# 8 Phylogenetic Relationships Using Restriction Site Variation of the Chloroplast DNA Inverted Repeat

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## 8.1 Introduction

Despite intensive multidisciplinary studies using nonmolecular characters, relationships among families comprising the Caryophyllales remain unclear. Reconstruction of phylogenies from molecular data has become an increasingly common approach in systematics and has often provided valuable insight into historical relationships. Of the three genomes present in plants, the chloroplast genome is now the most widely used for phylogenetic inference.

The chloroplast genomes of photosynthetic land plants are circular DNA molecules ranging in size from 120 to 217 kilobase pairs (kb) (Palmer 1985). Complete restriction maps are available for several species of Caryophyllidae; their chloroplast genomes range between 147 and 158 kb (Palmer 1982, 1985; Downie and Palmer 1992a). Chloroplast genomes contain, with few exceptions, two duplicate regions in reverse orientation known as the inverted repeat (IR). In a typical angiosperm chloroplast genome of 150 kb, each of the two IR copies is about 25 kb. These repeated regions separate the remainder of the molecule into large single-copy and small single-copy regions (Fig. 8.1). The expansion or contraction of the IR into, or out of, adjacent single-copy regions, and changes in sequence complexity due to insertion or deletion of unique sequences are largely responsible for variation in size of the molecule.

Recent studies of chloroplast DNA (cpDNA) genome evolution have revealed a high degree of conservatism in size, structure, primary sequence, gene content, and linear order of genes among major lineages of land plants (Palmer 1985, 1991; Palmer and Stein 1986; Downie and Palmer 1992a). This conservative mode of cpDNA evolution suggests that any change in primary sequence, structure, arrangement, or content of the chloroplast genome may have significant phylogenetic implications.

Mutations in cpDNA are of two general kinds: point mutations (single nucleotide pair substitutions) and structural rearrangements. Point mutations can be detected either indirectly, through restriction site mapping (when mutations occur within restriction endonuclease recognition sites), or directly,

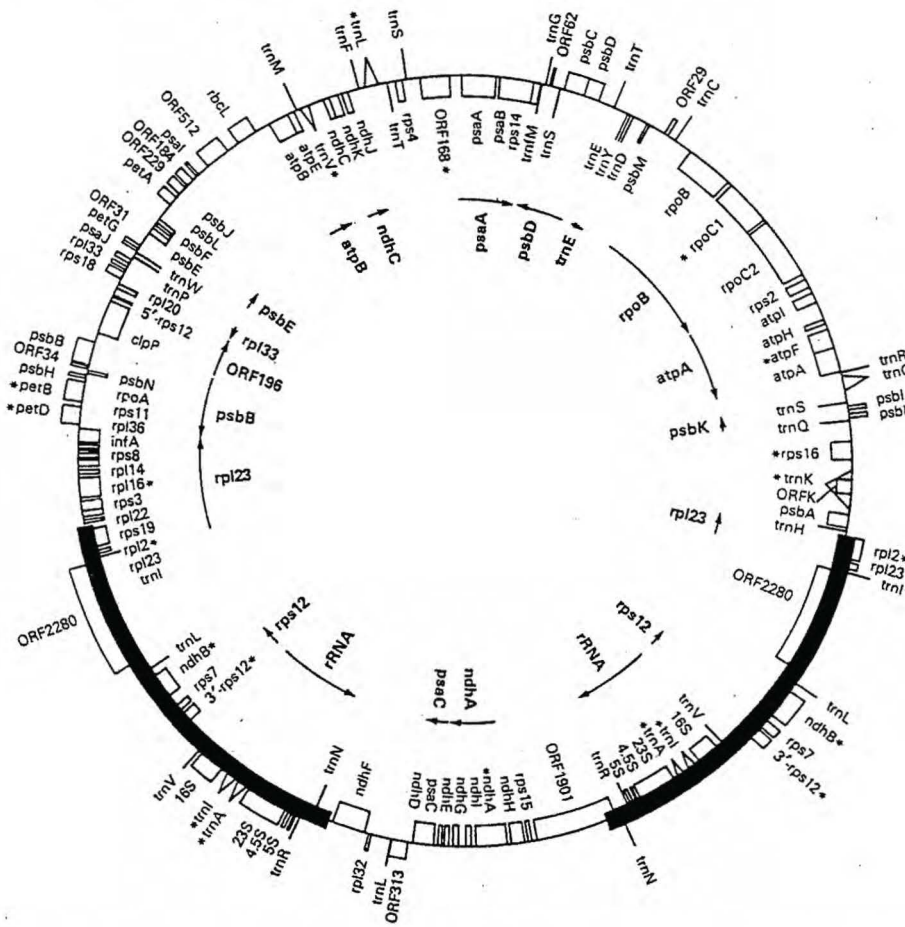


**Fig. 8.1.** Physical and gene map of the 156-kb *Nicotiana tabacum* chloroplast genome showing the densely packed arrangement of genes and open reading frames (ORFs). The genome is organized into large (87 kb) and small (18 kb) single-copy regions separated by two duplicate regions (about 25 kb each) in reverse orientation, known as the inverted repeat (indicated by the *thickened parts* of the circle). Genes transcribed clockwise are shown on the *inside* of the circle; those transcribed in the reverse direction are on the *outside*. *Arrows* on the inside of the circle indicate sets of genes thought to constitute operons; the operon names are indicated. *Asterisks* denote genes that contain introns

by DNA sequencing. Analyses of restriction site polymorphisms in cpDNA have almost invariably elucidated relationships among taxa at the rank of family or below (reviewed in Palmer et al. 1988). Recently, however, we have shown that by focusing on the highly conserved IR region, restriction site comparisons can be usefully extended to questions of relationships among families within a subclass (Downie and Palmer 1992b). Phylogenetic analyses of *rbc*L sequence data have been extensively used to examine relationships among several major lineages of angiosperms, including the

Caryophyllidae (Giannasi et al. 1992; Olmstead et al. 1992; Rettig et al. 1992; Chase et al. 1993), and will be discussed elsewhere (see Chap. 9). Structural rearrangements of the chloroplast genome (such as inversions and major deletions) are relatively infrequent events among photosynthetic land plants and can usually provide strong evidence of monophyly for a particular group (reviewed in Downie and Palmer 1992a).

In this chapter we present a preliminary analysis of the phylogenetic relationships of several caryophyllalean taxa based on restriction site variation in the highly conserved IR region of the chloroplast chromosome. This information ultimately will be synthesized with a second analysis, currently underway, with an emphasis on major genomic structural rearrangements, to identify the major lineages within the order.

## 8.2 Materials and Methods

Material of 24 species (Table 8.1), representing 13 families of Caryophyllales plus Polygonaceae and Plumbaginaceae, was field-collected or obtained from various sources as fresh leaf material. The isolation of cpDNA or total

**Table 8.1.** Species of Caryophyllidae examined for cpDNA IR restriction site and structural variation. A list of sources and voucher information for all taxa examined herein is available upon request

| | |
|---|---|
| Caryophyllales | Nyctaginaceae |
| Aizoaceae | *Bougainvillea glabra* |
| *Tetragonia tetragonioides* | *Mirabilis nyctaginea* |
| Amaranthaceae | Petiveriaceae |
| *Alternanthera dentata* | *Rivina humilis* |
| *Celosia plumosa* | Phytolaccaceae |
| Basellaceae | *Phytolacca heterotepela* |
| *Anredera cordifolia* | Portulacaceae |
| Cactaceae | *Claytonia perfoliata* |
| *Pereskia grandiflora* | *Portulaca oleracea* |
| Caryophyllaceae | Stegnospermataceae |
| *Agrostemma githago* | *Stegnosperma halimifolium* |
| *Corrigiola litoralis* | |
| *Silene schafta* | Polygonales |
| Chenopodiaceae | Polygonaceae |
| *Beta vulgaris* | *Rheum rhaponticum* |
| *Chenopodium murale* | *Polygonum persicaria* |
| *Spinacia oleracea* | Plumbaginales |
| Didiereaceae | |
| *Alluaudia montagnacii* | Plumbaginaceae |
| *Didierea madagascariensis* | *Limonium gmelinii* |
| Molluginaceae | |
| *Mollugo verticillata* | |

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 performed according to Palmer (1986) and Downie and Palmer (1992a). All DNAs were digested singly with each of ten restriction endonucleases: *Ava*I, *Bam*HI, *Ban*II, *Bcl*I, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hinc*II, and *Hin*dIII. Nineteen subclones from the cpDNA IR region of *Nicotiana tabacum* were used as hybridization probes to survey for restriction site variation. These probes ranged in size from 0.2 to 3.3 kb, averaging approximately 1 kb. This list of probes is available upon request. The conservative nature of land plant chloroplast genome structure and primary sequence allows the use of these hybridization probes across divergent subclasses of angiosperms.

Unambiguous restriction site maps for each of the ten endonucleases were constructed for the *N. tabacum* IR 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.

To assess the circumscription and possible monophyly of the Caryophyllales, three representatives of Polygonaceae and Plumbaginaceae were chosen as outgroups (Table 8.1). Among current classification systems, a consensus favors an association between the Caryophyllales and these two families. These two families are clearly excluded from the order, and several authors have even suggested that there is no strong evidence linking Polygonaceae and Plumbaginaceae to the Caryophyllales (e.g., Rodman et al. 1984; Giannasi et al. 1992). However, results from recent phylogenetic analyses of *rbc*L sequence data (Olmstead et al. 1992; Chase et al. 1993) strongly support the monophyly of the Caryophyllales and indicate that the Polygonaceae and Plumbaginaceae are their most appropriate outgroup.

## 8.3 Results and Discussion

The cpDNA IR sequences for each of 24 species (Table 8.1) are, with few exceptions, similar in structure and readily aligned with that of *N. tabacum* (and, thereby, also with the IRs of the majority of angiosperms so far examined). Differences in structure that were apparent included the previously documented loss of the *rpl*2 intron (Downie et al. 1991) and partial deletions of coding sequences within the gene ORF2280. We have greatly underestimated the actual extent of restriction fragment length variation in the species examined because: (1) most cpDNA length mutations (for the entire genome) are between 1 and 10 bp in size (Palmer 1985); (2) length variants less than 150 bp could not be detected on our gel systems; and (3)

we could only deal with the IR region of the genome because, at the interfamilial level, little or no alignment of restriction sites was possible in single-copy regions. With the exception of the *rpl*2 intron loss, which is discussed below, phylogenetic implications of the remaining deletions are discussed elsewhere (S. Downie and J. Palmer, unpubl. data).

### 8.3.1 *rpl*2 Intron Loss

Introns (intervening noncoding sequences within gene coding regions) are highly stable components of land plant chloroplast genomes, with no cases of intron gains and few cases of intron losses known during land plant evolution (Palmer 1991). Plant cpDNAs contain approximately 20 introns (Fig. 8.1), most, if not all, of which were present in the common ancestor of land plants (Palmer 1991).

The chloroplast gene *rpl*2, encoding the ribosomal protein L2, is of systematic interest because it is known to be interrupted by a single intron in most, but not all, land plants. DNA sequencing first revealed that this chloroplast intron is present in *Nicotiana debneyi* (Solanaceae) but absent from *Spinacia oleracea* (Chenopodiaceae) (Fig. 8.2; Zurawski et al. 1984). Utilizing an *rpl*2 intron-specific probe (Fig. 8.2), subsequent investigations revealed that this intron is absent from the chloroplast genomes of all examined taxa (ten families and 19 species) of Caryophyllales, yet present in cpDNAs of *Limonium gmelinii* (Plumbaginaceae) and three genera of Polygonaceae (*Polygonum*, *Rheum*, and *Rumex*) (Downie et al. 1991). Sequencing of the *rpl*2 gene in five genera of the Caryophyllales and in *Rumex* not only confirmed the filter hybridization results, but also showed that for all taxa lacking the intron, the *rpl*2 gene has undergone a precise

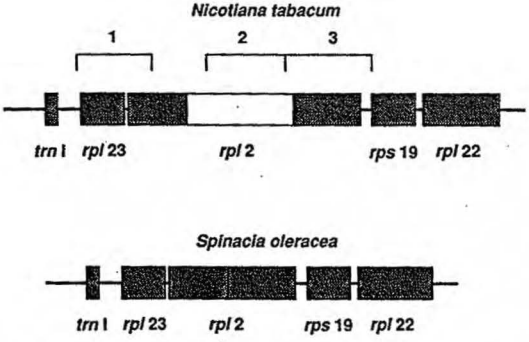


**Fig. 8.2.** Structural organization of the *rpl*2 gene and flanking regions in *Nicotiana tabacum* and *Spinacia oleracea* cpDNAs. Coding regions are indicated by *shaded boxes*; the *rpl*2 intron is indicated by an *open box*. The numbered *brackets* indicate the hybridization probes used to determine the presence or absence of coding regions (*1* and *3*) and the intron (*2*). Probe descriptions are presented in Downie et al. (1991)

deletion of the intron (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., Eckardt 1976; Ehrendorfer 1976; Mabry 1977; Cronquist 1981; Rodman et al. 1984).

The relationships between Polygonaceae and Plumbaginaceae, and between these two families and the Caryophyllales, are not wholly clear (Nowicke and Skvarla 1977; Rodman et al. 1984; Giannasi et al. 1992). The presence of the chloroplast *rpl2* intron in Polygonaceae and Plumbaginaceae further distinguishes them from the Caryophyllales but provides no information with respect to the controversy concerning closeness of relationships among these three groups.

### 8.3.2 Phylogenetic Analysis of Inverted Repeat Restriction Site Mutations

A total of 161 different restriction sites was identified using ten endonucleases among the 24 taxa included in the phylogenetic analysis. Of these 62 (39%) were shared by two or more taxa and were 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. The occurrence of many invariant restriction sites and the ability to identify readily homologous sites across 14 families (including *N. tabacum*) is notable, and similar to what we observed across widely divergent families in a previous investigation of the Asteridae (Downie and Palmer 1992b).

Cladistic analysis using Wagner parsimony (Swofford 1990) resulted in 79 equally most-parsimonious topologies requiring 163 steps (consistency index including autapomorphies = 0.62; excluding autapomorphies = 0.50). From these, a strict consensus tree was derived (Fig. 8.3). A bootstrap analysis was conducted with 100 replications to provide a measure of internal support for the clades identified in the consensus tree (Fig. 8.3).

Wagner parsimony (which permits an individual restriction site to be gained or lost with equal weight) is thought not to provide a biologically accurate model of character-state transformation given that, at any one position, the probability of a restriction site loss is much greater than a site gain. Character-state weighted parsimony, where parallel gains of a restriction site are permitted but biased against, is believed to be a more biologically sound method of analysis (detailed in Albert et al. 1992a). Four equally most-parsimonious trees resulted from the character-state weighted parsimony analysis (where gain/loss weights ranged from 1.1:1.0 to 1.3:1.0). These trees did not, however, offer any greater resolution among the taxa than those obtained from the Wagner parsimony analysis and, thus, are not presented.

Results of the phylogenetic analysis show that the order is split into two major clades, one consisting of a basal Chenopodiaceae and Amaranthaceae,

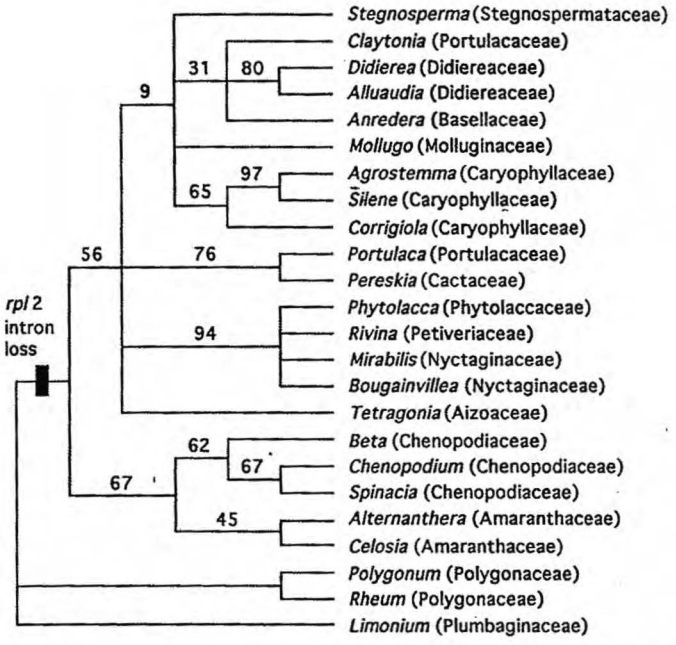


**Fig. 8.3.** Strict consensus of equally most-parsimonious Wagner trees based on cpDNA IR restriction site mutations. *Numbers* above nodes indicate the number of times that a monophyletic group occurred in 100 bootstrap replicates. This analysis produced 79 shortest trees of 163 steps and a consistency index of 0.62. The *rpl*2 intron character was not used in the phylogenetic analysis, but was subsequently mapped onto the consensus tree

and the other consisting of all remaining families. The close relationship between Chenopodiaceae and Amaranthaceae has been expressed by many. On the basis of this limited sampling, both Chenopodiaceae and Amaranthaceae appear to be monophyletic.

*Phytolacca* (Phytolaccaceae), *Rivina* (Petiveriaceae), and *Mirabilis* and *Bougainvillea* (Nyctaginaceae) are strongly supported as a distinct clade. The consensus is that Nyctaginaceae and Phytolaccaceae sensu stricto are closely allied (Cronquist 1981; Rodman et al. 1984; Rettig et al. 1992; see also Chap. 9). *Stegnosperma*, long associated with Phytolaccaceae (e.g., Heimerl 1934; Cronquist 1981), is recognized by many as belonging to its own family (Stegnospermataceae; Hutchinson 1973; Dahlgren 1980; Bedell 1980; Takhtajan 1980; Brown and Varadarajan 1985). In our results, *Stegnosperma* is clearly excluded from the Phytolaccaceae–Nyctaginaceae clade. *Stegnosperma* shares numerous similarities with Caryophyllaceae (Behnke 1976; Bedell 1980; 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.

The data strongly 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). The moderately high (76%) bootstrap value supporting this clade suggests that these two taxa are more closely related to each other than either is to any of the other genera examined.

Caryophyllaceae and Molluginaceae, the only anthocyanin-producing taxa in the order, occur in the same portion of the consensus tree and are not basal to the group. These two families form a monophyletic group 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.

Several lineages that are indicated in the cladogram (Fig. 8.3) exhibit a close correspondence with currently recognized families, but, in many cases, the branching patterns among them remain unresolved. Of the seven families for which more than one species was examined, and on the basis of this limited sampling, five (Polygonaceae, Amaranthaceae, Chenopodiaceae, Caryophyllaceae, and Didiereaceae) constitute monophyletic groups; one (Portulacaceae) appears polyphyletic; and one (Nyctaginaceae) is unresolved with the data at hand.

### 8.3.3 *Nepenthes* and the Caryophyllales

Results from a recent cladistic analysis of *rbc*L sequence data (Albert et al. 1992b; Chase et al. 1993) indicate that *Nepenthes* (Nepenthaceae) may be surprisingly closely allied to the Caryophyllales. As part of an ongoing investigation to look for cpDNA structural changes in angiosperms (S. Downie and J. Palmer, unpubl. data), mapping data were available for *Nepenthes alata*. However, because its cpDNA was digested with only four restriction endonucleases (*Bam*HI, *Eco*RV, *Bgl*II, and *Hin*dIII), and not the ten used in the comparative IR restriction site survey, it was not used in the cladistic analysis.

Our results indicate that the cpDNA IR of *Nepenthes alata* is both colinear in gene arrangement and readily alignable with that of *Nicotiana tabacum* (and, thereby, also with the IR of all other Caryophyllales examined). Comparison of all maps revealed low levels of restriction site divergence. For example, of 41 restriction sites compared for four restriction endonucleases, *Nepenthes* and *Bougainvillea* (Nyctaginaceae) differed by only five sites. Pairwise nucleotide sequence divergence estimates of cpDNA IR sequences (expressed as $100 \times p$; Nei and Li 1979) between *Nepenthes* and *Bougainvillea*, *Nepenthes* and *Chenopodium*, and *Bougainvillea* and

*Chenopodium* were 1.1, 2.1, and 2.3%, respectively. The high sequence similarity between *Nepenthes* and members of the Caryophyllales is highly intriguing and generally supportive of the *rbc*L results (Chase et al. 1993).

*Rbc*L sequence data show both *Drosera* and *Nepenthes* to be closely allied to Polygonaceae, Plumbaginaceae, and the Caryophyllales (Albert et al. 1992b; Chase et al. 1993). Filter hybridizations revealed that the chloroplast *rpl*2 intron is absent from *Drosera filiformis* and from all examined members of the Caryophyllales, but present in *Nepenthes alata* (Downie et al. 1991). Whether the loss of the *rpl*2 intron occurred in parallel in *Drosera* and in the Caryophyllales or in a common ancestor shared by these two groups (but not *Nepenthes*) is an interesting problem that deserves further study.

## 8.4 Conclusions

The data presented here represent an initial analysis of Caryophyllales phylogeny using cpDNA IR restriction site characters. The lack of resolution in many portions of the consensus tree is due to an insufficient number of characters. Future analyses should, therefore, include additional restriction endonucleases to increase the number of informative restriction sites sampled. Moreover, because the current sampling is rather limited, further analyses would also benefit by the inclusion of additional taxa, particularly those representing problematic families such as Portulacaceae, Phytolaccaceae, Molluginaceae, and Caryophyllaceae. Nevertheless, our results provide some explicit hypotheses about relationships in the Caryophyllales that can be tested as more evidence becomes available.

Phylogenetic analyses of cpDNA IR restriction site variation and the distribution of structural rearrangements provide a means of reassessing the traditional classifications of the Caryophyllales. Phylogenetic relationships based on these molecular data should help to assess the relative importance of nonmolecular characters (e.g., morphological, anatomical, ultrastructural, phytochemical, and palynological) currently used in delimiting taxa within the order. The possible polyphyly of Portulacaceae, the putative sister-group relationship between *Portulaca* and *Pereskia*, and the close relationship of *Nepenthes* and *Drosera* to the Caryophyllales suggest that a reevaluation of these nonmolecular characters is in order. Similarly, the molecular data, including those derived from *rbc*L sequencing, need to be scrutinized carefully for various biological factors that may influence the assumptions of phylogenetic analysis (reviewed in Doyle 1992). One approach of phylogenetic estimation that should be considered in future analyses is the integration of molecular with nonmolecular data. This might offer the best hope in resolving evolutionary issues within the order.

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