in resolving higher-level relationships in plants (Chase et al., 1993; Hamby & Zimmer, 1992; Palmer et al., 1988; Ritland & Clegg, 1987; Zurawski & Clegg, 1987). In particular, recent studies by a number of researchers employing comparative sequencing of the chloroplast gene encoding the large subunit of the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase (*rbc*L) indicate an appropriate size and rate of evolution for providing a sufficient number of characters for phylogenetic studies at the familial and ordinal levels (Donoghue et al., 1992; Kim et al., 1992; Olmstead et al., 1992; Soltis et al., 1990). In this paper we analyze nucleotide sequences for *rbc*L from representatives of the Asteraceae and eight putatively related families to determine evolutionary relationships among the families and identify the sister group of the Asteraceae.

Materials and Methods

New *rbc*L sequences were determined for six taxa in the Asteraceae and eight representatives of putatively closely related families in the subclass Asteridae. For these sequences, fresh leaf material obtained either from the field or seedlings was used to isolate DNA as purified chloroplast DNA by the sucrose gradient method (Palmer, 1986) or as total cellular DNA by a modified CTAB procedure (Doyle & Doyle, 1987) followed by CsCl gradient purification. The *rbc*L gene was isolated for cloning by one of two methods. (1) For most taxa, fragments containing the entire *rbc*L gene were gel-isolated from either Sac I or Sac I/BamHI digests and ligated into the plasmid vector Bluescript (Stratagene, Inc., LaJolla, California). Recombinant, *rbc*L-containing colonies were confirmed by Southern hybridization to cloned *rbc*L fragments from peas. The coding region was sequenced from a single-stranded template by the dideoxy chain termination method (Sanger et al., 1977) using a series of primers based on *rbc*L sequences from maize and spinach (obtained from G. Zurawski, DNAX). (2) Sequences from *Pentas* and *Boopis* were obtained following amplification and cloning of a double-stranded fragment using the polymerase chain reaction following the methods of Olmstead et al. (1992).

Preliminary analyses included 14 new sequences (see Table 1 and Appendix to this issue) and one previously published sequence from *Flaveria* in the Asteraceae (Hudson et al., 1990) and, to serve as outgroups, sequences from *Spinacia* in the Caryophyllidae (Zurawski et al., 1981) and *Nicotiana* in the Asteridae (Shinozaki et al., 1988). These analyses (Michaels & Palmer, 1990) on only a subset of the data reported here identified a closest sister group identical to the present expanded analysis. The results of concurrent studies of *rbc*L sequences in the Saxifragaceae (Soltis et al., 1990), Asteridae (Olmstead et al., 1992), and 499 angiosperms (see Chase et al., 1993) have motivated the inclusion of data from other outgroups and from taxa not previously suspected to be associated with the Asteraceae. Data for *Heuchera* (Soltis et al., 1990), *Magnolia* (Golenberg et al., 1990), *Villarsia*, *Menyanthes*, *Hedera*, and *Coriandrum* (Olmstead et al., 1992) were obtained from published reports, whereas an unpublished sequence for *Corokia* was made available by D. Morgan and D. Soltis.

Although the coding regions for these taxa ranged from 1428 to 1458 bp, only a 1428 bp region was analyzed because no major insertions or deletions were found in this region, allowing alignment by eye, and because homology of positions beyond 1428 is uncertain. Analyses over longer sequences did, however, produce virtually the same results.

TABLE 1. Species of Asteraceae, related families, and outgroups compared by *rbc*L sequence. * = New sequence obtained for this study.

Asteraceae	
Barnadesioideae	*Barnadesia caryophylla* (Vell.) S. F. Blake*
	Dasyphyllum dicanthoides (Less.) Cabrera*
Chicorioideae	*Lactuca sativa* L.*
	Carthamnus tinctorius L.*
Asterioideae	*Flaveria trinervia* Mohr
	Helianthus annuus L.*
	Senecio mikanioides Otto*
Related families	
Caprifoliaceae	*Viburnum acerifolia* L.*
Dipsacaceae	*Dipsacus sativus* Honck.*
Valerianaceae	*Valeriana officinalis* L.*
Rubiaceae	*Pentas lanceolata* K. Schum.*
Goodeniaceae	*Scaevola frutescens* Krause*
Campanulaceae	*Campanula ramosa* Sibth. & Smith*
Lobeliaceae	*Lobelia erinus* L.*
Calyceraceae	*Boopis anthemoides* Jussieu*
Apiaceae	*Hedera helix* L.
Araliaceae	*Coriandrum sativum* L.
Menyanthaceae	*Menyanthes trifoliata* L.
Menyanthaceae	*Villarsia calthifolia* F. Muell.
Cornaceae	*Corokia macrocarpa* T. Kirk
Outgroup families	
Chenopodiaceae	*Spinacia oleracea* L.
Saxifragaceae	*Heuchera micrantha* Douglas ex Lindl.
Solanaceae	*Nicotiana tabacum* L.
Magnoliaceae	*Magnolia macrophylla* Michx.

Analyses were also conducted in which transitions and transversions were differentially weighted (1.0: 1.3, Albert et al., 1993). Since weighted analyses did not alter the tree topologies, only the results of the equally weighted analyses are reported here. PAUP, Phylogenetic Analysis Using Parsimony, Version 3.0r (Swofford, 1991), was used to conduct Fitch parsimony analyses (Fitch, 1971). The HEURISTIC search option with 10 random replicates (Maddison, 1991) was used for tree building and branch swapping under the MULPARS and TBR options. Strict consensus trees were computed. To assess robustness of clades identified in the analyses, the bootstrap method (based on 500 replicates; Felsenstein, 1985) was used. In addition, a "decay" analysis was conducted (cf. Bremer, 1988; Donoghue et al., 1992; Hillis & Dixon, 1989) in which strict consensus trees were generated from all trees five steps longer than the most parsimonious ones. The FILTER TREES option was then used to identify all trees at each shorter tree length. Support for each clade was assessed by determining when each clade was no longer resolved in consensus trees from progressively less parsimonious solutions.

RESULTS

Of the 1,428 nucleotide positions compared among the 24 *rbc*L sequences in the analysis, 452 were variable. Exclusion of autapomorphies resulted in 278 potentially synapomorphous characters, 204 of which were at third-codon positions, 45 at first, and 29 at second. The third position characters are essential sources of phylogenetic information, as their elimination from the data set results in little resolution (data not shown). Initial analyses (results not shown) used four taxa [*Magnolia* (Magnoliidae), *Spinacia* (Caryophyllidae), *Heuchera* (Rosidae), and *Nicotiana* (Asteridae)] as designated outgroups. These analyses established *Nicotiana* as the sister group to an ingroup consisting of the taxa proposed as outliers to Asteraceae. In subsequent analyses, the more distant outgroups *Magnolia*, *Spinacia*, and *Heuchera* were deleted, and trees were arranged using *Nicotiana*

alone as the designated outgroup. The heuristic search found four equally parsimonious trees (results not shown) of 833 steps with consistency index (C.I.) of 0.48 (autapomorphies excluded) and a retention index (R.I.) of 0.502. The strict consensus of the four trees (Fig. 1) shows a large, nested monophyletic group consisting of the Asteraceae, Goodeniaceae, Calyceraceae, *Corokia* (Cornaceae sensu Cronquist, 1981), Menyanthaceae, Lobeliaceae, and Campanulaceae. The two polytomies exist in either a more basal portion of the tree or within the Asteraceae. Among the putative relatives of the Asteraceae examined here, *Pentas* (Rubiaceae) was placed as the most basal clade. As in our earliest analysis with only a subset of these data, the expanded analysis identifies a clade consisting of *Scaevola* (Goodeniaceae) and *Boopis* (Calyceraceae) in the most proximal position to the Asteraceae. This sister relationship is supported by eight shared nucleotide substitutions.

The robustness of the sister relationship of the Goodeniaceae–Calyceraceae clade to the Asteraceae is indicated by two subsequent analyses. First, the results from 500 bootstrap replications using the heuristic search option indicate that this relationship is one of the more robust areas of the tree topology, occurring in 84% of the trees (Fig. 2). Relationships are, with some exceptions, poorly resolved within the Asteraceae (also see Kim et al., 1992) and also outside the "Asterales" (Lobeliaceae–Campanulaceae through Asteraceae) clade. Within the "Asterales" are three other well-supported groups besides the Asteraceae–Goodeniaceae–Calyceraceae clade: (1) the Asteraceae emerge as monophyletic in 97% of the bootstrap trees, (2) Campanulaceae and Lobeliaceae occur together in all bootstrap replicates, (3) the Goodeniaceae and Calyceraceae clade is monophyletic 86% of the time. Finally, the "Asterales" group as a whole appears in 86% of the trees.

Second, the strength of the relationship of the Goodeniaceae–Calyceraceae clade to the Asteraceae is indicated in the decay analysis by the persistence of this group in less parsimonious solutions. The sister position of this clade is maintained in 127 trees up to two steps longer than the most parsimonious trees. The strict consensus tree at three additional steps places the Goodeniaceae–Calyceraceae clade within an unresolved polytomy together with various unresolved lineages of the Asteraceae, while the Asteraceae–Goodeniaceae–Calyceraceae group forms an unresolved polytomy with the rest of the "Asterales" (*Villarsia, Corokia, Menyanthes, Campanula–Lobelia*). When the 964 trees up to four steps longer are included, most of the resolution in the tree is lost, and all but the strongest clades (i.e., Goodeniaceae–Calyceraceae, Campanulaceae–Lobeliaceae, Valerianaceae–Dipsacaceae, and *Helianthus–Flaveria*) disintegrate.

Discussion

The *rbc*L data clearly indicate a close relationship among the Asteraceae and other families traditionally placed in the Asterales and Campanulales. This conclusion is supported not only in the local analysis presented here, but also in much broader analyses of the Asteridae (Olmstead et al., 1992, 1993) and in an analysis of 499 *rbc*L sequences from seed plants (Chase et al., 1993). The placement of the Campanulaceae, Lobeliaceae, Goodeniaceae, Calyceraceae, and Asteraceae in a single monophyletic group is consistent with the system of relationships proposed by Takhtajan (1987) and Thorne (1992), in which these groups (together with families in the Stylidiales) form the superorder Asteranae. This grouping is also distinguished in the system of Wagenitz (1977) and the recent review by Lammers (1992), but not in the systems proposed by Dahlgren (1975) or Cronquist (1981). Dahlgren aligned the Goodeniaceae and the Calyceraceae far from the Asteraceae, placing them in the Gentiananae and Cornanae, respectively. The topology inferred from the *rbc*L data is most divergent from the Cronquist system. Although Cronquist placed the Goodeniaceae together with the Campanulaceae and other families to form Campanulales, the Calyceraceae were assigned to the Dipsacalean group, while the Asteraceae were placed nearest the Rubiaceae. The latter association is clearly not supported by the *rbc*L data; *Pentas* (Rubiaceae) was consistently placed in the most distant position of all the putatively allied families under consideration as sister groups in this analysis.

The sister-group relationship of the Goodeniaceae–Calyceraceae clade to the Asteraceae is supported by evidence from a number of studies. The pollen-presentation mechanisms of the Goodeniaceae, Calyceraceae, and Asteraceae are similar, and they also resemble the mechanisms of the Campanulaceae, Lobeliaceae, and Brunoniaceae (Erbar & Leins, 1989; Leins & Erbar, 1990; Lammers, 1992; Wagenitz, 1992). Details of floral development (Harris, 1991; Erbar & Leins, 1989) also unite both Goodeniaceae and Calyceraceae with the Asteraceae. However, the particular development pattern they share is also found in Brunoniaceae, Campanulaceae, Stylidiaceae, Menyan-

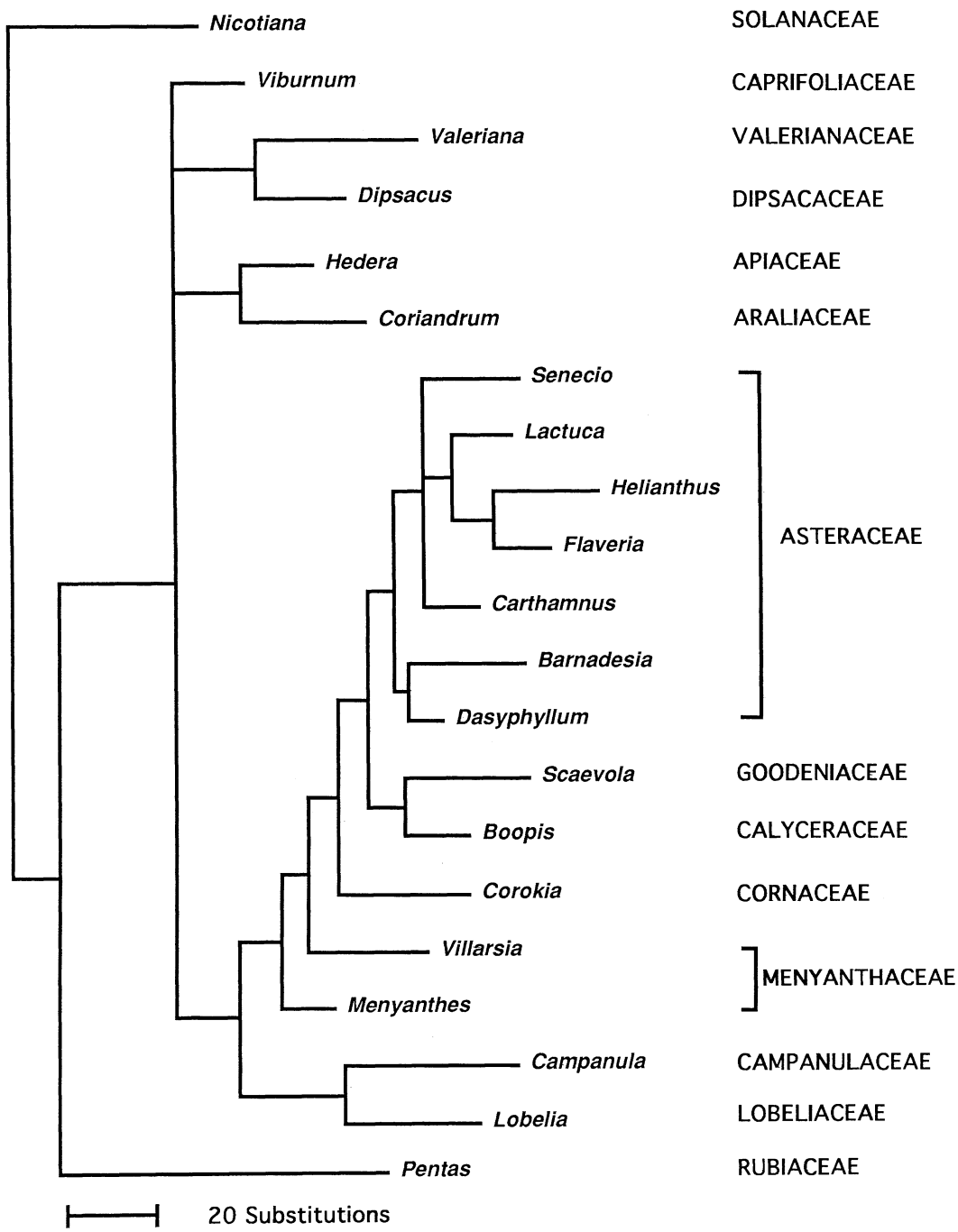

FIGURE 1. Strict consensus of four equally parsimonious trees (length = 833 steps, C.I. = 0.48, R.I. = 0.50) based upon *rbc*L sequences. Branch lengths are proportional to the number of nucleotide substitutions supporting a node or distinguishing a terminal lineage (note scale at bottom).

thaceae, Rubiaceae, and families of the Dipsacales (Erbar & Leins, 1989), and therefore this is probably a plesiomorphic character. Chemical characters such as carbohydrate storage as inulin (in Asteraceae, Calyceraceae, and Goodeniaceae, as well as Menyanthaceae, Campanulaceae, and Lobeliaceae; Pollard & Amuti, 1981) and phenolics (e.g., caffeic acid in Asteraceae, Calyceraceae, and

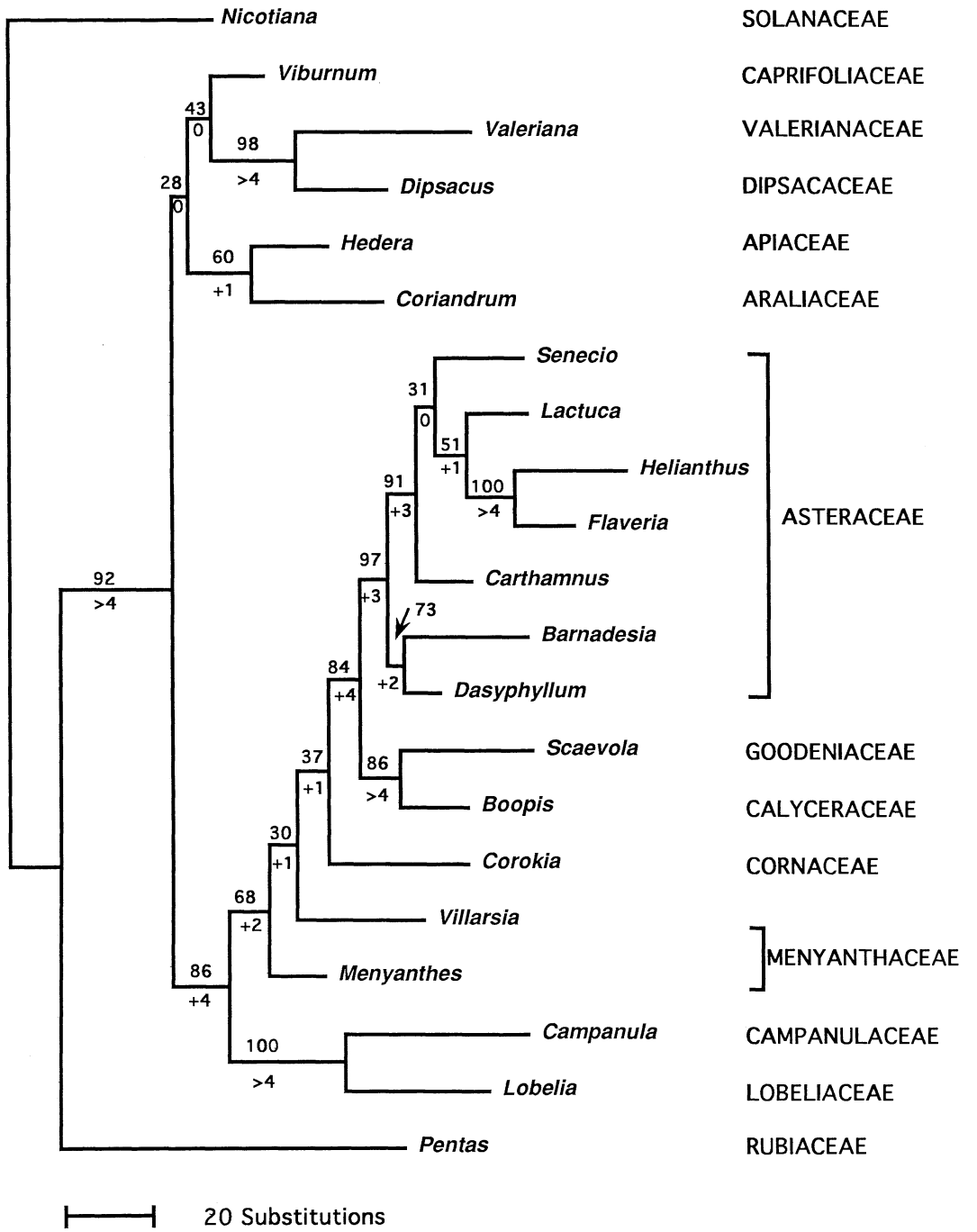

FIGURE 2. One of the four equally parsimonious trees based upon *rbc*L sequences with the results of the bootstrap and decay analyses. Branch lengths are proportional to the number of nucleotide substitutions supporting a node or distinguishing a terminal lineage (note scale at bottom). Bootstrap values from 500 replications are indicated above the branches. Numbers below indicate numbers of additional steps needed for a branch to collapse.

Goodeniaceae, as well as Menyanthaceae and Campanulaceae; Lammers, 1992) further support the position of the Goodeniaceae and Calyceraceae. Asteraceae, Goodeniaceae, and Calyceraceae also lack endosperm haustoria, have binucleate tapetal cells, and produce herbivore defenses through the mevalonate pathway, characters that are also shared with Menyanthaceae, but are absent from Campanulaceae and Lobeliaceae (Lammers, 1992).

Several other characters specifically support the sister placements of Goodeniaceae or Calyceraceae alone. Polyacetylenes, which are characteristically found in the Asteraceae, have also been reported from some Campanulaceae (Ferreira & Gottlieb, 1982), Lobeliaceae, and one Goodeniaceae (Lammers, 1992). Their distribution in Calyceraceae, Menyanthaceae, or other families that place near the Asteraceae in our analysis is unknown. The Calyceraceae were proposed as a sister group to the Asteraceae by Jeffrey (1977). Evidence in support of the placement of Calyceraceae seen in our *rbc*L analyses derives from chloroplast DNA restriction site data (Downie & Palmer, 1992a; unfortunately, due to their unusual genome organization, Goodeniaceae could not be included in this study) and floral development, as noted above, but also from pollen morphology, capitulum structure, and biogeography. Skvarla et al. (1977) concluded that the ultrastructure of Calyceraceae exine is nearly identical to that of the Asteraceae, while the Goodeniaceae are among five families listed with pollen similar enough to "suggest distinct linkages." As previously advocated by Turner (1977), the structurally homologous capitula and floral features, and congruent core distributions of the Asteraceae and Calyceraceae in South America are particularly compelling arguments supporting a shared phylogenetic history.

This analysis suggests a particularly strong sister-group relationship for the Goodeniaceae and Calyceraceae, occurring in 86% of the bootstrap trees. This clade also remained intact throughout the decay analysis, a level of association seen only in other groups that have traditionally been strongly linked (e.g., *Helianthus* and *Flaveria* in the Heliantheae; Campanulaceae and Lobeliaceae; Dipsacaceae and Valerianaceae). As noted above, the Goodeniaceae and Calyceraceae are united by several characters, including pollen-presentation mechanism, floral development, pollen embryology, and secondary chemistry (e.g., seco-iridoids; Lammers, 1992). The *rbc*L trees and above evidence supporting the placement of the Goodeniaceae (a small family of 325 species distributed primarily in Australia and Tasmania) and the South American Calyceraceae are consistent with Turner's (1977) proposal of a Gondwanaland origin for the Asteraceae. Indeed, all the closest groups from Menyanthaceae to Goodeniaceae are distributed primarily in the Southern Hemisphere.

The *rbc*L gene was recently sequenced from two families previously suggested to have affinities with the Asteraceae (Olmstead et al., 1992). Both the Apiaceae (*Hedera*) and Araliaceae (*Coriandrum*) have been proposed as close associates of the Asteraceae based upon shared phytochemistry (Hegnauer, 1964, 1977). However, Lammers (1992) has argued that the synthesis of sesquiterpene lactones in these groups is due to parallel evolution. Parsimony analyses of *rbc*L indicate that, although neither is closely related to the Asteraceae, these groups do belong in the Asteridae rather than in their more traditional position in the Rosidae (Olmstead et al., 1992, 1993; Chase et al., 1993).

Although neither Menyanthaceae nor Cornaceae have ever been allied with the Asteraceae based on traditional data, they were included in this study because other recent chloroplast DNA analyses suggested close relationships. Based on *rbc*L sequences, Olmstead et al. (1992) found an unexpected association of the Menyanthaceae with the families more typically suggested to have affinities with the Asteraceae. A survey of restriction sites in the chloroplast DNA inverted repeat (Downie & Palmer, 1992a) also places the Menyanthaceae along with the Calyceraceae and Asteraceae. The most parsimonious solutions from Olmstead et al. (1992, 1993), which are based on a much larger sampling of taxa within the Asteridae, are consistent with the results reported here. Although Menyanthaceae are nearer to the Asteraceae than either the Campanulaceae or Lobeliaceae in this study, Calyceraceae retain the sister position (Goodeniaceae were not included in the Asteridae analyses of either Olmstead et al., 1992, or Downie & Palmer, 1992a). Several features shared by Menyanthaceae, Calyceraceae, and Goodeniaceae (the production of seco-loganin, carbohydrate storage as inulin, presence of multinucleate tapetal cells, absence of endosperm haustoria, and chromosome numbers based upon x = 8 or 9; Lammers, 1992) are consistent with the placement of Menyanthaceae in these analyses.

In addition, the analyses of Morgan & Soltis (1993) and Chase et al. (1993) indicate an unsuspected affiliation of *Corokia* (Cornaceae sensu lato) with the asteralean clade. The association of *Corokia* with Asteraceae and its near relatives was

confirmed in our analyses, which are more likely to produce optimal solutions, while the computational difficulties of the much larger analyses (Chase et al., 1993; Morgan & Soltis, 1993) may have precluded discovery of shortest trees. This radical departure from previous placements of *Corokia* (in Escallonioideae, Saxifragaceae sensu lato, Engler, 1928; in Cornaceae, Eyde, 1966 and Cronquist, 1988; or in Araliaceae, Phillipson, 1967) is consistent with several other characters: (1) biogeography (*Corokia* is distributed primarily in New Zealand and Australia; Eyde, 1966), (2) some aspects of morphology (inferior ovary, locules 1–3 with a single, apical, unitegmic ovule, and multicellular, T-shaped trichomes, found elsewhere only in the Asteraceae and three other unrelated families; Eyde, 1966), and (3) chemistry (presence of iridoids in Escallonioideae; Dahlgren et al., 1981). See also Morgan & Soltis (1993) for further discussion of evidence linking genera of the Escallonioideae with Asteridae. These unanticipated associations not only illustrate the heuristic value of the broader analyses and the benefits of widespread data sharing, but also motivate the acquisition of additional data from other sources that may corroborate these new hypotheses of relationships. For example, studies of floral developmental patterns, pollen ultrastructure and development, and embryogeny in Menyanthaceae and *Corokia* would be a logical direction for future work.

Finally, although many families that have been previously considered as serious contenders for sister group to the Asteraceae have been investigated in this study, several others remain to be examined. In particular, Brunoniaceae and Stylidiaceae have been associated with Asteraceae and Campanulaceae in Takhtajan's (1987), Wagenitz's (1977), and Thorne's (1992) classifications. Both are distributed primarily in Australia and share the floral developmental features found in Asteraceae, Campanulaceae sensu lato, Goodeniaceae, and Calyceraceae (Erbar, 1991). Brunoniaceae also possess a similar pollen-presentation mechanism to those found in the first three of these families (Leins & Erbar, 1990). Any comprehensive attempt to further explore the origins of the Asteraceae should include several other, poorly understood small families that have also been associated with Campanulales (Pentaphragmataceae, Cyphiaceae, and Sphenocleaceae; Lammers, 1992). The addition of data from these groups is likely to provide new insights into the phylogeny of the Asteraceae and further resolve the current picture of relationships of this distinctive lineage.

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