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Systematic Botany, Vol. 19, No. 2. (Apr. - Jun., 1994), pp. 236-252.

Stable URL:
http://links.jstor.org/sici?sici=0363-6445%28199404%2F06%2919%3A2%3C236%3AACDPOT%3E2.0.CO%3B2-0

Systematic Botany is currently published by American Society of Plant Taxonomists.

Systematic Botany (1994), 19(2). pp 236–252

A Chloroplast DNA Phylogeny of the Caryophyllales Based on Structural and Inverted Repeat Restriction Site Variation

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ABSTRACT. Phylogenetic relationships among 25 representatives of the Caryophyllales and three outgroup taxa from Polygonaceae and Plumbaginaceae were assessed using structural variation in the chloroplast genome and restriction site variation in the highly conserved inverted repeat region of the chloroplast genome. In addition to the previously reported loss of the chloroplast *rpl*2 intron in the common ancestor of the Caryophyllales, observed structural mutations include: 1) the loss of the *rpl*16 intron in the chloroplast genome of *Limonium* (Plumbaginaceae); 2) three large, phylogenetically informative deletions within the gene ORF2280, and 3) parallel, 6-kb inversions in the large single-copy region of the chloroplast genomes in *Pereskia* (Cactaceae) and in *Atriplex* and *Chenopodium* (Chenopodiaceae). Sixty-two of the 161 restriction sites scored were phylogenetically informative. Parsimony analyses of the structural and restriction site characters indicate that: 1) the Caryophyllales consist of two major clades, one comprising Amaranthaceae and Chenopodiaceae, and the other all remaining families; 2) two families, Phytolaccaceae and Portulacaceae (*Portulaca* and *Claytonia*), are polyphyletic, with elements of the former (*Phytolacca* and *Rivina*) strongly linked with Nyctaginaceae; 3) *Pereskia* (the only examined representative of Cactaceae) is, surprisingly, strongly linked to *Portulaca*, and 4) Caryophyllaceae and Molluginaceae, the only anthocyanin-producing taxa in the order, occur in the same portion of the trees and are not basal to the group. Relationships among several families are poorly resolved.

Although the Caryophyllales (or Centrospermae) are clearly monophyletic (Cronquist 1981; Eckardt 1976; Hershkovitz 1989; Rodman et al. 1984), phylogenetic relationships within the order are quite uncertain. This is surprising considering that perhaps no other order of flowering plants of its size has been as thoroughly investigated morphologically, ultrastructurally, and chemically (Mabry 1977). The delimitation of clades within the order and their relationships have been largely influenced by the choice of character(s) examined, with little consensus of relationships among the varying lines of taxonomic evidence (e.g., compare Behnke 1994; Cronquist 1981; Mabry 1976; Rettig et al. 1992; Rodman et al. 1984; Takhtajan 1980; Thorne 1992).

Beginning with Rodman et al.'s (1984) work on the familial relationships within the Caryophyllales, much attention during the past decade has focused on understanding relationships using explicit cladistic approaches to classification and/or detailed analyses of specific characters (e.g., Behnke 1994; Brown and Varadarajan 1985; Carolin 1987; Hershkovitz 1991a, 1991b; Nowicke 1994; Rettig et al. 1992; Rodman 1994). However, despite these and other analyses, several major points of contention remain within the Caryophyllales and involve, but are not limited to: 1) the relationship between the anthocyanin- and betalain-producing plants; 2) the alleged primitiveness of Phytolaccaceae within the order; 3) the naturalness of several families [e.g., Caryophyllaceae, Chenopodiaceae, Phytolaccaceae s.l., and Portulacaceae may each be paraphyletic (Rodman 1990)]; and 4) the placement of problematic genera, such as *Stegnosperma* and *Corrigiola*.

We have chosen to reassess phylogenetic relationships within the Caryophyllales using several classes of molecular characters from the chloroplast genome. Mutations in chloroplast DNA (cpDNA) are fundamentally of two kinds: point mutations (single nucleotide pair substitutions) and structural rearrangements. Point mutations can be detected either indirectly, through restriction site mapping (as in this study), or directly, by DNA sequencing [as in the recent study by Rettig et al. (1992) of *rbc*L sequence variation within the Caryophyllales]. Analyses of restriction site polymorphisms in cpDNA have almost invariably been limited to studies at the rank of family or below. Recently, however, we have shown that by focusing ex-

clusively on the highly conserved inverted repeat (IR) region, where rates of nucleotide substitution at silent (synonymous) sites are about 4–5 times lower than in single-copy regions (Wolfe et al. 1987), comparative restriction site mapping can be extended to greater evolutionary depths than those to which it has been applied previously when the whole genome was taken into consideration (Downie and Palmer 1992a). Structural rearrangements of the chloroplast genome (such as inversions and gene deletions) are relatively infrequent events among photosynthetic land plants and usually can provide strong evidence of monophyly for a particular group (reviewed in Downie and Palmer 1992b). Previous studies have demonstrated the utility of cpDNA rearrangements as molecular characters for elucidating evolutionary relationships among taxa at a variety of taxonomic levels (Bruneau et al. 1990; Downie et al. 1991; Jansen and Palmer 1987; Lavin et al. 1990; Palmer et al. 1988; Raubeson and Jansen 1992; Stein et al. 1992).

We undertook this study of Caryophyllales phylogeny with three goals in mind: 1) to investigate chloroplast genome structure within the Caryophyllales and related Plumbaginaceae and Polygonaceae; 2) to demonstrate the potential of comparative restriction site mapping of the highly conserved IR region of the chloroplast genome for resolving phylogenetic relationships within the group, and 3) to formulate more precise hypotheses about relationships among the diverse clades of the Caryophyllales, and, in so doing, to assess the relationships of taxa whose phylogenetic position is questionable.

Materials and Methods

Plant Material and Analysis of cpDNA's. Fresh leaf material from 25 species representing 11 families (sensu Cronquist 1981) of Caryophyllales, two species of Polygonaceae, and one species of Plumbaginaceae, was obtained from various sources (Table 1). The isolation of cpDNA or total cellular DNA, restriction endonuclease digestion, agarose gel electrophoresis, bidirectional transfer of DNA fragments from agarose gels to nylon filters, labeling of recombinant plasmids with ^{32}P by nick-translation or random priming, filter hybridization, and autoradiography were conducted following the methods of Palmer (1986) and Downie and Palmer (1992b).

The structure of the entire chloroplast genome was investigated in 14 species of Caryophyllidae (Table 1). All DNA's were digested singly with two restriction enzymes (*Bam*HI and *Hin*dIII), with the exception of *Limonium gmelinii*, which was digested singly with four restriction enzymes (*Bam*HI, *Eco*RV, *Bgl*II, and *Hin*dIII). Filter-bound digests were probed with 109 subclones, ranging in size from 0.1 to 3.5 kb, which together represent virtually the entire chloroplast genome of *Nicotiana tabacum* L. The positions of seven of these subclones, used to diagnose the presence of the 6-kb inversion described below, are shown in Fig. 1.

To investigate restriction site and structural variation within the IR, DNA's of 24 species from the Caryophyllidae (Table 1) were digested singly with each of the 10 restriction enzymes shown in Fig. 2. Filter blots of these digests were hybridized with 19 subclones (Fig. 2) from the cpDNA IR region of *Nicotiana tabacum*. Unambiguous restriction site maps for each of the ten enzymes were constructed for the *N. tabacum* IR (Fig. 2) by computer analysis of its completely known cpDNA sequence (Shinozaki et al. 1986). Because many restriction sites and fragment sizes among the taxa examined coincided with those known in *N. tabacum*, mapping efforts were greatly facilitated by scoring our data against these maps.

Phylogenetic Analysis. All phylogenetic reconstructions were performed using PAUP version 3.0s (Swofford 1990) on a Macintosh Quadra 700 computer. All analyses were replicated 10 times with RANDOM addition sequence using the HEURISTICS, TREE BISECTION-RECONNECTION, MULPARS, and COLLAPSE options with the ACCTRAN optimization. Wagner parsimony (which weights site gains and site losses equally) was invoked by assigning each restriction site character ORDERED status. Additionally, the character-state weighting method of Albert et al. (1992) was used with a range of weight ratios from 1.1:1.0 to 1.5:1.0 for gains over losses. Character-state weighting was accomplished using the USERTYPE STEPMATRIX option of PAUP. Ancestral states (ANCSTATES) were either not specified (i.e., coded as ?'s) or coded as site absences (0's), and ingroup monophyly was forced by implementing the command "enforcing topological

TABLE 1. Species of Caryophyllidae (sensu Cronquist 1981) examined for cpDNA variation. Species without asterisks indicate those taxa surveyed for both structural variation throughout the entire chloroplast genome and IR restriction site mutations. Single asterisks indicate those taxa used only in the structural rearrangement study of the entire chloroplast genome. Double asterisks indicate those taxa used only in the IR comparative restriction site mapping study (and in assessing major structural variation within this region). Voucher specimens, unless otherwise indicated, have been deposited at ILL.

Taxon	Source and voucher
Caryophyllales	
Aizoaceae	
Tetragonia tetragonioides (Pallas) Kuntze**	W. J. Beal Botanical Garden 89B423, *Downie 1070*
Amaranthaceae	
Alternanthera dentata (Moench) Scheygrond	Matthaei Botanical Garden, *Downie 164*
Celosia argentea L.**	Brooklyn Botanical Garden, *Downie 1049*
Basellaceae	
Anredera cordifolia (Ten.) Steenis	Matthaei Botanical Garden 840353, *Downie 163*
Cactaceae	
Pereskia grandiflora Haw.	Matthaei Botanical Garden, no voucher
Caryophyllaceae	
Agrostemma githago L.**	W. J. Beal Botanical Garden 89B659W, *Downie 1041*
Cerastium arvense L.*	JDP, no voucher
Corrigiola littoralis L.**	W. J. Beal Botanical Garden, *Downie 1035*
Silene schafta Gmel.**	W. J. Beal Botanical Garden, *Downie 1033*
Chenopodiaceae	
Atriplex hastata L.*	JDP, no voucher
Beta vulgaris L.	JDP, no voucher
Chenopodium murale L.	JDP, no voucher
Kochia sp.*	JDP, no voucher
Spinacia oleracea L.	JDP, no voucher
Didiereaceae	
Alluaudia montagnacii Rauh var. *ascendens* Drake**	Brooklyn Botanical Garden, *Downie 1055*
Didierea madagascariensis Baillon**	Missouri Botanical Garden 821268, *Downie 1063*
Molluginaceae	
Mollugo verticillata L.**	W. J. Beal Botanical Garden, *Downie 1068*
Nyctaginaceae	
Bougainvillea glabra Choisy	Matthaei Botanical Garden 22746, *Downie 220*
Mirabilis nyctaginea (Michx.) MacMillan**	W. J. Beal Botanical Garden B87137, *Downie 1067*
Phytolaccaceae	
Phytolacca heteropetala H. Walt.	JDP, no voucher
Rivina humilis L.**	Missouri Botanical Garden 894531, *Downie 1062*
Stegnosperma halimifolium Benth.**	Missouri Botanical Garden 720287, *Downie 1061*
Portulacaceae	
Claytonia caroliniana Michx.*	*Doyle 920* (BH)
Claytonia perfoliata Donn**	W. J. Beal Botanical Garden 90B1242W, *Downie 1031*
Portulaca oleracea L.**	Matthaei Botanical Garden, *Downie 282*
Polygonales	
Polygonaceae	
Rheum rhaponticum L.	JDP, no voucher
Polygonum persicaria L.**	Indiana University Greenhouse, *Downie 1022*

TABLE 1. Continued.

Taxon	Source and voucher
Plumbaginales	
Plumbaginaceae	
Limonium gmelinii Kuntze	W. J. Beal Botanical Garden, *Downie 465*

constraints" (Albert et al. 1992; Holsinger and Jansen 1993). Bootstrap (Felsenstein 1985) and decay (Donoghue et al. 1992) analyses were performed using PAUP to evaluate support for particular branches of the cladograms uncovered by the Wagner parsimony analysis. The number of additional steps required to force particular taxa into a monophyletic group was examined using the CONSTRAINTS option of PAUP.

The trees computed by PAUP were rooted by positioning the root along the branches connecting the outgroups *Polygonum* and *Rheum* (Polygonaceae) and *Limonium* (Plumbaginaceae) to the rest of the network (Maddison et al. 1984). Among current classification systems, a consensus favors an association between the Caryophyllales and these two families (Cronquist 1981; Dahlgren 1980; Takhtajan 1980). These two families are clearly excluded from the order, and several authors have even suggested that

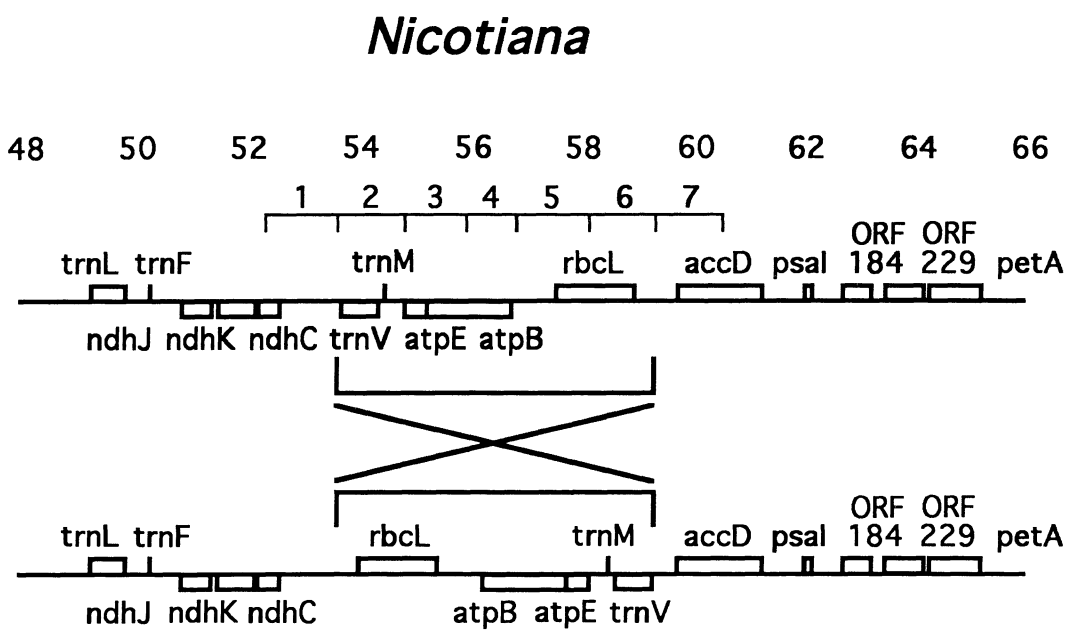


FIG. 1. Structural organization of a portion of the large single-copy region showing the location of 6-kb inversions in cpDNA's of *Atriplex hastata, Chenopodium murale,* and *Pereskia grandiflora* relative to *Nicotiana tabacum* cpDNA. The numbered square brackets indicate the seven *N. tabacum* cpDNA fragments used as hybridization probes to determine the presence of the inversion. Sequence coordinates in kb (scale on top) and gene locations for *N. tabacum* are modified from Shinozaki et al. (1986). Gene locations for *Atriplex, Chenopodium* and *Pereskia* are inferred from the hybridization results obtained using as probes the seven numbered *H. tabacum* fragments and also flanking fragments.

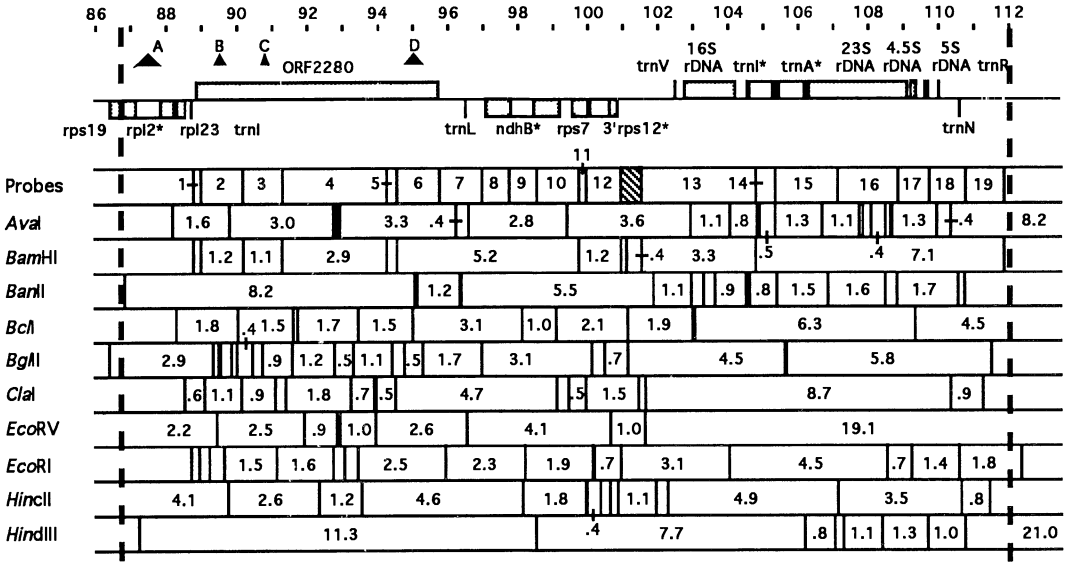


FIG. 2. Gene and restriction site maps of the IR and adjacent single-copy regions of *Nicotiana tabacum* cpDNA. Cleavage sites, gene locations, and sequence coordinates in kb (scale on top) are from Shinozaki et al. (1986). Asterisks indicate genes containing introns; for these genes, filled boxes indicate exons and open boxes indicate introns. Restriction fragment sizes are indicated in kb; fragment sizes less than 400 bp are not labeled. The subclones used as hybridization probes are numbered from 1 to 19. The region between probes 12 and 13 has not been subcloned. The boundaries of the *N. tabacum* IR are indicated by the vertical dashed lines. The locations of DNA deletions greater than or equal to 200 bp in size are indicated by triangles and are as follows: A = *rpl*2 intron deletion in all examined taxa of Caryophyllales; B = 300 bp deletion in *Beta, Chenopodium* and *Spinacia;* C = 200 bp deletion in Nyctaginaceae and Phytolaccaceae s. str.; and D = 500 bp deletion in *Pereskia* and *Portulaca.*

there is no strong evidence linking Polygonaceae and Plumbaginaceae to the Caryophyllales (e.g., Giannasi et al. 1992; Rodman et al. 1984). Although the former study involved an analysis of sequences of the chloroplast gene *rbc*L, subsequent analyses involving much more extensive taxonomic sampling of *rbc*L sequences strongly support the monophyly of the Caryophyllidae and indicate that the Polygonaceae and Plumbaginaceae are the most appropriate outgroup for the Caryophyllales (Chase et al. 1993; Olmstead et al. 1992). The selection of any single outgroup or combination of the three outgroup taxa did not affect the ingroup tree topology.

RESULTS AND DISCUSSION

Structural Variation. An analysis of structural variation within Caryophyllales cpDNA's was carried out at two levels: global (whole genome) and local (inverted repeat). The global analysis permitted the diagnosis of four kinds of structural rearrangements: 1) gene losses (as 49 of the 109 hybridization probes used are gene-specific); 2) intron losses (intron-specific probes are available for genes *rpl*2 and *rpl*16); 3) inversions, transpositions and any other gene order changes; and 4) expansion or contraction of the IR. Rearrangements were detected in the global analysis by arranging the autoradiograms according to the order in the *Nicotiana* chloroplast genome of the hybridization probes [*Nicotiana* cpDNA has the ancestral gene order for vascular plants (Palmer 1991; Palmer and Stein 1986)] and by observing both fragment number and size as one "walks" along the chloroplast chromosome from one hybridization probe to the next. Restriction site maps were not constructed. Any anomaly in the number of fragments detected, their size, or the intensity or pattern of hybridization would be suggestive of a rearrangement (see Downie and Palmer 1992b for a detailed discussion of this

approach and its limitations). Although this method allows for the rapid diagnosis of major structural mutations, the use of only two restriction enzymes for all taxa except *Limonium* and the lack of restriction site maps make it possible that not all rearrangements may have been detected.

The results of the global analysis reveal that the chloroplast genomes of all examined members of Caryophyllidae are, with few exceptions, similar in content and structure to that of *Nicotiana tabacum* and, thus, to the vast majority of angiosperms examined to date (Palmer 1991; Palmer and Stein 1986). Differences in structure that were apparent include inversions and the loss of introns; these structural mutations are described below.

The local analysis permitted greater insight into structural variation within the cpDNA IR. Although the same IR-specific probes were used as in the global analysis, 10 enzymes were used instead of two, and detailed restriction site maps for all taxa were constructed for each enzyme. Moreover, these maps could be aligned readily with the completely sequenced IR of *Nicotiana tabacum* owing to the conservative nature of this region (Wolfe et al. 1987). Consequently, analysis at this level was more sensitive than the global analysis in detecting small rearrangement events, such as length mutations and intron losses. For example, although intron-specific probes were not available for four of the five intron-containing genes within the IR (Fig. 2), the absence of these introns could be detected readily by the availability of these detailed maps. Thus, altogether, six of the 21 introns known from the entire *Nicotiana* chloroplast genome were surveyed (in genes *rpl*2, *rpl*16, 3′*rps*12, *trn*I, *trn*A, and *ndh*B). In addition to the loss of the *rpl*2 intron, results from the local analysis revealed three major length mutations within the gene ORF2280; these length variants are described below.

INTRON LOSSES. Introns are highly stable components of land plant chloroplast genomes, with no cases of intron gain and few cases of intron loss known during land plant evolution (Palmer 1991). A previous study has shown that the *rpl*2 intron is absent from the chloroplast genomes of all examined members of the Caryophyllales but present in the cpDNA's of *Limonium gmelinii* (Plumbaginaceae) and the three genera examined of Polygonaceae (*Polygonum*, *Rheum*, and *Rumex*) (Downie et al. 1991). This suggests that the intron was lost in the common ancestor of the order and supports the order as monophyletic, a concept in accordance with nonmolecular evidence (e.g., Cronquist 1981; Eckardt 1976; Mabry 1977; Rodman et al. 1984).

The chloroplast gene *rpl*16, encoding the ribosomal protein L16, is interrupted by an intron in *Spinacia* (Zhou et al., unpubl. data) and most other land plants examined (Hiratsuka et al. 1989; McLaughlin and Larrinua 1987; Ohyama et al. 1986; Shinozaki et al. 1986; Downie and Palmer, unpubl. data). We report here, however, that the *rpl*16 intron is absent from the chloroplast genome of *Limonium gmelinii* (Plumbaginaceae). A 528 bp *Eco*RI fragment from *Nicotiana tabacum* (coordinates 84087–84615 in Shinozaki et al. 1986) was used to test for the presence or absence of the intron. Adjacent probes (a 280 bp *Bam*HI-*Eco*RI fragment, coordinates 83807–84087, and a 635 bp *Eco*RI-*Xba*I fragment, coordinates 84615–85250) were used to test for the presence of *rpl*16 exon sequences and their linkage to flanking genes. Using this method, the intron was determined to be present in all other examined members of Caryophyllidae. Loss of this intron is otherwise known only from Geraniaceae (Downie, Logsdon, and Palmer, unpubl. data) and must have occurred independently in these two distantly related dicot groups. Additional study should indicate whether the loss of the *rpl*16 intron in Plumbaginaceae cpDNA circumscribes taxa at familial or infrafamilial levels.

INVERSIONS. Inversions in the chloroplast genomes of *Atriplex hastata* and *Chenopodium murale* (Chenopodiaceae) and *Pereskia grandiflora* (Cactaceae) relative to *Nicotiana tabacum* were detected. The locations of these inversion endpoints, as determined by filter hybridizations, lie in the large single-copy region somewhere in the intergenic spacers between genes *rbc*L and *acc*D and between *trn*V and *ndh*C (Fig. 1). The inversion was revealed by the hybridization of probes 2 and 7 (Fig. 1) to the same fragment(s) for many of the restriction enzymes. Hybridization of these seven small tobacco probes to *Bam*HI and *Hin*dIII digests of *Atriplex*, *Chenopodium*, and *Pereskia* cpDNA's and the construction of restriction site maps in this region for these two enzymes indicate that the inversion is approximately 6 kb in size.

When Chenopodiaceae the inversion was de-

tected in only two of five genera examined. The chloroplast genomes of *Beta*, *Kochia*, and *Spinacia* do not possess the inversion and are colinear with that of *Nicotiana tabacum*. Although the subfamilial taxonomy of Chenopodiaceae is controversial (Blackwell 1977; Cronquist 1981; Williams and Ford-Lloyd 1974), *Atriplex* and *Spinacia* have been either treated together in the same subtribe (e.g., Ulbrich 1934) or with *Chenopodium* in the same tribe (e.g., Eckardt 1964). On the basis of trichome characters, *Atriplex*, *Spinacia* and *Chenopodium* are closely related (Carolin 1983). *Beta* and *Kochia*, more diverse anatomically and morphologically, are placed in separate tribes (Ulbrich 1934; Eckardt 1964). The common possession of the inversion in *Atriplex* and *Chenopodium* suggests that these two genera may be more closely related to each other than either is to *Spinacia* (or to any of the other genera examined). The distribution of the inversion in Cactaceae apparently circumscribes the entire family, because the inversion has recently been detected in representative taxa from all three subfamilies including all currently recognized tribes within subfamily Cactoideae (R. Wallace, pers. comm.).

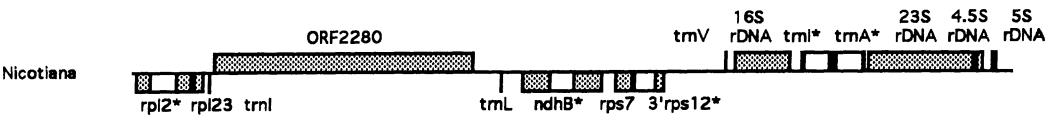
Sequencing the regions of the inversion endpoints in *Atriplex*, *Chenopodium* and *Pereskia* should show whether the two inversions occurred at the same or slightly different breakpoints. Phylogenetic analyses of nonmolecular characters (Rodman 1994; Rodman et al. 1984), *rbc*L sequences (Rettig et al. 1992), and cpDNA IR restriction sites (described below) show that Chenopodiaceae are monophyletic and are not closely related to Cactaceae. Therefore, regardless of how similar its endpoints are revealed to be by DNA sequencing, the inversion almost certainly occurred twice. Since at current levels of resolution these two inversions appear to be the same mutation, they are thus homoplastic. This, along with recent work by Hoot and Palmer (1994) on Ranunculaceae, is the first reported example of homoplasy in a cpDNA inversion.

RESTRICTION FRAGMENT LENGTH VARIANTS. Three major length variants (other than the loss of the *rpl*2 intron discussed above), ranging in size from 200 to 500 bp, were detected in the IR sequences of nine taxa. These variants were repeatedly seen in the restriction fragment arrays produced by different restriction enzymes, and can be classified as deletions based on outgroup comparisons. All deletions occur within the region identified as ORF2280 in Fig. 2. This gene is known to be absent from *Oryza sativa* cpDNA (Hiratsuka et al. 1989), and major deletions within the gene are known in several other taxa (Downie and Palmer 1992b; Downie et al. 1994; Kellogg 1992; Manos et al. 1993; Zhou et al. 1988). Because length variants less than 200 bp in size could not be detected on our gel systems, we have greatly underestimated the actual extent of restriction fragment length variation in the species examined. The absence of any other detectable length variant elsewhere in the IR, particularly in the intergenic spacer regions that account for 23% of this region in *Nicotiana tabacum* (Shinozaki et al. 1986), is surprising and similar to what we observed in Asteridae cpDNA (Downie and Palmer 1992b).

Each of the three ORF2280 deletions was shared by two or more taxa and, thus, are phylogenetically informative. A 200 bp deletion unites Nyctaginaceae with Phytolaccaceae s. str. The consensus is that these two taxa are closely allied (Cronquist 1981; Manhart and Rettig 1994; Rodman et al. 1984). A 300 bp deletion serves as a synapomorphy uniting all three examined genera of Chenopodiaceae (*Atriplex* and *Kochia* were used in the structural rearrangement survey only). A 500 bp deletion unites *Pereskia* (Cactaceae) with *Portulaca* but not *Claytonia*; phylogenetic implications of this deletion are discussed below.

Restriction Site Variation. A total of 161 different restriction sites was identified using ten endonucleases and 24 taxa. This represents sequence information for 966 nucleotides, or about 3.8% of the entire IR and approximately 0.6% of the entire chloroplast genome. *Anredera* and *Tetragonia* cpDNA's were not cut with the endonuclease *Cla*I; restriction sites for this endonuclease were scored as missing data. Sixty-two (39%) restriction sites were shared by two or more taxa and were potentially informative for phylogenetic analysis; 60 (37%) of the remaining sites were unvarying, and 39 (24%) were unique to individual taxa and, therefore, provided no phylogenetic information. Altogether, 3.8% of the 62 informative characters were scored as missing data among the 24 taxa (the complete data matrix is available upon request from SRD).

Alignment of all available *Spinacia oleracea* nucleotide sequences contained within the IR



Region	rpl2 3'ex	rpl2 5'ex	rpl23	trnI	ORF 2280	trnL	trnV	trnI 5'ex	trnI in	trnI 3'ex	trnA 5'ex	trnA in	trnA 3'ex	4.5S rDNA	5S rDNA
Length (bp)	434	391	282	74	6888	81	72	37	732	35	38	825	35	103	121
Percent DNA identity	97	97	97	100	95	99	99	100	96	100	97	96	100	97	100

FIG. 3. Results of a nucleotide sequence alignment of *Nicotiana tabacum* (Shinozaki et al. 1986) and *Spinacia oleracea* (Audren and Mache 1986; Audren et al. 1987; Briat et al. 1982; Massenet et al. 1987; Zhou et al. 1987; Zurawski et al. 1984) cpDNA IR homologous sequences. The four regions being compared are indicated. Nucleotide sequence identities and the lengths of the sequences being compared are provided for 15 genes or gene portions, open reading frames of unknown function (ORFs), or introns. Percentage sequence identities for each of these regions were calculated manually by direct pairwise comparisons. Gaps were excluded from these calculations. Chloroplast DNA sequences were aligned using CLUSTAL (Higgins and Sharp 1988) and adjusted by eye.

(Audren and Mache 1986; Audren et al. 1987; Briat et al. 1982; Massenet et al. 1987; Thomas et al. 1988; Zhou et al. 1987, 1988; Zurawski et al. 1984) with homologous sequences from the IR of *Nicotiana tabacum* (Shinozaki et al. 1986) reveals that many regions are highly conserved between the two taxa (Fig. 3). Overall, a total of 14,268 base pairs or about 56% of the total length of the *N. tabacum* cpDNA IR was compared. Nucleotide sequence identities (with gaps excluded) for the various exons and introns shown in Fig. 3 ranged between 95% and 100%. Relative to *N. tabacum*, three within-gene length variants greater than 200 bp are seen in the *Spinacia* IR and correspond to: 1) the loss of the *rpl*2 intron (666 bp); 2) a 297 bp deletion near the 5′ end of ORF2131 (the *Spinacia* homolog of tobacco ORF2280); and 3) an insertion of 253 bp in the spacer region between genes ORF2131 and *trn*L. The first two length variants were detected in our comparative restriction site study and were described above; the third length variant was not detected. In the vicinity of the third length variant, an insertion ranging between 200 and 300 bp in size was seen in the restriction fragment arrays produced by the enzymes *Cla*I, *Hin*cII, *Bgl*II and *Eco*RV. For these enzymes, our maps suggest that the presence of this mutation might circumscribe all caryophyllalean taxa. This mutation was not seen, however, in the fragment arrays produced by the other six enzymes because of the occurrence in this region of large fragments for several of these enzymes. Additional analysis is therefore warranted to ascertain the distribution of this 253-bp length mutation in the Caryophyllales.

The alignment also reveals high conservation in restriction endonuclease cleavage sites. For example, 35 of the 49 sampled restriction sites for 10 enzymes in the *Spinacia oleracea* homolog of ORF2280 (i.e., ORF2131) are shared with those found in *Nicotiana tabacum* ORF2280. This conservation increases confidence in our ability to score the shared presence of a restriction site as a homologous character and in ascertaining homologous length variants. Approximately half of the 101 variable restriction sites sampled in this investigation were contained within ORF2131, a region that encompasses only 28% of the length of the IR.

Estimates of nucleotide sequence divergence (Nei and Li 1979) between selected cpDNA's are presented in Table 2. Interfamilial comparisons range from 0.3% (between *Mollugo* and *Alluaudia*) to 3.2% (between *Polygonum* and *Pereskia*), with an average of 1.9%. This range of nucleotide sequence divergence among species belonging to at least seven families of Caryophyllidae is similar to those values reported

TABLE 2. Estimated nucleotide sequence divergence of the cpDNA IR among *Polygonum* and selected species of Caryophyllales. Complete names of species and their familial placement are presented in Table 1. The upper right portion of the matrix indicates the number of IR restriction site differences between two taxa as determined by direct pairwise comparisons. Pairwise nucleotide sequence divergence estimates (Nei and Li 1979) are expressed as $100 \times p$ in the lower left portion of the matrix. The number of restriction sites examined for each species ranged from 111 to 120.

Species	*Pol*	*Spi*	*Phy*	*Sil*	*Ste*	*Mol*	*All*	*Per*
Polygonum	—	29	25	30	25	27	27	27
Spinacia	2.9	—	24	25	18	16	16	28
Phytolacca	2.6	2.1	—	23	12	14	15	23
Silene	3.0	2.1	2.0	—	16	16	17	26
Stegnosperma	2.6	1.5	0.9	1.5	—	6	7	15
Mollugo	2.7	1.4	1.1	1.4	0.5	—	5	17
Alluaudia	2.9	1.4	1.2	1.5	0.5	0.3	—	15
Pereskia	3.2	2.7	2.4	2.9	1.7	1.8	1.7	—

for several inter- and intrageneric studies in which the entire chloroplast genome or most of it was examined (e.g., Duvall and Doebley 1990; Jansen and Palmer 1988; Palmer et al. 1983; Wallace and Jansen 1990). Overall, comparative restriction site mapping of the chloroplast DNA IR region, with its low levels of molecular divergence both in restriction site and length mutations, offers much potential for resolving phylogenetic relationships within the Caryophyllales.

Phylogenetic Analysis of Structural and Inverted Repeat Restriction Site Mutations. Cladistic analysis using Wagner parsimony and the restriction site data resulted in 79 equally most parsimonious topologies requiring 163 steps (consistency index including autapomorphies = 0.62, excluding autapomorphies = 0.50; retention index = 0.68). From these, a strict consensus tree was derived (Fig. 4). A bootstrap analysis was conducted with 100 replications to provide a measure of internal support for the clades identified in the consensus tree (Fig. 4). A decay analysis, in which trees longer than the most parsimonious ones were examined, was also performed to assess the robustness of the monophyletic groups. A total of 2252 trees resulted when trees one step longer than the most parsimonious trees (i.e., 164 steps) were saved; branches in Fig. 4 that collapse at this level are indicated with asterisks. For analyses with tree lengths equal to or greater than 165 steps, more than 25,000 trees were found before the computer run was aborted due to overflow of the tree buffer. Adding the structural rearrangement data resulted in the same topology as with the restriction site data alone but with stronger (bootstrap) support for many of the clades (Fig. 4). This combined analysis produced 79 shortest trees of 170 steps, a consistency index (including autapomorphies) of 0.63, and a retention index of 0.69. Because not all species used in the IR restriction site study were surveyed for the presence of the 6-kb inversion, these taxa were subsequently examined using the seven probes illustrated in Fig. 1.

Figure 5 shows one randomly chosen most parsimonious tree to illustrate the distribution of character support (unique mutations, and homoplastic losses, gains, and reversals) for branches on the tree. Of the 105 homoplastic character-state changes required in the depicted tree, 44 (42%) involve homoplastic losses, 43 (41%) involve homoplastic gains, and 18 (17%) involve reversals.

The same four equally most parsimonious topologies resulted from character-state weighting in which gain : loss weight ratios were either 1.1:1.0, 1.2:1.0, or 1.3:1.0 (results not shown) and ANCSTATES were coded as site absences. These four trees represent a subset of the 79 most parsimonious Wagner trees. A strict consensus of these four trees, however, differs only slightly from the topology exhibited by the Wagner strict consensus tree in presenting *Claytonia* and *Anredera* as sister taxa. Ten trees each resulted when gain/loss weight ratios were 1.4:1.0 or 1.5:1.0. The strict consensus trees at each of these weights differed from the Wagner consensus tree only in the collapse of the clade

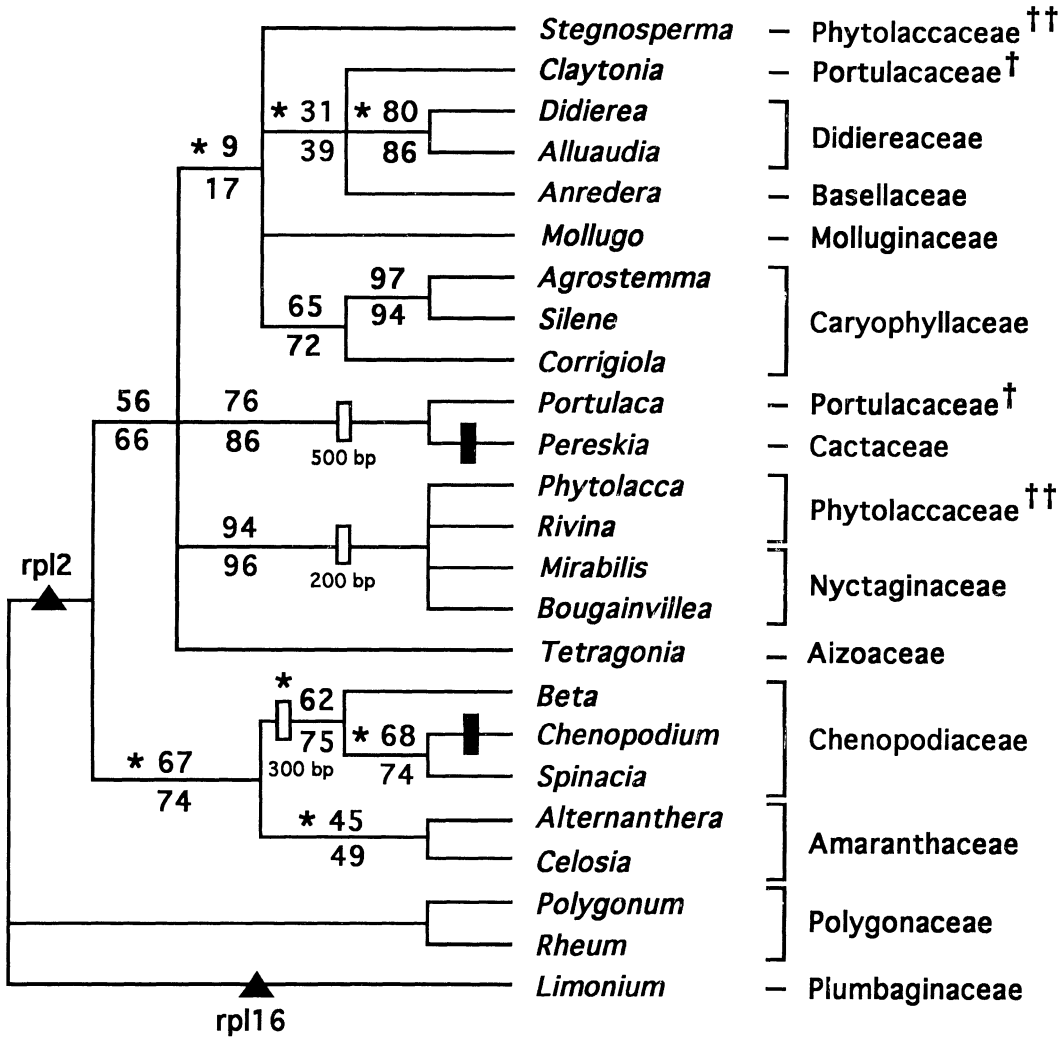


FIG. 4. Strict consensus of equally most parsimonious Wagner trees based on structural and IR restriction site variation. Numbers above the nodes indicate the number of times a monophyletic group occurred in 100 bootstrap replicates when only the restriction site data were used in the analysis. This analysis produced 79 shortest trees of length 163 steps, a consistency index (including autapomorphies) of 0.62, and a retention index of 0.68. Branches that collapsed at a tree length one step longer than these most parsimonious trees are indicated by asterisks. Decay analyses with tree lengths equal to or greater than 165 steps could not be done because of computational constraints. Numbers below the nodes indicate the number of times a monophyletic group occurred in 100 bootstrap replicates when both restriction site and structural rearrangement data were included in the analysis. This analysis produced 79 shortest trees of 170 steps, a consistency index (including autapomorphies) of 0.63 and a retention index of 0.69. Major cpDNA structural mutations are as follows: 6-kb inversion = solid bars; intron losses = triangles; deletions in ORF2280 = open bars. Single (†) and double (‡) daggers indicate the polyphyly of Portulacaceae and Phytolaccaceae, respectively.

consisting of *Stegnosperma, Claytonia,* Didiereaceae, *Anredera, Mollugo,* and Caryophyllaceae. When ANCSTATES were not specified (i.e., coded as ?'s) and character-states were weighted as 1.3:1.0 for gain : loss, eleven most parsimonious trees resulted. The strict consensus of these trees was similar to that presented in Fig. 4 except that Chenopodiaceae were nested within

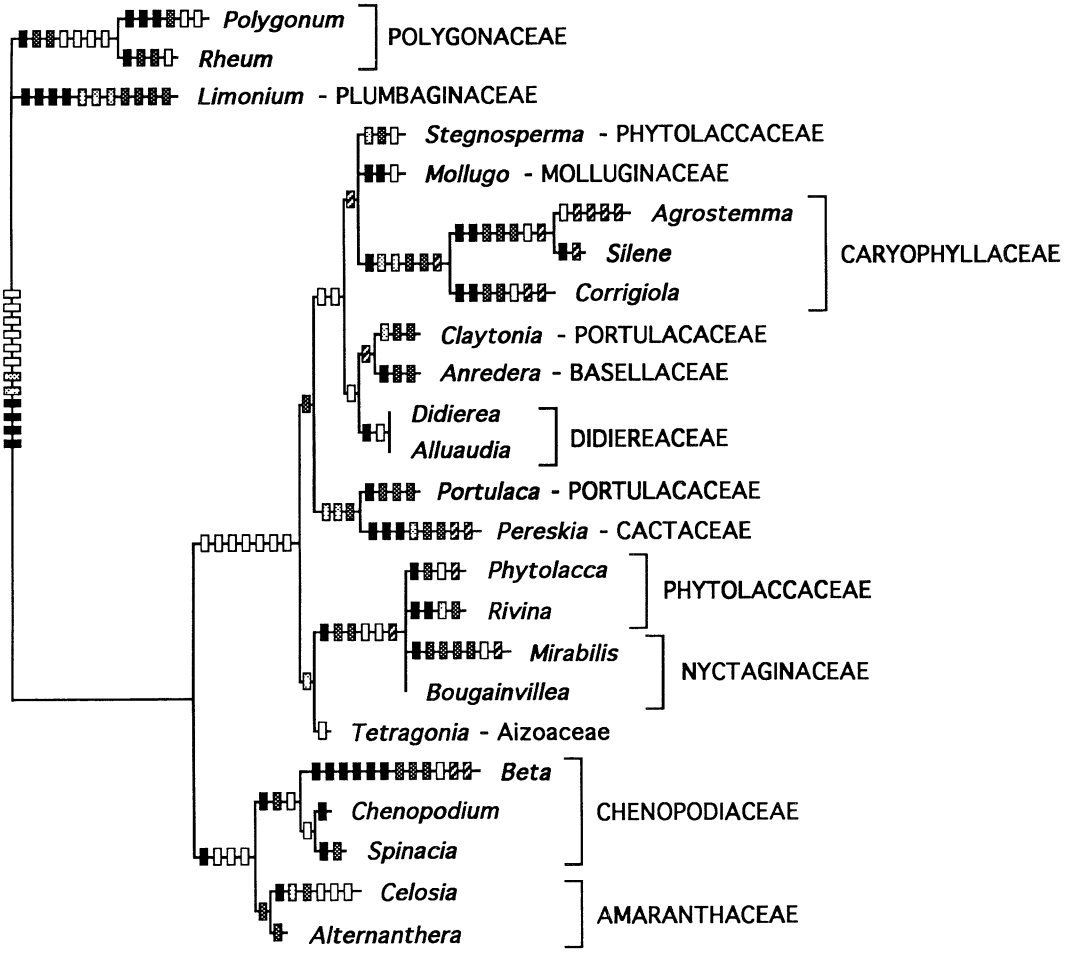


FIG. 5. One of 79 equally most parsimonious Wagner trees based on restriction site mutations in the cpDNA inverted repeat. The tree has a length of 163 steps, a consistency index of 0.62, and a retention index of 0.68. Branch lengths are proportional to the number of supporting character states. Nonhomoplastic gains = solid bars; nonhomoplastic losses = lightly shaded bars; homoplastic gains = open bars; homoplastic losses = darkly shaded bars; reversals = bars with diagonal stripes.

a paraphyletic Amaranthaceae. Character-state weighting of 1.1:1.0 or 1.2:1.0 each resulted in the same single tree that represents one of the 79 most parsimonious Wagner trees.

Phylogenetic Relationships within the Caryophyllales. Several lineages closely correspond with historical generic groupings (Cronquist 1981; Takhtajan 1980) or with relationships proposed on the basis of previous phenetic and cladistic analyses (Rodman et al. 1984), but in many cases the branching patterns among them remain unresolved. Moreover, the results presented here are generally consistent with those obtained from a recent phylogenetic analysis of *rbc*L sequence data (Fig. 6; Rettig et al. 1992). With the exception of Caryophyllaceae, the two molecularly-derived trees are quite congruent. The *rbc*L tree, however, is more fully resolved and more robust (as ascertained by the generally higher bootstrap values) than the tree constructed from structural rearrangements and restriction site mutations. This higher resolution in the *rbc*L tree is likely due to the greater number of informative characters examined. A total of 230 informative sites was obtained for 1407 bp of *rbc*L sequence compared to 62 informative sites for 966 bp of sequence sampled in this study. The consistency index (autapomorphies

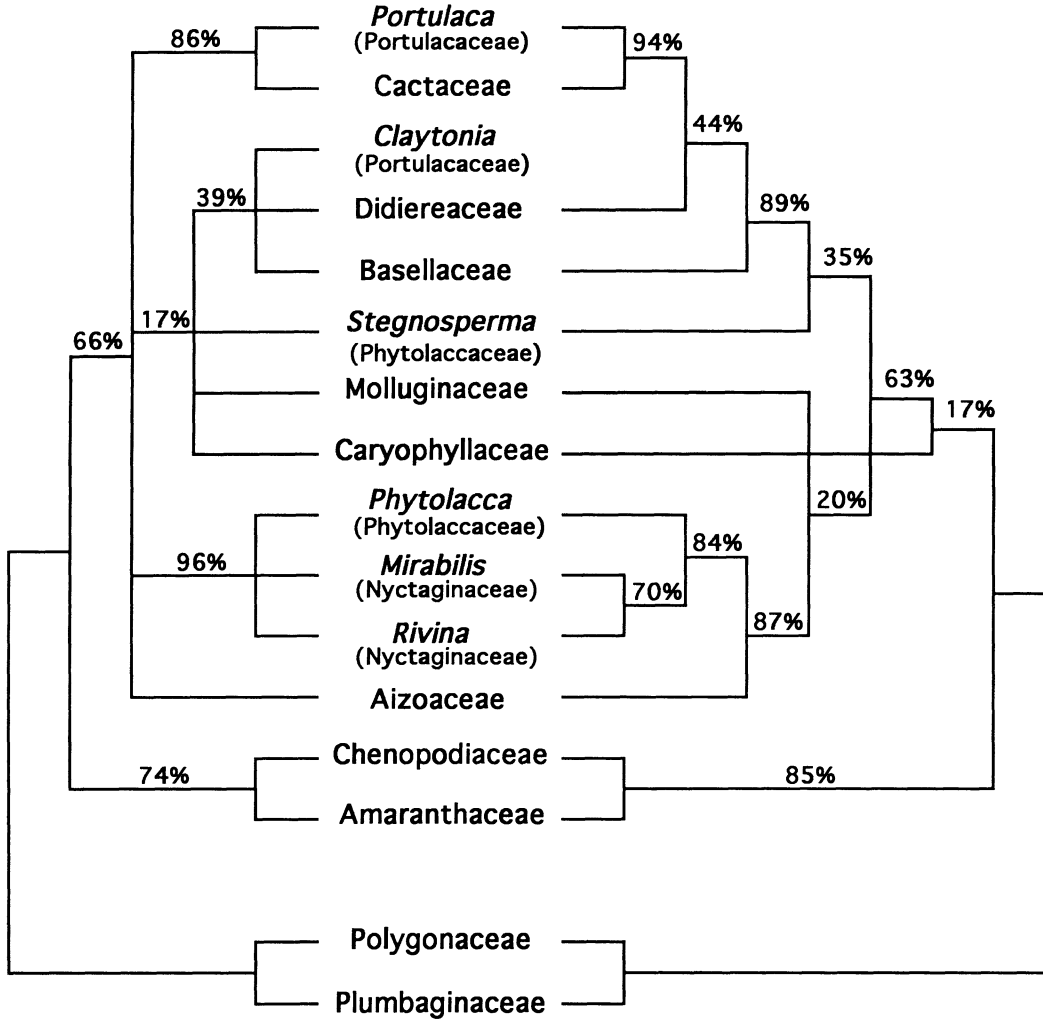


FIG. 6. Caryophyllales phylogeny based on cpDNA structural rearrangements and IR restriction site mutations (this study) compared with that of Rettig et al. (1992) based on *rbc*L sequences. Numbers above branches indicate the percentage of bootstrap replicates in which each grouping occurred. *Claytonia* was not examined in the Rettig et al. analysis.

excluded) and retention index calculated for this study were 0.50 and 0.68, respectively, and for the *rbc*L analysis were 0.52 and 0.61, respectively (Rettig et al. 1992; J. Manhart, pers. comm.). The CI is highly correlated with the number of taxa (Sanderson and Donoghue 1989). Although the number of taxa differed (21 taxa in the *rbc*L study versus the 24 taxa examined

here), both of these figures are close to the expected estimated CI's calculated from the equation provided in Sanderson and Donoghue (1989).

Both the Wagner (Fig. 4) and weighted consensus trees provide support for two major clades, a basal one consisting of Chenopodiaceae and Amaranthaceae, and another consisting of all other families. The close relationship between Chenopodiaceae and Amaranthaceae has been expressed by many on the basis of several characters, including floral morphology, a unique sieve-element plastid type, DNA-RNA hybridization data, and the common possession of pantoporate pollen (Behnke 1976; Cronquist 1981; Mabry 1976; Rodman et al. 1984; Skvarla and Nowicke 1976; Takhtajan 1980). These two families are also supported as monophyletic in the comparative *rbc*L sequence analyses of Rettig et al. (1992; Fig. 6) and Manhart and Rettig (1994); however, only in the former analysis do these families occur as basal elements of the order. The basal position of this clade is essentially the reverse of traditionally-held concepts of relationships within the order, although there is some support for their early emergence in the recent phylogenetic analysis by Rodman (1994).

All three examined members of Chenopodiaceae possess a 300-bp deletion in their cpDNA IR's; no such deletion is apparent in Amaranthaceae cpDNA. The possibility that Amaranthaceae may be nested within a paraphyletic Chenopodiaceae has been postulated by Rodman (1990) and illustrated by Carolin (1983). These molecular data, however, provide no evidence for paraphyly of the Chenopodiaceae. Nevertheless, the inclusion of additional generic representatives from these families is warranted in order to best estimate phylogenetic relationships between and within each family.

Phytolacca and *Rivina* (Phytolaccaceae) and *Mirabilis* and *Bougainvillea* (Nyctaginaceae) are supported as a distinct clade in the IR restriction site analysis and by the shared loss of a 200-bp sequence. These taxa are also closely allied in the *rbc*L analyses of Manhart and Rettig (1994), Chase et al. (1993), and Rettig et al. (1992), and in the cladistic analyses of Rodman et al. (1984) and Rodman (1994) using chemical, chromosomal, morphological, phytochemical, and anatomical data. Although the close association among these taxa is well known, most authors (e.g., Behnke 1976; Cronquist 1981; Takhtajan 1980) agree that the Nyctaginaceae are probably derived from the Phytolaccaceae. This is reflected in the *rbc*L-based phylogeny of Manhart and Rettig (1994) where Nyctaginaceae (i.e., *Mirabilis* and *Bougainvillea*) are nested within a paraphyletic Phytolaccaceae (i.e., *Phytolacca* and *Rivina*). Although Phytolaccaceae have been generally regarded as a primitive or 'basic' family within the order (Cronquist 1981, 1988; Nowicke 1975; Takhtajan 1980), cladistic analyses of molecular characters (this study and Rettig et al. 1992) indicate that at least *Phytolacca* and *Rivina* are likely derived.

The three examined genera of Caryophyllaceae also constitute a monophyletic group. Owing to the large size of Caryophyllaceae, however, it is premature to use these data to support the monophyly of the entire family. *Corrigiola*, included in either Caryophyllaceae or Molluginaceae (discussed in Gilbert 1987), falls alongside Caryophyllaceae in Fig. 4, but with very weak bootstrap support. The placement of *Corrigiola* in the Caryophyllaceae is favored by Behnke (1993) on the basis of sieve-element plastid characteristics. Caryophyllaceae and Molluginaceae, the only anthocyanin-producing taxa in the order, occur in the same portion of the consensus tree (along with several other taxa) and are not basal to the group, a position suggested by several authors (e.g., Cronquist 1981, 1988; Ehrendorfer 1976; Rodman 1990; Rodman et al. 1984). Forcing Caryophyllaceae and Molluginaceae together at the base of the Caryophyllales would involve generating trees five steps longer than the most parsimonious ones. These anthocyanin-producing taxa have generally been considered to form a distinct clade within the order (Cronquist 1981; Mabry 1977; Rodman 1990). These two families are sister groups in 28 of the 79 equally most parsimonious Wagner trees. Consequently, as few as one coupled reversal (i.e., loss of betalain synthesis and regain of anthocyanin synthesis) may be necessary to generate anthocyanin production in Molluginaceae and Caryophyllaceae from a betalain-producing ancestor. It has been suggested that the process leading to the absence of anthocyanins and presence of betalains may represent one interrelated step (likely a blockage at a terminal step in flavonoid biosynthesis), with the loss of one character influencing the appearance of another (Giannasi 1978;

Giannasi and Crawford 1986); however, the circumstances responsible for the loss of betalain synthesis and the regain of anthocyanin production are not altogether clear.

Stegnosperma, long associated with Phytolaccaceae (e.g., Cronquist 1981; Heimerl 1934), is recognized by many as belonging to its own family (Bedell 1980; Brown and Varadarajan 1985; Dahlgren 1980; Hutchinson 1973; Takhtajan 1980; Thorne 1992). In our results, *Stegnosperma* is excluded from the Phytolaccaceae-Nyctaginaceae clade. This lack of relationship is also illustrated by *rbc*L sequence data (Manhart and Rettig 1994; Rettig et al. 1992, and Fig. 6). Six additional steps are necessary to force the monophyly of *Stegnosperma, Phytolacca* and *Rivina*. *Stegnosperma* shares numerous similarities with Caryophyllaceae (Bedell 1980; Behnke 1976; Narayana and Narayana 1986). Our results place, but with very weak bootstrap support, *Stegnosperma* in the same derived portion of the consensus tree as Caryophyllaceae (and several other taxa) but are equivocal in determining sister group relationships. *Stegnosperma, Mollugo*, and the three examined members of Caryophyllaceae constitute a monophyletic group in 11 of the 79 shortest Wagner trees.

A recent survey of foliar structure, particularly vasculature and epidermal patterning, in the Caryophyllales (Hershkovitz 1991b, and unpubl. data) has indicated that the families Basellaceae, Cactaceae, and Didieriaceae are internested among eastern American/African Portulacaceae. The possible paraphyly of Portulacaceae has also been suggested by Rodman (1990). While our sample size is too small to adequately address this issue of paraphyly and the relationships among these succulent centrospermous families, it does indicate that Portulacaceae are not monophyletic. *Claytonia* and *Portulaca* fall out in different portions of the consensus tree, with the latter allied with *Pereskia* (Cactaceae). A tree of four more steps than the most parsimonious trees was needed to force the monophyly of Portulacaceae. *Pereskia* and *Portulaca* share a 500-bp deletion that is absent in *Claytonia*. The common possession of this deletion and the moderately high bootstrap value supporting the clade obtained from the restriction site data suggest that these two taxa are more closely related to each other than either is to any of the other genera examined. Cladistic analyses of *rbc*L sequences treat *Portulaca* and *Schlumbergera* (Cactaceae) as sister taxa (Rettig et al. 1992) or *Portulaca* and a clade consisting of *Pereskia* and *Schlumbergera* as sister taxa (Manhart and Rettig 1994). A *rbc*L sequence for *Claytonia* is not yet available. Moreover, a cladistic analysis of Portulacaceae foliar data (Hershkovitz 1991b, and unpubl. data) reveals that Cactaceae (and Basellaceae and Didiereaceae) are nested among eastern American and southern African Portulacaceae (which includes *Portulaca* but not *Claytonia*). The shared presence of pantocolpate pollen in *Portulaca* and *Pereskia* (Nowicke 1994) also supports this relationship, although this character can be found in several other centrospermous families. Pending the inclusion of additional taxa and a more comprehensive sampling of restriction sites, the positions of *Portulaca, Claytonia, Pereskia*, and related families will remain uncertain.

The results presented here represent an initial attempt to formulate more precise hypotheses about relationships within the Caryophyllales. The phylogenetic results presented here, however, are best regarded as preliminary in the sense that the number of taxa sampled is small and does not best represent the diversity found within the order. Nevertheless, they do provide a set of explicit hypotheses about relationships in the Caryophyllales that can be tested as the data set is enlarged and more evidence, both molecular and nonmolecular, become available for comparative analysis.

ACKNOWLEDGMENTS. Financial support for this study was provided by National Science Foundation grant BSR-8717600 to JDP and as laboratory start-up funds from the University of Illinois to SRD. We thank the Missouri Botanical Garden, the W. J. Beal Botanical Garden (Michigan), Matthaei Botanical Garden (Michigan), and the Brooklyn Botanic Garden (New York) for generously providing us with leaf material used in this study. We also thank Daniel J. Crawford and an anonymous reviewer for helpful comments on the manuscript.

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