PHYLOGENETIC RELATIONSHIPS OF DIPSACALES BASED ON *rbc*L SEQUENCES[1]

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ABSTRACT

Nucleotide sequences of the chloroplast gene *rbc*L were obtained from *Lonicera*, *Sambucus*, *Adoxa*, and *Cornus*. Phylogenetic analyses of these sequences, along with previously sequenced Dipsacales and placeholders for other Asteridae and Rosidae, lead to the following conclusions: (1) the genera of Caprifoliaceae (in any traditional sense) do not form a monophyletic group; (2) *Symphoricarpos* and *Lonicera* (representing Caprifoliaceae sens. str.) are related and are united with Valerianaceae and Dipsacaceae; and (3) *Adoxa* and *Sambucus* are directly linked and are possibly related to *Viburnum*. Our analyses also cast doubt on the monophyly of Dipsacales and Asteridae. These findings are generally congruent with other molecular results and with hypotheses based on morphological data.

Uncertainty has long surrounded the phylogenetic relationships of taxa traditionally assigned to the angiosperm order Dipsacales (Donoghue, 1983). It has been unclear, for example, whether *Adoxa* is related to Dipsacales, whether *Sambucus* and *Viburnum* are closely related to one another or to genera of Caprifoliaceae sens. str., or whether some Caprifoliaceae are more closely related to Valerianaceae and/or Dipsacaceae than they are to each other. The broader phylogenetic relationships of these plants have also been debated. It is still uncertain whether Dipsacales were derived from (i.e., are nested within) Rubiales, as implied by Cronquist (1981, 1988), or whether they (and possibly Asterales) are more closely related to rosid groups such as Cornales, as suggested recently by Takhtajan (1987; also see Dahlgren, 1983; Thorne, 1983).

Donoghue (1983) considered morphological evidence bearing on these questions, especially the relationships of *Viburnum*. However, owing to uncertainty over broader phylogenetic relationships, he was able to reach only tentative conclusions, conditional on particular assumptions. For example, *if* Caprifoliaceae sens. lat. and Dipsacales are both monophyletic, *then* morphological data directly unite *Viburnum* with *Sambucus* plus *Adoxa*, whereas under some other assumptions (e.g., Dipsacales polyphletic) this connection is unclear. Substitution of different outgroup arrangements (sensu Donoghue & Cantino, 1984) yielded few unequivocal conclusions. Happily, however, the position of *Adoxa* was an exception. If *Adoxa* is assumed to be related in some way to other Dipsacales (as opposed, for example, to Saxifragaceae), then it is invariably linked with *Sambucus* on the basis of several morphological features, including *Adoxa*-type embryo sac development.

Here we present a preliminary analysis of the phylogenetic relationships of dipsacalean taxa based on molecular data. In particular, we report the results of parsimony analyses of chloroplast *rbc*L sequences (see Ritland & Clegg, 1987; Palmer et al., 1988; Soltis et al., 1990; Doebley et al., 1990; Olmstead et al., 1992) of various Dipsacales and possibly related groups, including new sequences

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from representatives of four critical genera: *Adoxa*, *Sambucus*, *Lonicera*, and *Cornus*. Although we are able to make some comparisons between the molecular results and those based on morphological characters, a detailed comparison must await the completion of an expanded morphological study (Donoghue, in prep.) and sequencing of additional Dipsacales (in progress). In the meantime, we highlight conclusions that are most strongly supported by *rbc*L data, so that these can be taken into account in formulating a phylogenetic system of Asteridae.

Materials and Methods

*rbc*L sequences were newly obtained from four taxa: *Adoxa moschatellina* L. of Adoxaceae, *Cornus mas* L. of Cornaceae, and *Sambucus racemosa* subsp. *sieboldiana* (Miquel) Hara, and *Lonicera prolifera* Rehder of Caprifoliaceae sens. lat. The Wisconsin Department of Natural Resources kindly permitted the collection of *Adoxa* specimens in Vernon County, Wisconsin (*Donoghue, Smith, & Kuetchenreuther 1*, WISC). Material of the other species was obtained by M. Donoghue at the Arnold Arboretum, Jamaica Plain, Massachusetts (where vouchers are deposited). In each case, fresh leaves were collected on ice and then stored at −80°C, and DNA extractions were carried out using a modified CTAB method (Doyle & Doyle, 1987; Smith et al., 1992).

A segment of the chloroplast genome containing the *rbc*L gene was amplified by the polymerase chain reaction (PCR), using the primers described in Olmstead et al. (1992; see their fig. 1). This procedure yielded double-stranded segments of approximately 1550 bp. Each amplification product was then digested with the restriction endonucleases EcoRV and XbaI, known to recognize other Asteridae *rbc*L at nucleotide positions 103–108 and 1269–1274, respectively. As expected, this procedure yielded three fragments in *Adoxa*, *Lonicera*, and *Cornus*: a 5′ segment of 102 bp, an internal segment of 1172 bp including most of the *rbc*L gene, and a 3′ segment of approximately 290 bp (minor variation owing to length difference downstream of *rbc*L). However, in the case of *Sambucus* the double digest yielded four fragments due to an additional EcoRV site present at nucleotide positions 1018–1023 bp. This site is also found in *Vibrunum plicatum*, and more extensive restriction site studies indicate its presence in other (but not all) species of *Viburnum* (Donoghue & Sytsma, unpublished).

The fragments obtained were cloned in the plasmid vector BlueScript Sk+ (Stratagene, Inc.) as described by Olmstead et al. (1992), and single stranded DNAs were prepared (phenol-chloroform extraction) from selected colonies screened for inserts of the larger EcoRV-XbaI fragments (or the equivalent fragments in *Sambucus*). Sequencing was carried out using the dideoxy chain termination technique, with internal primers kindly provided by G. Zurawski (DNAX Research Institute, Palo Alto, California). In some cases the smaller 5′ and 3′ fragments were recovered and sequenced, so that the entire *rbc*L sequence was obtained. However, the analyses reported below are based solely on the internal 1172 bp segment. Using the known tobacco sequence as a reference, differences in our sequences were easily scored; length differences were never encountered in the region of interest. The sequences utilized in the present study will be available in GenBank, and may also be obtained directly from M. Donoghue or R. Olmstead.

A total of twelve taxa were included in the phylogenetic analyses reported here. These were selected so as to represent Dipsacales and other major lines of Asteridae and relevant Rosidae, as well as to include suggested relatives of the dipsacalean taxa, especially *Adoxa*. In addition to the four newly sequenced taxa discussed above, seven sequences were obtained from workers in J. Palmer's laboratory: R. Olmstead provided *Hedera helix* L. (Araliaceae) and *Symphoricarpos albus* Blake (Caprifoliaceae sens. str.) (see Olmstead et al., 1992), as well as *Spigelia marilandica* L. (Loganiaceae) and *Menyanthes trifoliata* L. (Menyanthaceae) (R. Olmstead, unpublished); and H. J. Michaels (pers. comm.; Michaels et al., in prep.) is responsible for *Viburnum acerifolium* L. (Caprifoliaceae sens. lat.), *Dipsacus sativus* Honckeny (= *D. fullonum* L.) (Dipsacaceae), and *Valeriana officinalis* L. (Valerianaceae). The published sequence (Soltis et al., 1990) of *Heuchera micrantha* Dougl. (Saxifragaceae) was also included.

Elimination of invariant nucleotide positions left 194 variable sites, and by omission of autapomorphies this was further reduced to 88 potentially informative characters (Table 1). Of the latter, 74 are binary, 12 are unordered 3-state, and 2 are unordered 4-state characters, so there are a minimum of 104 character state changes (steps) in the absence of homoplasy. Note that the vast majority of potentially informative sites occur at third positions within codons; only eight are at first positions and six at second positions.

Character state changes were equally weighted in most of our analyses, but we did experiment with several differential weighting schemes, includ-

TABLE 1. Twelve taxa scored for 88 potentially informative *rbc*L characters (invariant sites and autapomorphies removed), with characters numbered by base position within the gene. All positions are third positions within codons, unless otherwise indicated above the character number. The complete set of potentially informative nucleotides is given for *Heuchera* (used to root trees); a nucleotide is shown for other taxa only if it differs from *Heuchera* (a dot indicates that the same nucleotide is present). Missing information (due to ambiguity in scoring) is indicated by "?."

	138	147	159	168	177	186	213	225	227(2)	255	267	271(1)	283(1)	284(2)	290(2)	312	345	357	363	393	412(1)	420	426	441	450	459	462	495	501	507	540	555	561	564	579	582	606	612	624	648	672	673(1)	677(2)	682(1)	687	711	732
HEUCHERA	T	A	G	G	T	T	C	C	A	T	C	G	A	G	T	C	C	C	G	C	C	C	T	T	G	T	C	T	T	A	A	C	A	A	C	T	T	A	C	C	A	C	A	G	A	G	A
HEDERA	.	.	A	.	C	.	.	.	G	C	.	.	.	.	.	A	.	.	A	T	.	.	.	.	C	.	.	.	.	G	.	.	.	G	.	.	C	G	.	T	.	.	.	.	G	.	G
CORNUS	.	.	A	.	C	.	.	T	G	C	.	.	.	A	.	.	T	.	.	T	.	T	.	.	C	.	.	.	.	G	.	.	.	.	A	.	.	G	.	.	.	A	T	T	G	.	.
SPIGELIA	C	?	.	A	C	.	.	T	G	C	T	C	G	A	A	.	.	.	A	.	T	.	.	C	.	C	G	.	A	G	.	.	G	G	T	.	.	.	.	T	.	.	T	.	.	A	.
MENYANTHES	.	.	A	A	C	.	.	G	G	.	.	C	.	.	.	A	.	.	A	T	T	.	.	.	C	C	.	.	.	G	.	.	.	T	T	.	.	.	.	T	G	.	.	.	G	.	G
ADOXA	.	.	A	.	C	.	T	.	.	C	.	.	.	C	.	A	.	.	.	.	.	T	.	.	C	.	T	.	.	G	.	.	.	G	.	C	C	G	T	T	C	.	.	.	.	.	G
SAMBUCUS	.	.	A	.	C	.	T	.	G	C	.	.	.	.	.	A	.	.	.	.	.	T	.	?	C	.	T	.	.	G	.	.	.	G	.	C	C	G	T	T	G	.	.	.	.	.	G
VIBURNUM	.	.	A	.	C	.	T	.	G	C	.	.	.	.	.	A	.	.	.	?	.	T	.	.	C	.	.	.	A	G	.	.	.	G	.	C	C	G	.	T	.	A	.	.	.	.	G
LONICERA	C	G	.	.	.	A	.	.	G	C	.	.	.	C	.	A	.	T	.	.	.	.	C	.	C	.	T	C	.	G	.	.	G	G	A	.	.	G	.	T	C	A	T	.	.	.	.
SYMPHORICARPOS	.	G	A	.	.	A	.	.	G	C	.	C	.	C	.	A	.	T	.	.	.	.	C	C	C	.	T	C	C	G	.	.	G	G	A	.	.	G	.	T	C	A	.	.	G	.	.
VALERIANA	G	.	A	.	C	A	.	G	G	C	T	.	G	C	.	A	T	T	.	T	.	.	.	.	C	C	T	C	.	.	G	T	.	G	A	.	.	G	.	T	C	A	.	T	.	.	G
DIPSACUS	C	.	A	A	C	A	.	.	G	C	.	.	.	A	A	A	.	T	.	.	.	T	C	.	C	.	.	.	A	G	G	T	.	.	A	.	.	G	.	T	C	.	.	.	.	A	.

	741	747	753	759	763(1)	771	801	804	808(1)	816	834	836(2)	840	846	885	927	930	950(2)	982(1)	984	990	1020	1032	1047	1050	1059	1095	1116	1128	1131	1137	1149	1164	1185	1194	1197	1209	1224	1242	1245	1266
HEUCHERA	C	A	C	G	G	C	C	T	T	G	T	G	A	T	T	A	C	C	T	T	C	G	C	A	T	C	T	A	C	G	A	T	T	A	T	A	T	A	A	A	T
HEDERA	.	.	G	.	.	.	T	.	.	.	.	C	G	.	C	G	.	G	.	C	.	C	.	G	.	.	C	.	.	.	G	C	.	G	C	.	.	.	.	C	A
CORNUS	.	.	G	.	.	.	T	C	.	.	.	.	G	.	.	G	T	.	G	.	T	.	.	G	.	T	.	.	.	.	G	C	.	G	.	.	.	.	.	G	A
SPIGELIA	.	.	G	A	.	T	T	C	.	T	.	C	.	.	.	G	T	G	G	.	T	C	.	.	C	T	.	.	T	.	.	C	.	G	.	.	.	.	G	.	A
MENYANTHES	.	.	G	.	.	.	T	C	C	T	.	C	G	.	C	.	.	.	.	C	.	.	.	G	.	T	.	.	.	.	.	C	.	G	C	.	.	G	.	C	A
ADOXA	.	.	G	A	A	.	.	C	C	T	.	C	G	.	C	.	.	.	.	C	.	.	T	.	C	T	.	.	GA	G	C	.	G	C	.	.	.	.	.	G	A
SAMBUCUS	.	C	G	A	A	.	.	C	.	.	.	C	G	.	C	G	.	.	G	C	.	T	T	G	C	T	.	.	G	.	G	C	.	G	C	.	.	.	.	C	A
VIBURNUM	.	T	G	.	.	.	T	C	.	.	.	C	G	.	C	G	.	.	G	C	.	.	.	G	C	T	.	.	.	.	G	C	C	G	C	.	.	.	.	C	A
LONICERA	T	C	G	.	.	.	.	C	.	.	C	C	G	C	C	G	.	.	G	C	.	A	.	G	.	T	.	G	.	.	G	C	?	.	C	C	C	.	.	T	C
SYMPHORICARPOS	T	C	G	.	.	.	.	C	.	.	C	C	G	C	C	G	.	.	.	C	.	A	.	G	?	T	.	G	.	.	G	.	C	.	C	C	A	.	.	C	C
VALERIANA	T	.	.	.	.	.	.	C	C	.	.	.	G	.	C	.	.	.	.	C	.	.	.	G	C	T	C	G	T	A	G	C	C	G	.	C	C	G	G	T	C
DIPSACUS	T	.	.	.	.	T	T	.	.	.	.	.	G	.	C	.	.	.	?	?	.	.	.	G	C	T	.	G	.	.	G	C	.	G	.	C	C	.	G	C	C

ing omission or down-weighting of various positions within codons and weighting of transversions (Tv) versus transitions (Ts). The Tv : Ts weighting of 1.3 : 1.0 used in the present study is based on calculations by V. A. Albert (pers. comm.; see Albert & Mishler, in press), which in turn rely on substitution rates and Tv : Ts ratios for *rbc*L obtained from a 5-taxon tree of land plants (Albert et al., 1992).

In view of the small number of taxa under consideration, parsimony analyses were conducted using the Branch and Bound option in PAUP for the Macintosh computer (version 3.0L; Swofford, 1990), which guarantees finding all most parsimonious trees. PAUP was also used in computing consensus trees (including "combinable component" trees: Bremer, 1990), optimizing character state changes (Swofford & Maddison, 1987), performing bootstrap analyses (100 replicates: Felsenstein, 1985; Sanderson, 1989), differentially weighting characters (including use of a step matrix in implementing the Tv : Ts weighting), and calculating character and tree statistics, such as consistency index (CI; Kluge & Farris, 1969) and retention index (RI; Farris, 1989; also see Archie, 1989a).

The lengths and implications of alternative tree topologies were explored using the Topological Constraints option in PAUP, and using finalized portions of MacClade test version 2.99.11 (to be published as MacClade 3.0; Maddison & Maddison, 1992). MacClade was also used to calculate character transformations of various types on trees of interest (e.g., transversions versus transitions) and the numbers of changes at different codon positions.

To evaluate further the relative robustness of clades found in the most parsimonious trees, strict consensus trees were constructed of all trees up to one step longer than the shortest trees, then of all trees two steps longer, and so forth, until the consensus tree eventually collapsed to an unresolved bush. This procedure yields a "decay" index, indicating the number of steps that must be added before each clade present in the minimum length trees is no longer unequivocally supported (cf. Bremer, 1988; Hillis & Dixon, 1989).

In order to explore more fully the structure in

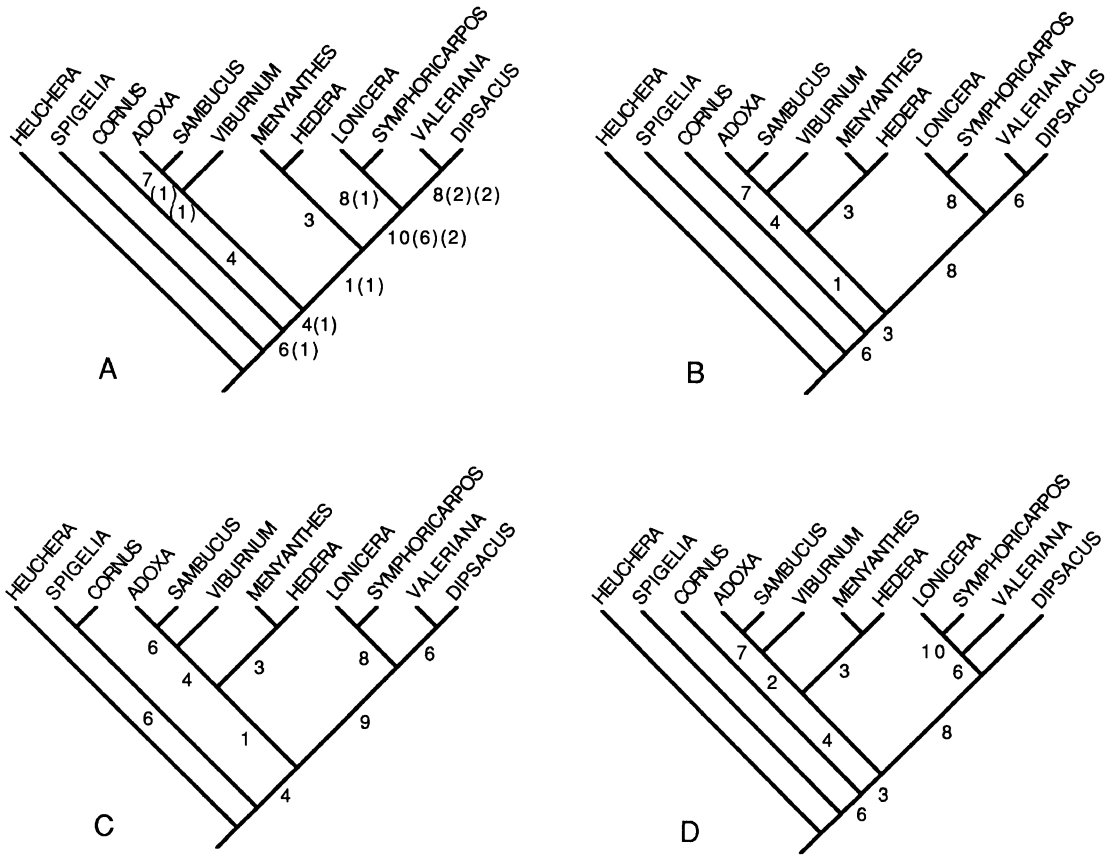

FIGURE 1. Four most parsimonious trees of 202 steps each, rooted by *Heuchera*. The number associated with each internal branch indicates the number of unambiguous nucleotide changes along the branch. In 1A the number of transversions is shown in the first set of parentheses, and changes in first and second positions within codons are indicated in the second set of parentheses: (1) = one first position change; (2) = one second position change.

the data set, two other procedures were utilized. First, the length of the most parsimonious trees for the *rbc*L data was compared to the distribution of most parsimonious trees based on 50 "random" data sets generated under the null model of Archie (1989b). This was accomplished using the "shuffle" option in MacClade, which maintains the same number of states for each character observed in the real data set, but randomly assigns these states to the taxa. To save time, analysis of each of the 50 random data sets was carried out using a heuristic search strategy in PAUP ("closest" add sequence, one tree held with each addition, TBR branch-swapping, and MULPARS). Second, PAUP was used to generate a histogram of tree lengths of 10,000 randomly sampled trees (there being too many taxa in the analysis to examine all trees). This allowed us to determine whether the observed distribution showed significantly greater left-hand skewness than tree-length distributions obtained from randomly generated data matrices (Hillis, 1991; Huelsenbeck, 1991). The comparison is based on the g_1 skewness statistic (i.e., the third central moment divided by the cube of the standard deviation; Sokal & Rohlf, 1981), for which critical values ($P = 0.05$) were provided by David Hillis (pers. comm.).

Rooted trees were obtained by positioning the root along the branch connecting *Heuchera* to the rest of the network (simultaneous resolution; Maddison et al., 1984). Initially, we planned to position the root along the branch connecting the three rosid taxa to the rest of the network, under the assumption that asterid taxa would join the network along a single branch and that the root should not be placed within Asteridae. However, it emerged that the rosids were not attached by a single branch to the asterid taxa in the analysis (see below). Our decision to root the tree along the *Heuchera* branch assumes only that Saxifragaceae is a more basal rosid clade than Cornaceae and/or Araliaceae.

RESULTS

Parsimony analysis of the matrix in Table 1 resulted in the four trees of 202 steps shown in

Figure 1. The strict consensus of these trees (identical to the combinable component tree) is shown in Figure 2. The CI of 0.515 (RI = 0.470) indicates that there are about twice as many character state changes as there would be in the absence of homoplasy. Although this is a rather low CI for 12 taxa, there is still not a statistically significant departure from the expected value of 0.64 based on an analysis of 60 published data sets (Sanderson & Donoghue, 1989).

Note that in Figure 1 we have indicated the number of character state changes that can be unambiguously assigned to each internal branch, and for one tree (Fig. 1A) we also show the number of unambiguous changes that are anything other than third-position transitions. As expected from the matrix, the vast majority of changes in the most parsimonious trees occur at third positions. Separate analyses of characters at diffeent codon positions confirm that the basic structure seen in the most parsimonious trees is primarily determined by these third position characters. When third positions are analyzed by themselves, one tree of 163 steps (CI = 0.546) is obtained, which is identical to Figure 1B. In contrast, when only the 14 first and second position characters are analyzed the consensus of the 55 trees of 31 steps (CI = 0.484) is completely unresolved. Weighting first and second position changes twice as much as third position changes allows discrimination among the four trees in Figure 1 (A: 240 steps; B: 241; C: 238; D: 239), but we see no justification for such weighting in this case.

Also as expected, the number of transitions far exceeds the number of transversions on the most parsimonious trees, yet the percentages of the two sorts of changes on the trees differ somewhat from the expectation based on the matrix. From the matrix it can be seen that there are a minimum of 76 transitions and 28 transversions, which means that we might expect about ¾ of the changes on the tree to be transitions. However, the tree shown in Figure 1A necessitates 102 unambiguous transitions and 45 unambiguous transversions. Thus, only about ⅔ of the unambiguous changes are transitions, meaning that there are relatively fewer transitions, and relatively more transversions, than one might have expected based on the matrix. Despite the different numbers of changes of the two types, differential weighting of transversions versus transitions had little effect on the outcome. Assigning transversions a weight of 1.3 (see above) yielded 3 trees, corresponding to Figures 1A, 1C, and 1D. The consensus of these trees is identical to the consensus based on equal weighting (Fig. 2). In other words, this weighting scheme has the effect

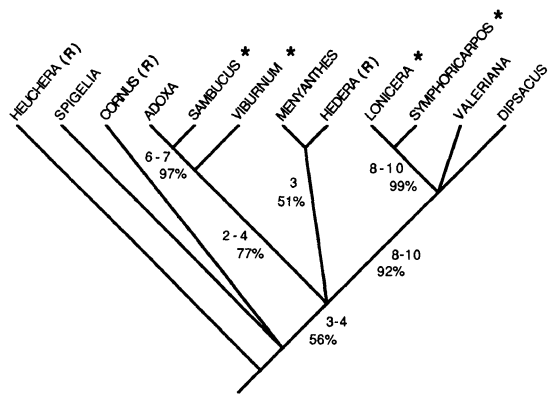

FIGURE 2. Strict consensus of the four most parsimonious trees in Figure 1. Numbers associated with the internal branches indicate the range of unambiguous character changes, and the percentage of times that the branch was recovered in 100 bootstrap samples. Taxa marked by an asterisk are traditionally placed in Caprifoliaceae; taxa marked by (R) are assigned to Rosidae.

of rendering one of the most parsimonious trees slightly less parsimonious (see Albert et al., 1992, for a similar example in *Clarkia*). Even a weighting of 2 : 1 had no effect on the consensus result.

Along each branch of the consensus tree we indicate the range of unambiguous character changes and the percentage of times the branch was recovered in 100 bootstrap replicates. Only one branch not seen in the strict consensus appeared in over 50% of the bootstrap runs: *Valeriana* and *Dipsacus* were directly united in 52% of the trees. Two clades are seen in over 95% of the samples: *Lonicera* plus *Symphoricarpos* (99%) and *Sambucus* plus *Adoxa* (97%). The connection of *Lonicera* and *Symphoricarpos* with *Valeriana* and *Dipsacus* is not far behind, being found in 92% of the replicates.

The order of "decay" of the six components present in the most parsimonious trees is shown in Figure 3. Two clades are lost when the six trees just one step longer are considered, and the remaining four clades are maintained with the addition of 21 trees of length 204 and 57 trees of 205. When the 182 trees less than or equal to 206 steps are considered, the connection between *Viburnum* and the *Sambucus–Adoxa* clade is lost. With the addition of the 142 trees of 207 steps the consensus tree no longer maintains the connection between *Sambucus* and *Adoxa*, although this link is still found in 99% of the 324 trees. Only the connection between *Lonicera* and *Symphoricarpos* is still present in the consensus of all 594 trees of 208 steps or less, and all structure is lost at 209 steps, only 7 steps from the most parsimonious trees.

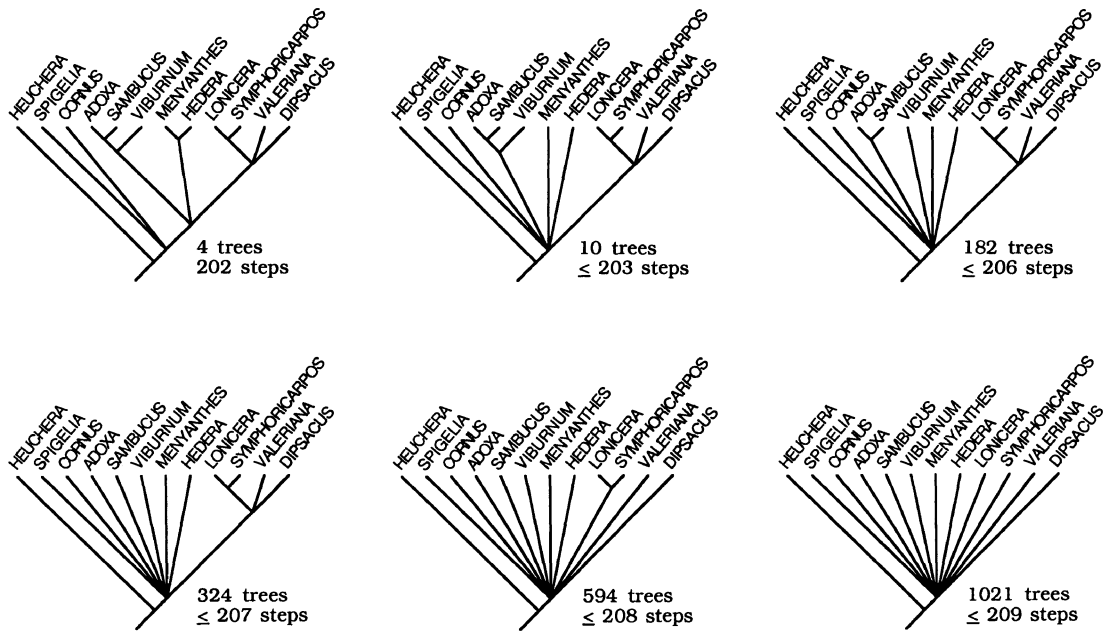

FIGURE 3. Trees showing the order of "decay" of clades. Each tree is the strict consensus of the trees that are less than or equal to the number of steps shown. The basal branch in all trees is an artifact of rooting the network along the branch connecting *Heuchera*.

In Figure 4 the tree lengths of 50 randomized data sets are compared to the length (202) of the most parsimonious trees obtained for the *rbc*L data. Clearly, the real data set falls far (several standard deviations) outside the normal distribution for data sets randomized using the method suggested by Archie (1989b). Whether this result will hold for other null models is uncertain, but it may suggest that there is phylogenetic structure in the *rbc*L data.

The distribution of the lengths of 10,000 randomly generated trees is shown in Figure 5. Note that the shortest random tree recovered was of length 220—18 steps longer than the shortest trees for the *rbc*L data. Although this means that none of the 1,021 trees known (from the decay experiments) to require 209 steps or less were actually found in the sample of random trees, it is presumed that 10,000 trees is sufficient to reveal the shape of the underlying distribution for all trees (D. Hillis, pers. comm.). The skewness of this distribution, measured by g_1, is -0.78, which far

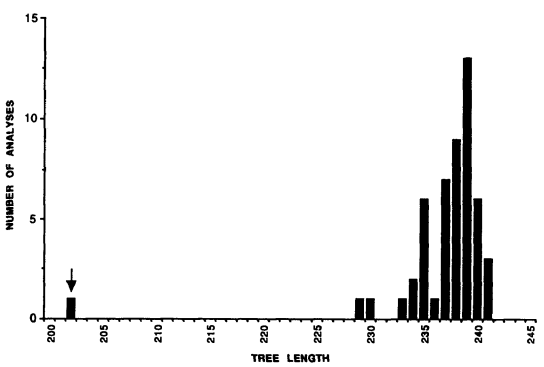

FIGURE 4. Histogram of tree lengths showing the position of the most parsimonious trees (202 steps) for the *rbc*L data in relation to parsimonious trees for 50 randomized data sets (sensu Archie, 1989b); arrow indicates the most parsimonious trees found with the real data set.

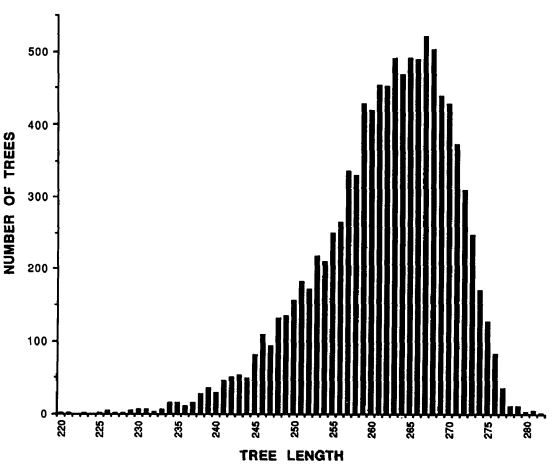

FIGURE 5. Histogram of tree lengths for 10,000 randomly generated trees, showing the degree of left-hand skewness ($g_1 = -0.78$).

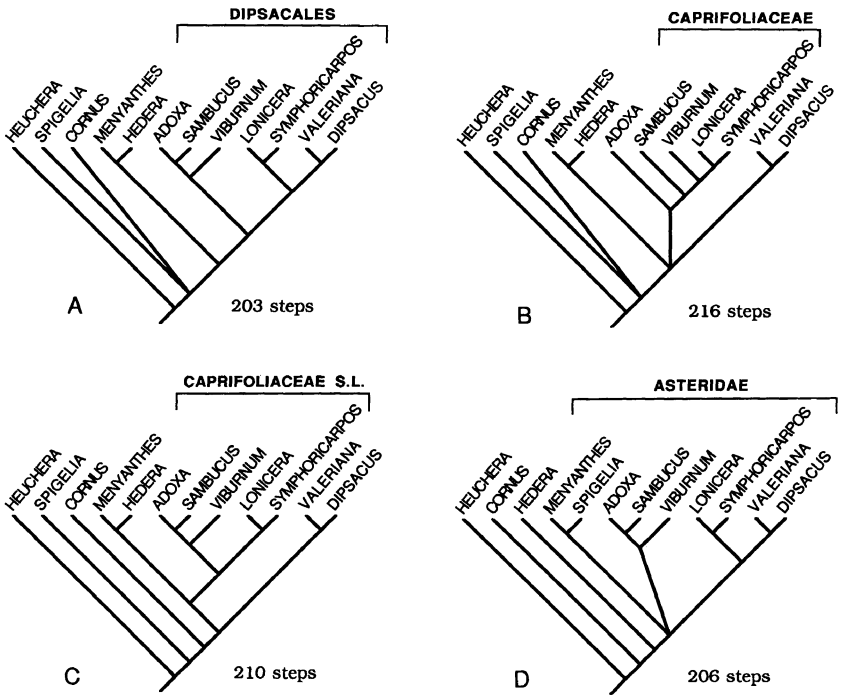

FIGURE 6. Lengths of alternative trees, with the following topological constraints imposed: (A) Dipsacales monophyletic; (B) Caprifoliaceae (*Sambucus, Viburnum, Lonicera, Symphoricarpos*) monophyletic; (C) Caprifoliaceae plus *Adoxa* monophyletic; (D) Asteridae monophyletic. When more than one tree was obtained, the strict consensus is shown.

exceeds the $P = 0.05$ critical value for data sets of 10 to 15 taxa with 50 to 100 characters (D. Hillis, 1991, pers. comm.). Again, this appears to indicate significant phylogenetic signal in the *rbc*L data.

DISCUSSION

DIPSACALES AND CAPRIFOLIACEAE SENS. LAT.

The Dipsacales have generally been considered a "homogeneous," natural element within Asteridae (Wagenitz, 1977, 1992). However, none of our most parsimonious trees support the monophyly of Dipsacales (Fig. 1), even though the consensus of these trees (Fig. 2) seems to allow this possibility. Instead, the clade consisting of *Menyanthes* (representing Asterales sens. lat.; Olmstead et al., 1992) and *Hedera* (representing Apiales, Rosidae) is intercalated between two clades of dipsacalean taxa, namely a *Viburnum–Sambucus–Adoxa* clade and a line comprising *Lonicera, Symphoricarpos, Valeriana,* and *Dipsacus.*

Taken at face value, this result supports the idea, based on immunology (Hillebrand & Fairbrothers, 1970a, b) and morphology, that these two lines of Dipsacales are not closely related (Donoghue, 1983). It is also generally consistent with restriction site studies of the inverted repeat (Downie & Palmer, 1992) and other regions of the chloroplast genome (Sytsma, Smith, & Donoghue, unpublished). However, it is important to recognize that the non-monophyly of Dipsacales is not a strongly supported result of our analysis. In fact, trees in which Dipsacales are monophyletic (Fig. 6A) require only a single additional step. Note that in this case the *Menyanthes–Hedera* clade appears as the sister group of Dipsacales, an arrangement that is more consistent with the broader analysis of *rbc*L sequences of Olmstead et al. (1992).

None of our most parsimonious trees are consistent with the monophyly of Caprifoliaceae in the traditional sense (that is, excluding *Adoxa,* Valerianaceae, and Dipsacaceae), and this conclusion is very strongly supported in the present analysis. In particular, *Viburnum* and *Sambucus* are united with *Adoxa,* and this clade is separated from *Lonicera* and *Symphoricarpos* (of Caprifoliaceae sens. str.), which are united instead with *Valeriana* and *Dipsacus.* Trees in which *Sambucus, Viburnum, Lonicera,* and *Symphoricarpos* are united in a clade require a minimum of 216 steps, 14 more steps than the shortest trees (Fig. 6B). Even if *Adoxa* is considered part of Caprifoliaceae (as it sometimes has been; see Donoghue, 1983), it would

still be better to consider the latter to be paraphyletic, because trees with this expanded Caprifoliaceae forming a clade require a minimum of 210 steps (Fig. 6C).

Regarding the status of Caprifoliaceae our results are congruent with morphological characters. Donoghue (1983) summarized earlier evidence, and research available since that time—for example, on pollen (Böhnke-Gütlein & Weberling, 1981; Donoghue, 1985), nectaries (Wagenitz & Laing, 1984), and the tapetum (Weberling & Hildenbrand, 1986)—has strengthened the idea that there are independent and unrelated lines of Caprifoliaceae. This view is also consistent with a phenetic analysis of morphological data (Hsu, 1983), with chromosome structure (Sax & Kribs, 1930), with secondary chemistry (Bohm & Glennie, 1971), and with immunological evidence (Hillebrand & Fairbrothers, 1970a, b). Finally, our results are congruent in this respect with the results of other molecular studies (Olmstead et al., 1992; Downie & Palmer, 1992).

CAPRIFOLIACEAE SENS. STR., VALERIANACEAE, AND DIPSACACEAE

The result that genera of Caprifoliaceae (in the traditional sense) do not form a monophyletic group is partly a function of the solid connection between Caprifoliaceae sens. str., Valerianaceae, and Dipsacaceae. Indeed, the *Lonicera–Symphoricarpos–Valeriana–Dipsacus* clade is one of the best supported in the entire analysis, judging by the number of unambiguous character changes, the bootstrap value of 92%, and the high "decay" index (collapsing in trees 6 steps longer than the shortest trees). This connection is also supported by many morphological features, including reduction of carpellary vascular traces (Wilkinson, 1949), aspects of pollen morphology (Donoghue, 1985), and nectary structure (Wagenitz & Laing, 1984).

Within this clade there is very strong support for a direct connection between *Lonicera* and *Symphoricarpos*. These genera are united by 8–10 unambiguous changes in the most parsimonious trees, and they appeared together in 99% of the bootstrap samples. Furthermore, this link lasted the longest in the decay analyses, appearing in all 594 trees less than or equal to 208 steps. At 209 steps it is possible to find trees in which *Symphoricarpos* and *Lonicera* are dissociated, with either one united with Valerianaceae and Dipsacaceae.

The strong connection between *Lonicera* and *Symphoricarpos* is somewhat surprising. These two genera are not unequivocally connected by morphological characters and they have generally been placed in separate tribes (see Wilkinson, 1949; Donoghue, 1983). *Lonicera* is usually allied with *Leycestaria* in the tribe Lonicereae. More importantly, *Symphoricarpos* has often been placed in the tribe Linnaeeae (e.g., Fukuoka, 1972; Hara, 1983), whose other members appear to be more closely related to Valerianaceae and Dipsacaceae than they are to other genera of Caprifoliaceae sens. str. (Donoghue, 1983, in prep.). If *Symphoricarpos* really belonged with Linnaeeae, and if Linnaeeae are more closely related to Valerianaceae and Dipsacaceae, then it should not have attached directly to *Lonicera* in our analysis, but instead to *Valeriana* and *Dipsacus*. Other molecular data seem to confirm that *Symphoricarpos* is more closely related to *Lonicera* than it is to genera of Linnaeeae. The two genera are united in the trees of Downie & Palmer (1992), and it appears that they may also be united (along with *Triosteum*) by the loss of the chloroplast gene *clp*P (Downie, Donoghue, Sytsma, & Palmer, unpublished).

Our results for *Lonicera* and *Symphoricarpos* might be considered evidence of the monophyly of Caprifoliaceae sens. str., but this conclusion would be premature in view of the limited sample of taxa included in the analysis. As we noted above, one of the strongest results of morphological analyses is that Caprifoliaceae sens. str. are paraphyletic, with the tribe Linnaeeae more closely related to Valerianaceae and Dipsacaceae than to other Caprifoliaceae sens. str. (Donoghue, 1983, in prep.). Some of the strongest evidence is provided by floral anatomy, especially the abortion of carpels during the development of the ovary (Wilkinson, 1949). The link between Linnaeeae and Valerianaceae and Dipsacaceae is also evident in the restriction site studies of Downie & Palmer (1992), although in this case the one representative of Linnaeeae (*Kolkwitzia*) is actually connected directly with *Valeriana*. It seems likely that if representatives of Linnaeeae were included they would be linked with *Valeriana* and *Dipsacus* rather than with *Lonicera* and *Symphoricarpos*. Sequencing of representatives of Linnaeeae (*Dipelta, Kolkwitzia, Linnaea*) is in progress.

In our analysis the position of *Valeriana* is uncertain. In three of the four most parsimonious trees (Fig. 1) it is united directly with *Dipsacus*, but in the fourth it is linked with *Lonicera* plus *Symphoricarpos*. Morphological data support a close connection between Valerianaceae and Dipsacaceae (e.g., Hofmann & Göttmann, 1990; Don-

oghue, in prep.), as does the more inclusive *rbc*L analysis of Olmstead et al. (1992), which again suggests that there may be a problem of limited sampling of taxa. Inclusion of additional Caprifoliaceae sens. str. might strengthen the connection between Valerianaceae and Dipsacaceae.

VIBURNUM, SAMBUCUS, AND *ADOXA*

Viburnum is linked with *Sambucus* plus *Adoxa* in all of our most parsimonious trees, but this connection is not especially srong: 2–4 unambiguous character changes mark this branch, it appeared in 77% of the bootstrap trees, and it decayed at 206 steps. Morphological data also tend to unite *Viburnum* with *Sambucus* and *Adoxa* (under most outgroup arrangements), but by itself the evidence does not inspire great confidence (Donoghue, 1983, in prep.). It is certainly encouraging that the molecular and morphological data point to the same conclusion.

The connection between *Adoxa* and *Sambucus* is quite robust. There are 6–7 unambiguous character changes below this node, and it appeared in 97% of the bootstrap samples. In the decay analyses this connection lasted until trees of 207 steps were added, making it the third most robust result in our analysis. Curiously, this clade decayed before the *Lonicera–Symphoricarpos–Valeriana–Dipsacus* clade, even though the latter had a somewhat lower bootstrap value.

In addition to Caprifoliaceae (e.g., Eichler, 1875; Hooker, 1873), *Adoxa* has been allied with Saxifragaceae (Hutchinson, 1973; Warming & Mobius, 1929), Araliaceae (de Candolle, 1830), and Cornales (Dahlgren, 1975), although it has generally been placed in its own family (reviewed in Wagenitz, 1977; Donoghue, 1983). The *rbc*L data argue against these other connections. Thus, the shortest trees in which *Adoxa* is linked directly with *Hedera* require 213 steps (11 steps longer than the most parsimonious trees), and it takes 214 steps to connect *Adoxa* with *Heuchera.*

Some authors have noted similarities between *Adoxa* and *Sambucus* (e.g., Eichler, 1875; Hallier, 1912; Takhtajan, 1980), and this connection has been supported by preliminary cladistic analyses (Donoghue, 1983, in prep.). One morphological character in particular has seemed compelling, namely the presence of *Adoxa*-type embryo sac development in *Adoxa* and *Sambucus.* This condition is extremely rare in angiosperms and is otherwise very widely scattered in occurrence (Maheshwari, 1946; Donoghue, 1983). Its distribution within *Sambucus* is still somewhat unclear, and it is possible that it will be discovered in some *Viburnum* species, inasmuch as several species are reported to have bisporic development (Sunesson, 1933). Other possible indicators of a close relationship include compound leaves (depending on outgroups) and extrorse anthers (although there is some variation in *Sambucus* and the condition in *Adoxa* is difficult to score). Nevertheless, most authors (e.g., Sprague, 1927) have hesitated to connect the two genera, largely because *Adoxa* is so different (presumably highly derived) in habit and in floral morphology. However, Thorne (1983) adopted Donoghue's (1983) suggestion that *Adoxa* and *Sambucus* (and possibly *Viburnum*) be treated as the family Adoxaceae.

One outstanding problem concerns the relationships of recent additions to Adoxaceae (Wu, 1981; Wu et al., 1981; Hara, 1981, 1983). Although *Tetradoxa omeiensis* (=*Adoxa omeiensis*) is very similar to *Adoxa moschatellina,* and is probably closely related, *Sinadoxa* differs in many respects and may be more closely related to Araliales than to *Adoxa.*

ASTERIDAE AND ROSIDAE

Conclusions regarding the broader relationships of dipsacalean plants are tenuous, because much of the relevant structure seen in the most parsimonious trees disappears when slightly longer trees are taken into consideration. Nevertheless, it is interesting to note that the most parsimonious trees support a clade including the dipsacalean taxa, along with *Hedera* plus *Menyanthes.* The former is a placeholder for Araliales, and the latter represents an Asterales sens. lat. clade (comprising Asteraceae, Menyanthaceae, Goodeniaceae, Calyceraceae, Campanulaceae, and Lobeliaceae), which is consistently supported in the more inclusive *rbc*L analyses of Olmstead et al. (1992). Although the exact arrangement of these groups in relation to dipsacalean taxa is uncertain, the present analysis is consistent with the view that Asterales sens. lat. and Araliales are closely related, and that together they are related either to the *Viburnum–Sambucus–Adoxa* clade or to the Caprifoliaceae sens. str.–Valerianaceae–Dipsacaceae clade.

This result is especially significant in light of the inclusion of *Spigelia* (Loganiaceae) in the analysis, which is intended to represent the remainder of the Asteridae, based on the broader analyses of Olmstead et al. (1992). If Asteridae were monophyletic, *Spigelia* would be linked with *Menyanthes* and the dipsacalean taxa, to the exclusion of the rosids. The shortest trees of this type require

206 steps, four steps more than our most parsimonious trees (Fig. 6D). Instead, *Cornus* and *Hedera* are variously nested among Asteridae in our most parsimonious trees (Fig. 1), which implies that the Asteridae, as they are generally constituted, are not monophyletic. This conclusion is consistent with the larger analyses of *rbc*L (Olmstead et al., 1992).

Although the standard view of morphological systematists has been that Asteridae form a natural group, Takhtajan (1987) proposed that it be subdivided into two major lines: Asteridae sens. str. (Asterales sens. lat. and Dipsacales) and Lamiidae (the remaining Asteridae). These lines, he believes, originated independently from separate lines of Rosidae. Phylogenetic analyses of the morphological evidence are needed before molecular and morphological results can be compared critically.

In the meantime, it is interesting to note that while the results of the present study are consistent with many aspects of other molecular analyses, they also differ in several ways. In trees presented by Olmstead et al. (1992) Araliales are linked directly with Dipsacales, rather than with Asterales sens. lat. Similarly, the trees of Downie & Palmer (1992) show Araliales connected to Caprifoliaceae sens. str., Valerianaceae, and Dipsacaceae. Although these studies include more taxa outside of the groups of immediate concern here, they also contain fewer potentially close relatives. The resolution of this issue requires a more complete sample of relevant taxa as well as additional molecular and morphological evidence.

It is important to recognize that all three of the molecular studies suggest that Asteridae in their traditional sense are not monophyletic, and they imply that some "asterid" features such as sympetaly may have evolved independently in Asterales sens. lat., Dipsacales, and Lamiidae, or may have been lost in Araliales and possibly Cornales. Here, the ontogenetic studies of Erbar (1991) are of special interest. She has demonstrated a difference between "early sympetaly" (corolla tube initiated before petal primordia) and "late sympetaly" (corolla lobes initiated as separate primordia and connected later), which largely corresponds with major clades of Asteridae implied by molecular data. In particular, early sympetaly is found in Asterales sens. lat. (including Campanulales and Menyanthaceae) and Dipsacales, while late sympetaly occurs in virtually all other Asteridae (i.e., in Lamiidae sensu Takhtajan, 1987). Rubiaceae, Oleaceae, and several Acanthaceae are exceptions to this correspondence in having early sympetaly. Unfortunately, the development of sympetaly has not yet been studied in *Viburnum*, *Sambucus*, or *Adoxa*. These observations are consistent with (but do not necessitate) the idea that sympetaly evolved independently at least twice.

Erbar (1991) also noted evidence (see Erbar & Leins, 1988) that in some Araliales the corolla is initiated as a ring primordium. Because this ring does not develop into a conspicuous tube, however, the mature flowers of these plants appear to have separate petals. This similarity to early sympetaly can be interpreted in several ways. It may be that the condition in Araliales is a precursor of early sympetaly in Asterales sens. lat. and Dipsacales. Alternatively, the apparently separate petals of Araliales may have been derived from the early sympetalous condition (i.e., sympetaly was "lost"). The latter hypothesis will be favored if phylogenetic studies continue to nest Araliales among sympetalous groups (see Olmstead et al., 1992).

Conclusions

The analyses reported here suggest a number of changes in the circumscription of taxa. First, it is clear that the genera of Caprifoliaceae do not form a monophyletic group, and the traditional concept of this family must be abandoned if we hope to achieve a truly phylogenetic system of angiosperms (Donoghue & Cantino, 1988). In particular, the genera of Caprifoliaceae sens. str. are more closely related to Valerianaceae and Dipsacaceae than they are to *Viburnum* and *Sambucus*, and *Sambucus* (very probably) and *Viburnum* (with less certainty) are more closely related to *Adoxa*. The smallest monophyletic group that would include the genera traditionally assigned to Caprifoliaceae would also include the rest of the Dipsacales (Valerianaceae, Dipsacaceae, and Adoxaceae), and possibly also the Asterales sens. lat. and Araliales.

The connection between Caprifoliaceae sens. str., Valerianaceae, and Dipsacaceae should be formally represented, but a decision on the best way to do this must await the publication of the morphological results and additional molecular analyses. The *rbc*L sequence data are still too limited to evaluate properly the morphological hypothesis that Caprifoliaceae sens. str. are paraphyletic, with the tribe Linnaeeae more closely related to Valerianaceae and Dipsacaceae. If this is borne out by further studies, it may be best to merge Caprifoliaceae sens. str., Valerianaceae, and Dipsacaceae into a single family.

Although the status of Caprifoliaceae sens. str. is still uncertain, *Lonicera* and *Symphoricarpos* seem to be more closely related than one might have expected based on previous taxonomic treatments of the family (see discussion above). It should

be noted, however, that because ovary development in *Symphoricarpos* does not correspond exactly with Lonicereae or with Linnaeeae (Wilkinson, 1949), its exact relationships based on morphology are still uncertain (Donoghue, in prep.). Our analyses strongly suggest that *rbc*L sequences of representatives of additional genera of Caprifoliaceae sens. str. would be very valuable and might result in tribal realignments.

The close relationship between *Sambucus* and *Adoxa* should be represented in the phylogenetic system, perhaps by expanding Adoxaceae. This has been suggested based on morphological evidence (Donoghue, 1983, in prep.), but these data have not been entirely convincing, owing especially to the extreme modification/reduction of *Adoxa*. The *rbc*L data provide strong support for this connection and argue against alternative hypotheses that *Adoxa* may be related to Araliales or Saxifragaceae. Recently described Adoxaceae, especially *Sinadoxa*, must be evaluated, as should additional representatives of *Sambucus*. This phylogenetic information will be especially critical in understanding the nature of the genetic and developmental changes associated with morphological shifts in the evolution of *Adoxa*-like plants.

The position of *Viburnum* is still somewhat uncertain. It is connected with *Sambucus* and *Adoxa* in our most parsimonious trees, and there is also some morphological support for this arrangement. Although the evidence is not overwhelming in either case, the congruence is satisfying. *Viburnum* might be placed in Adoxaceae along with *Sambucus* and *Adoxa*, but until its relationships are more confidently resolved there may be some merit in placing *Viburnum* in its own family, the Viburnaceae (see Dahlgren, 1980). In the end, the assignment of rank is arbitrary.

Our results are encouraging as regards the quality of *rbc*L data. Although the consistency index is quite low for a data set of this size, randomized data sets and tree-length distributions both suggest that considerable phylogenetic information is present in the data. The bootstrap and decay analyses suggest that this signal may reflect the presence of several very well supported clades: especially the connections between *Lonicera* and *Symphoricarpos*, between these genera and *Valeriana* and *Dipsacus*, and between *Sambucus* and *Adoxa*. Most of the rest of the connections are not strongly supported and decay with the addition of only a single step. Overall, these results are especially encouraging with respect to potential resolution of the relationships of dipsacalean taxa using *rbc*L sequences.

Our analyses also bear on the general issue of differentially weighting characters or character state changes. Our main phylogenetic results are supported by changes at third positions within codons; there are few changes at first and second positions and these show considerable conflict with one another. We see no reason to disregard or even to downweight third sites in this case, and we caution against a priori judgements of this sort. Likewise, we found that much of the phylogenetic structure resided in the much more numerous transition substitutions, and saw no evidence that transitions were less reliable than transversions.

Comparison of this study with the more inclusive *rbc*L analysis of Olmstead et al. (1992) raises important issues concerning the effects of the sampling of taxa in phylogenetic studies. The smaller sample considered here allows a much more detailed examination of the structure in the data set. Exact parsimony solutions can be obtained using branch-and-bound algorithms, which also ensure an accurate picture of the rate of decay of clades, and so on. Studies using randomized data sets and tree-length distributions are also much easier to carry out with smaller data sets. Some of these sorts of analyses can be extended to larger analyses, and we hope that exploration of the robustness of clades (beyond the use of the bootstrap) will soon become routine. In the meantime, it is difficult to ascertain the reliability of the results of the larger analyses, and one must be especially cautious in drawing evolutionary conclusions based on such studies (see Maddison, 1991).

On the other hand, smaller studies are subject to error by virtue of leaving out relevant taxa (Donoghue et al., 1989; Olmstead et al., 1992). In particular, in any analysis of a small portion of Asteridae (for example) one may not discover globally more parsimonious solutions, which might become apparent with the addition of taxa outside the group of interest. Of course, this warning applies to larger studies as well, as does the problem of leaving out taxa within the group of interest that might have an important impact on the outcome. If the sample of taxa within the group is too sparse, errors might arise by virtue of highly divergent branches "attracting" one another due to the chance accumulation of mutations (see Felsenstein, 1978). Increasing the sample of taxa might effectively "shorten" such long branches, thereby decreasing the likelihood of such errors. This sort of consideration could perhaps be translated into a scheme for selecting a set of taxa in the first place.

In our analysis there is a better representation of critical dipsacalean taxa, which probably allows our study to reflect more accurately relationships among Dipsacales than the broader analysis of Olm-

stead et al. (1992). On the other hand, our representation of other major lines is much poorer than in the analysis of Olmstead et al. (1992). Our failure to achieve any clearer picture of the broader relationships may be largely a function of the limited sample of taxa aside from Dipsacales, rather than any fundamental problem with the use of *rbc*L sequences in addressing the more inclusive phylogenetic questions.

The congruence between molecular and morphological evidence on the phylogeny of Dipsacales is gratifying, if not very surprising (Donoghue & Sanderson, 1992). As is often the case, it is clear that the different data sets provide consistent results, but that the results show different levels of resolution. For example, in our *rbc*L analysis the position of *Valeriana* is unresolved, whereas several morphological characters link Valerianaceae with Dipsacaceae. On the other hand, *rbc*L sequences strongly connect *Symphoricarpos* with *Lonicera*, whereas the relationships of these genera are not clearly resolved in morphological analyses (Donoghue, in prep.). Under these circumstances it will be especialy valuable to combine data sets, so that all of the evidence can be considered at once (Barrett et al., 1991; Donoghue & Sanderson, 1992).

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