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SIX INDEPENDENT LOSSES OF THE CHLOROPLAST DNA *rpl*2 INTRON IN DICOTYLEDONS: MOLECULAR AND PHYLOGENETIC IMPLICATIONS

STEPHEN R. DOWNIE,[1,6] RICHARD G. OLMSTEAD,[1,5] GERARD ZURAWSKI,[2]
DOUGLAS E. SOLTIS,[3] PAMELA S. SOLTIS,[3] JOHN C. WATSON,[4] AND JEFFREY D. PALMER[1]
[1]*Department of Biology, Indiana University, Bloomington, IN 47405 USA*
[2] *Department of Molecular Biology, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304 USA*
[3] *Department of Botany, Washington State University, Pullman, WA 99164 USA*
[4] *Department of Botany, University of Maryland, College Park, MD 20742 USA*

Abstract.—Previous studies have shown that in several angiosperms and the liverwort *Marchantia* the chloroplast gene *rpl*2, encoding ribosomal protein L2, is interrupted by an intron, but that in spinach (*Spinacia oleracea*, Caryophyllales) this intron has been lost. We have determined the distribution of the *rpl*2 intron for 390 species representing 116 angiosperm families. Filter hybridizations reveal that the intron is absent from the chloroplast genomes of all examined families of the Caryophyllales, suggesting that the intron was lost in the common ancestor of the order. Sequencing of the *rpl*2 gene in five genera of the Caryophyllales and in *Rumex* (Polygonales) not only confirms the filter hybridization results, but also shows that for all taxa lacking the intron, the *rpl*2 gene has undergone a precise deletion of the intron. In all cases, it is the original *rpl*2 gene that has sustained loss of its intron. This implies that in chloroplast DNA, integration of exogenous genes (e.g., a reverse transcript of a spliced mRNA) occurs mainly by homologous, replacement recombination, rather than by illegitimate recombination elsewhere in the genome. Filter hybridizations also reveal that the *rpl*2 intron was lost independently in the common ancestors of at least five other lineages of dicotyledons: Saxifragaceae (*s.s.*), Convolvulaceae (including *Cuscuta*), Menyanthaceae, two genera of Geraniaceae, and one genus of Droseraceae. The molecular and phylogenetic implications of these independent intron losses are discussed.

Key words.—Chloroplast DNA, dicotyledons, molecular systematics, phylogeny, *rpl*2 intron, structural rearrangement.

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Chloroplast genomes of land plants are evolutionarily conserved, varying little in gene content, gene order, structure, and size (Palmer, 1985, 1991). Because of their rarity, major structural mutations of the chloroplast genome (such as inversions, insertions or deletions of genes and introns, and the loss of one copy of the inverted repeat) usually can provide strong evidence of monophyly for a particular group of plants (see reviews in Palmer et al., 1988*a*, and Downie and Palmer, 1991). Previous studies have demonstrated the utility of chloroplast DNA (cpDNA) rearrangements as molecular characters for elucidating evolutionary relationships among taxa at various levels (Jansen and Palmer, 1987; Palmer et al., 1988*a*, 1988*b*; Bruneau et al., 1990; Lavin et al., 1990).

The chloroplast gene *rpl*2, encoding the large subunit 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) (Zurawski et al., 1984). Subsequent investigations revealed that this intron is present in cpDNAs of each of six other land plants examined, including two dicots, three monocots, and the liverwort *Marchantia polymorpha* (Ohyama et al., 1986; Posno et al., 1986; Shinozaki et al., 1986*a*; Larrinua and McLaughlin, 1987; Moon and Wu, 1988; Spielmann et al., 1988). The homology of the intron in these six land plants is indicated by its conserved position within the gene and its highly conserved primary sequence. Thus, it is clear that the intron was present in the common

[5] Present Address: Department of Environmental, Population and Organismal Biology, University of Colorado, Boulder, CO 80309 USA.
[6] To whom correspondence should be addressed.

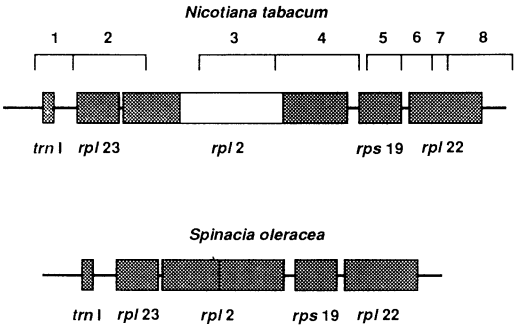

FIG. 1. Structural organization of the *rpl*2 gene and flanking regions in *Nicotiana tabacum* and *Spinacia oleracea* cpDNAs. Coding regions are indicated by shaded boxes; the *rpl*2 intron is indicated by an open box. The numbered square brackets indicate the hybridization probes used in this survey (see Table 1 for probe description).

ancestor of angiosperms, and probably land plants, and subsequently lost in a lineage leading to *Spinacia oleracea.*

Here we present the results of DNA sequencing and a large scale filter hybridization survey to determine the taxonomic distribution of this rare chloroplast DNA structural mutation. We report that the *rpl*2 intron has been lost at least six times independently in dicotyledons and we discuss the molecular and phylogenetic implications of these losses.

MATERIALS AND METHODS

A total of 390 species from 116 angiosperm families, comprising members of all subclasses of monocotyledons and dicotyledons, were surveyed for the presence or absence of the *rpl*2 intron (Appendix 1). Voucher information is available from the senior author upon request.

The isolation of cpDNA or total cellular DNA from fresh leaf material was accomplished using the sucrose gradient technique of Palmer (1986) or the modified CTAB procedure of Doyle and Doyle (1987), respectively. For the Geraniaceae, a modification of the sucrose gradient procedure was used (Calie and Hughes, 1987). All DNAs, except those of the Saxifragaceae (which were prepared and analyzed in the laboratory of D. Soltis and P. Soltis), were further purified by centrifugation in cesium chloride/ethidium bromide gradients.

TABLE 1. Characteristics of *Nicotiana tabacum* cpDNA fragments used as hybridization probes.

Number	Fragment[a] (bp)	Coordinates[b]
1	243 *Cla*I/*Bam*HI	88535–88778
2	494 *Cla*I	88041–88535
3	520 *Hin*dIII/*Pst*I	87225–87745
4	544 *Hin*dIII	86681–87225
5	234 *Xba*I/*Hin*dIII	86381–86615
6	209 *Pst*I/*Xba*I	86172–86381
7	106 *Xho*I/*Pst*I	86066–86172
8	428 *Sal*I/*Xho*I	85632–86060

[a] Tanaka et al., 1986
[b] Shinozaki et al., 1986*a*

Restriction endonuclease digestions, agarose gel electrophoresis, uni- or bidirectional transfer of DNA fragments from agarose gels to nylon filters (Zetabind®, AMF Cuno), labeling of recombinant plasmids with ^{32}P by nick-translation or random priming, filter hybridizations, and autoradiography were performed according to Palmer (1986) and Sambrook et al. (1989). Two or more single digests of a given DNA were done to confirm the reproducibility of the hybridization assays that were used to diagnose the presence or absence of the intron. The DNAs of Saxifragaceae were digested with at least two of the following restriction endonucleases: *Eco*RI, *Hin*dIII, and *Xba*I. All cpDNAs of the Asteridae were digested with each of the following four enzymes: *Bam*HI, *Eco*RV, *Bgl*II, and *Hin*dIII; all remaining cpDNAs were digested with *Bam*HI and *Hin*dIII. The latter four or two digests for any given DNA were placed together on the same gel to enable rapid diagnosis of the intron presence or absence. Filters were washed twice for 5–15 min at room temperature in 2× SSC, 0.5% SDS wash buffer followed by two 30–60 min washes at 65°C prior to autoradiography.

Eight cloned restriction fragments from *Nicotiana tabacum* cpDNA were used as hybridization probes against all taxa (Table 1, Fig. 1). All eight probes were hybridized serially to the same set of filters. In most instances, plants possessing the intron were on the same filters as those without it and served as positive controls.

The *rpl*2 gene was sequenced in six taxa (Table 2). For the sequence analysis, *Bam*HI-*Hin*dIII fragments containing the *rpl*2 gene were cloned in either M13 mp11/

12 or pUC11/12 vectors following standard methods (Sambrook et al., 1989). We employed both single- and double-stranded dideoxy sequencing methods (Hattori and Sakaki, 1986) using universal primers and a set of synthetic oligonucleotide primers based on the *Spinacia oleracea rpl2* coding sequence and the *Nicotiana tabacum rpl2* intron sequence. Data were verified by sequencing both strands of the *rpl2* clones.

Results

Heterologous filter hybridizations were performed using the eight probes described in Table 1 and illustrated in Figure 1. Probe 3 was used to test for the presence or absence of the *rpl2* intron. Probe 4 was used to test for the presence of exon 2 of the *rpl2* gene and its linkage to the intron. The six remaining probes were used to test for linkage between the *rpl2* gene and the genes that normally flank it.

The taxa surveyed for the presence or absence of the *rpl2* intron (Appendix 1) include both monocots and dicots and represent angiosperm lineages that may have diverged as much as 200 million years ago (Wolfe et al., 1989). The hybridization of heterologous probes to plants that have been separated for this length of time was carried out at standard stringency conditions (65°, 2× SSC wash). The strong hybridization of probes for coding regions (probes 1, 2 and 4–8) to all taxa is not surprising given the high sequence conservation among many chloroplast genes (e.g., the coding region of the *rpl2* gene has 90% amino acid identity between the dicot *Nicotiana* and the monocot *Oryza* [Sugiura, 1989]). What is startling, however, is the high degree of conservation in *rpl2* intron size and sequence. This intron varies little in size among angiosperms (661–667 bp; Table 2) and the intron in *Nicotiana* is 96% identical in nucleotide sequence to that of the monocot *Oryza* and the dicot *Rumex*. This extraordinary conservation in intron sequence is reflected in the strong hybridization signals observed (Fig. 2) and permits the use of an intron probe across widely divergent lineages. This striking sequence conservation may be due to the gene's location within the inverted repeat (IR), whose mutation rate is at least three times slower than that of single-copy sequences (Wolfe et al., 1987), and may also reflect unusually strong functional constraints on the evolution of this particular intron.

Since the gene *rpl2* is located within the IR in most angiosperms it is present in two identical copies. All mutations completely within the IR, such as the loss of the *rpl2* intron, are observed to occur symmetrically in both copies (Palmer, 1985). Because *rpl2* lies near the margin of the large single-copy region in most angiosperms, the *rpl2* exon and intron probes often hybridized to the same pair of restriction fragments, ones that overlap this margin (e.g., Fig. 2, lanes 4, 16, 17, 20, 21). Such a fragment pair is defined by a common site that occurs symmetrically within each of the IR segments and by different sites that occur asymmetrically within the ends of the adjacent single-copy region. Probes 2 and 4 generally hybridized to the same set (either one or two) of fragments for a given enzyme. In all cases in which the intron probe (probe 3) did hybridize, its hybridization was to this same set of fragments, i.e., in all cases probe 3 hybridization was consistent with there being an intron in the *rpl2* gene.

The filter hybridization survey revealed that most angiosperms contain the *rpl2* intron. The intron was found in three of five examined genera of the Geraniaceae and in all examined members of 100 of the remaining 115 families. The intron was judged to be absent, based on failure to obtain hybridization to the *rpl2* intron probe, in all examined representatives from 15 families: 10 families (19 species) of the Caryophyllales (Caryophyllidae); 24 genera (50 species) of Saxifragaceae *s.s.* (Rosidae); four genera (five species) of Convolvulaceae (Asteridae) plus *Cuscuta* sp. (Cuscutaceae, Asteridae); four genera (five species) of the Menyanthaceae (Asteridae); and *Drosera filiformis* (Droseraceae, Dilleniidae) (Appendix 1). The absence of the *rpl2* intron circumscribes putatively monophyletic groups at ordinal, familial, and intrafamilial levels. Hybridization results for 21 dicotyledons, illustrating the presence or absence of the *rpl2* intron, are presented in Figure 2.

DNA sequencing of the *rpl2* gene in six genera of the Caryophyllales and in *Rumex* (Polygonales) (Table 2) confirmed that the

TABLE 2. Comparison of *rpl*2 exon-intron boundary sequences for the chloroplast genomes of 12 species of land plants.

Taxon	5′ exon	Intron
Intron present		
Marchantia polymorpha[a]	CCTACCTTTGA	GTGCGGTTT···CTACTTCAA
Oryza sativa[b]	CCTACCTTTGA	GTGCGGTTT···CTCCTTCAA
Rumex sp.[c]	CCTACCTTTGA	GTGCGGTTT···CTACTTCAA
Epifagus virginiana[d]	CCTACCTTTGA	GTGCGGTTT···CTACTTCAA
Nicotiana debneyi[e]	CCTACCTTTGA	GTGCGGTTT···CTACTTCAA
Nicotiana tabacum[f]	CCTACCTTTGA	GTGCGGTTT···CTACTTCAA
Intron absent		
Amaranthus salicifolius[c]	CTTACCTTTGA	
Cerastium arvense[c]	CCTACCTTTGA	
Beta vulgaris[c]	CCTACCTTTGA	
Chenopodium murale[c]	CCTACCTTTGA	
Kochia americana[c]	GCTACCTTTGA	
Spinacia oleracea[e]	CCTACCTTTGA	

[a] Ohyama et al., 1986.
[b] Moon and Wu, 1988.
[c] This study.
[d] K. Wolfe, unpubl. data.
[e] Zurawski et al., 1984.
[f] Shinozaki et al., 1986*a*.

intron is indeed absent in all members of the former group and present in the latter. In those taxa lacking the intron, sequence results show that the *rpl*2 gene has undergone a precise deletion of the intron with the two exons juxtaposed into a single, uninterrupted gene. The *rpl*2 genes in the other lineages judged to lack the intron by filter hybridizations were not investigated at the DNA sequence level.

The results from the hybridization of probes 1, 2, and 5–8 reveal that the genes illustrated in Figure 1 are present in all taxa and occur in exactly the same order, i.e., that shown in Figure 1 for *Nicotiana*. Thus, the *rpl*2 gene is in the same position in all taxa regardless of whether they have the intron.

DISCUSSION

Molecular Implications

Introns 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, most, if not all, of which were present in the common ancestor of land plants (Shinozaki et al., 1986*a*; Ohyama et al., 1988; Hiratsuka et al., 1989). Besides the six (see next section) *rpl*2 intron losses described in this paper, six other cases of intron loss have been found in angiosperms: the *rpo*C1 intron in *Oryza sativa* (Hiratsuka et al., 1989), two introns of *clp*P (ORF196) in *O. sativa* (Hiratsuka et al., 1989) and other grasses (Downie and Palmer, 1991), the *trn*I intron in *Campanula garganica* (S. Downie and J. Palmer, unpubl. data), and the *rpl*16 intron in *Limonium gmelinii* and the common ancestor of the Geraniaceae (J. Logsdon, S. Downie, P. Calie and J. Palmer, unpubl. data).

A comparison of the *rpl*2 exon-intron boundary sequences reveals that these sequences are highly conserved among all taxa examined and that the position of the intron is unvarying within the gene (Table 2). Alignment of these conserved exon sequences with the uninterrupted *rpl*2 genes of members of the Caryophyllales shows that these sequences are maintained upon loss of the intron (Table 2). The conserved boundary sequences of the intron are similar to the boundary sequences found in other Group II introns (Shinozaki et al., 1986*b*; Sugiura et al., 1987; Michel et al., 1989) and are presumably essential for intron splicing.

The juxtaposition of what are, in most plants, two exons into a single, uninterrupted *rpl*2 gene shows that the intron has been precisely removed from this gene in the Caryophyllales. In all other cases of chloroplast intron loss that have been confirmed by sequencing (i.e., *rpl*16, *rpo*C1, and *clp*P (2 introns); see above) the introns have

TABLE 2. Extended.

3′ exon	Intron size (bp)
CCAATATGCCC	545
CCGATATGCCC	667
CCGATATGCCC	664
CCGATATGCCC	661
CCGATATGCCC	666
CCGATATGCCC	666
CCGATATGCCC	
CCGATATGCCC	
CCGATATGCCC	
CCGATATGCCC	
CCGATATGCCC	
CCGATATGCCC	

also been precisely removed from the gene. We presume this is also the case for the five lineages of intron-lacking *rpl*2 genes that have not been sequenced. The precise deletion of an entire intron sequence is generally thought to involve a reverse-transcriptase mediated mechanism. Reverse transcription of a spliced transcript, followed by homologous recombination between the intron-less complementary DNA (cDNA) and the original gene could account for such a precise intron loss without any alteration in gene position (Fink, 1987; Dujon, 1989). Other, undocumented processes that theoretically could effect intron removal include DNA-level deletion and gene conversion between an intron-containing gene and its spliced transcript.

All cases of intron loss known among angiosperm cpDNAs involve processing of the original gene, as opposed to creation of a duplicate gene via integration of a processed cDNA elsewhere in the genome (this report; Hiratsuka et al., 1989; J. Logsdon, S. Downie, P. Calie and J. Palmer, unpubl. data). Thus, as in the nucleus of yeast but unlike that of vertebrates (Fink, 1987), most integration of exogenous genes in cpDNA can be inferred to occur by homologous, replacement recombination, as opposed to illegitimate, nonreplacement recombination at more or less random sites in the genome.

Phylogenetic Implications

The evolutionary polarity of the *rpl*2 intron loss is unambiguous within the angiosperms. The presence of the *rpl*2 intron in the liverwort *Marchantia polymorpha* (Ohyama et al., 1986), in the gymnosperm *Ginkgo biloba* (J. Palmer, unpubl. data), in all examined monocots, and in most dicot families, including representatives from the putatively ancestral subclasses Magnoliidae and Hamamelidae (Appendix 1), strongly implies that the intron was present in the common ancestor of angiosperms, if not all land plants, and that its absence in various dicotyledons is a derived feature.

Because of their extreme rarity and the success of initial studies employing them in phylogenetic reconstruction (Jansen and Palmer, 1987; Bruneau et al., 1990; Lavin et al., 1990), major structural rearrangements of the chloroplast genome have been considered a relatively infallible class of phylogenetic characters. Indeed, it has been proposed that structural features should be weighted more heavily than more frequently changing characters such as single nucleotides or restriction sites (Palmer et al., 1988*a*; Lloyd and Calder, 1991). Here, however, we report a structural mutation that has occurred at least six times independently in the dicotyledons, but show that the distribution of homoplasy in this character allows its essentially unambiguous use as a phylogenetic marker.

The six groups of taxa lacking the *rpl*2 intron represent a diverse assemblage of plants and have been assigned to four subclasses of dicots (Appendix 1; Cronquist, 1981). We consider it much more likely that the loss of the *rpl*2 intron occurred independently in the common ancestors of each of these six groups than that it occurred only once, in which case it would unite all the intron-lacking taxa as a monophyletic group to the exclusion of all intron-containing taxa. This conclusion follows from two independent lines of evidence regarding angiosperm relationships. The six groups lacking the intron are distantly related to one another both in classification systems based largely on morphology (Cronquist, 1981; Takhtajan, 1987) and in a phylogeny based on *rbc*L sequence data (R. Olmstead, H. Michaels and J. Palmer, unpubl. data). The intron is present in each of the immediate respective sister groups to the six groups lacking the intron as implied by both conventional clas-

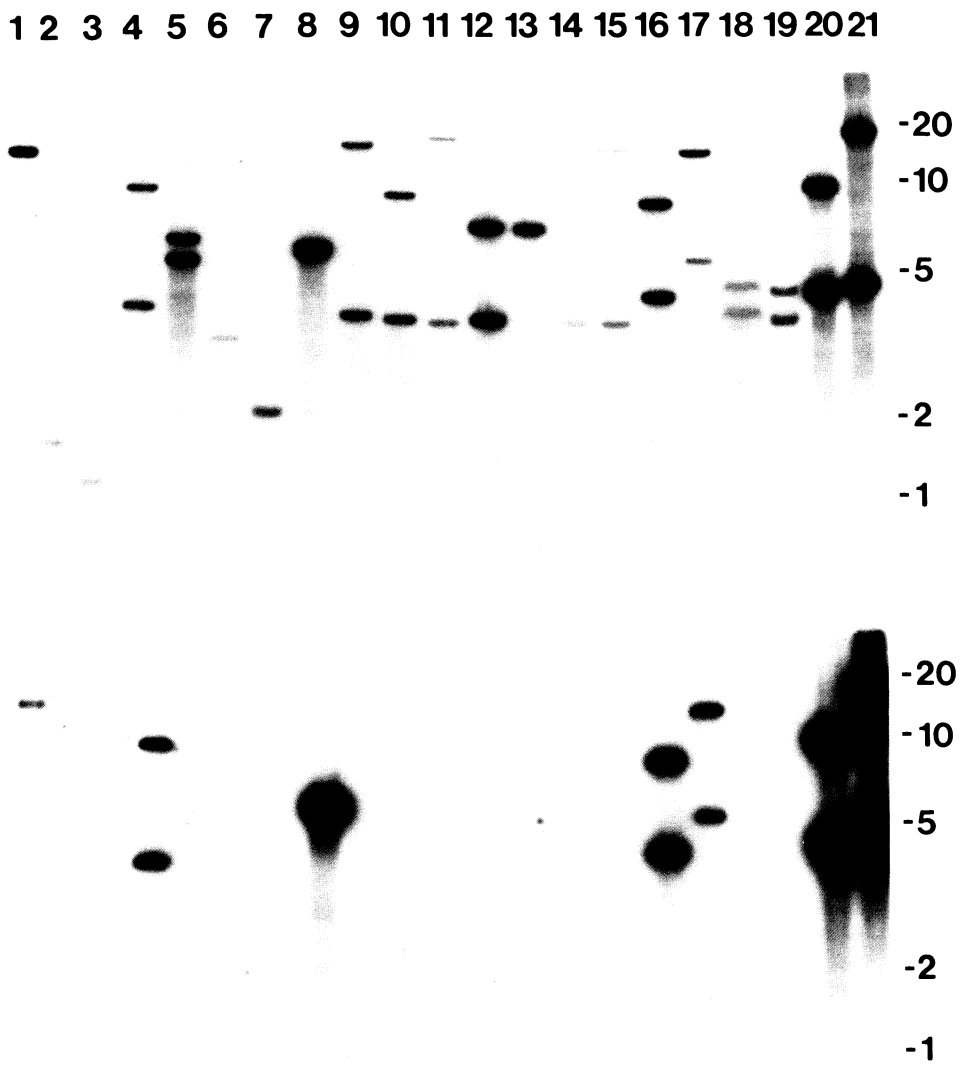

FIG. 2. Filter hybridizations showing the presence or absence of the *rpl*2 intron in 21 representative dicotyledons. CpDNA fragments produced by digestion of all taxa with *Bam* HI were electrophoresed in a 1.0% agarose gel. (Top panel) Hybridization with a 544-bp fragment (probe 4) containing 433 bp of the 3′exon of *rpl*2 and 51 bp of the *rpl*2 intron. (Bottom panel) Hybridization with a 520-bp fragment (probe 3) internal to the *rpl*2 intron. The identification of taxa in each lane is as follows: Lanes 1–3 *Pelargonium cotyledonis, Monsonia speciosa, Sarcocaulon vanderietiae* (Geraniaceae); Lane 4, *Gentiana dahurica* (Gentianaceae); Lane 5, *Menyanthes trifoliata* (Menyanthaceae); Lanes 6–7, *Convolvulus* sp., *Calonyction aculeatum* (Convolvulaceae); Lane 8, *Nicotiana tabacum* (Solanaceae); Lanes 9–15, *Alternanthera dentata, Anredera cordifolia, Pereskia grandifolia, Spinacia oleracea, Atriplex triangularis, Bougainvillea glabra, Claytonia caroliniana* (Caryophyllales); Lane 16, *Rheum rhaponticum* (Polygonaceae); Lane 17, *Limonium gmelinii* (Plumbaginaceae); Lanes 18–19, *Boykinia rotundifolia, Tellima grandiflora* (Saxifragaceae); Lane 20, *Ribes americanum* (Grossulariaceae); Lane 21, *Sedum laxum* (Crassulaceae). Numbers at right of panels indicate fragment sizes in kb.

sifications and the *rbc*L sequence phylogeny. The consistent absence of the *rpl*2 intron among the many species surveyed in the Caryophyllales, Saxifragaceae *s.s.*, Convolvulaceae, and Menyanthaceae indicates that it is an evolutionarily stable mutation and not prone to reversal. This is not surprising, as reversal here would mean the precise insertion of a segment of DNA into a site formerly containing it, a highly improbable event in general.

Recent investigations into chloroplast genome structure have revealed other homoplastic rearrangements. The loss of one copy of the IR, for example, characterizes five clearly unrelated lineages of land plants (Strauss et al., 1988; Lavin et al., 1990; S. Downie, P. Calie and J. Palmer, unpubl. data), and several chloroplast genes have been lost on multiple independent occasions during angiosperm evolution (Downie and Palmer, 1991). To date, inversions are the only class of structural rearrangements that are consistently uniquely derived (Jansen and Palmer, 1987; Bruneau et al., 1990). Structural molecular characters are thus similar to traditional characters (such as morphological features and secondary chemicals) that have been used to infer phylogeny at higher levels in being afflicted with some level of homoplasy.

We feel it is not yet possible to rigorously evaluate the relative homoplasy of structural molecular characters and traditional ones because equivalent taxa have not been adequately examined for the different kinds of characters. However, it is possible, indeed worthwhile, to examine the relative variation and homoplasy of different types of molecular characters. We take as our example a comparative restriction site mapping study of the Solanaceae (R. Olmstead and J. Palmer, unpubl. data), which affords special insight into the occurrence of intron and gene loss, owing to the availability of a complete genome sequence for *Nicotiana tabacum,* one of the included species (Shinozaki et al., 1986*a*). Among 1,055 restriction sites compared, the number of mutations for each site inferred from the most parsimonious tree ranged from 0 to 12 with a mean of 1.5. In contrast, not one of the 20 introns known to exist in the *Nicotiana* chloroplast genome was lost and only a single case of gene loss was evident among the more than 50 chloroplast genes surveyed. Thus, we conclude that intron and gene losses occur at significantly lower frequency and with less homoplasy than restriction site mutations when measured within the same set of taxa.

The phylogenetic implications of the *rpl*2 intron loss for each of the six groups of dicotyledons are discussed below.

Caryophyllales.—The Caryophyllales (or Centrospermae) consist of approximately 11 core families. Although the delimitation of some families is uncertain and their phylogenetic relationships are a contentious issue, it is accepted widely that the order is monophyletic (Ehrendorfer, 1976; Mabry, 1977; Rodman et al., 1984; Hershkovitz, 1989). The occurrence of PIII-type sieve element plastids and bound ferulic acids in unlignified cell walls unites the members of the order. A host of other embryological, palynological and phytochemical characters, although not universally present, further attests to the close relationship of the families. The absence of the *rpl*2 intron in all examined members of the Caryophyllales (Appendix 1) suggests that it was lost in the common ancestor of the order.

The Caryophyllales are sometimes associated with two small orders, the Polygonales and Plumbaginales (each consisting of a single family), and together with them comprise the subclass Caryophyllidae (Takhtajan, 1980; Cronquist, 1981, 1988). However, the relationships between the Polygonaceae and Plumbaginaceae, and between these two families and the Caryophyllales, are not clear (Behnke, 1976; Ehrendorfer, 1976; Thorne, 1976; Nowicke and Skvarla, 1977; Rodman et al., 1984). The presence of the *rpl*2 intron in these two families further distinguishes them from the Caryophyllales but is neutral with respect to the controversy concerning closeness of relationships among these three orders.

Saxifragaceae.—Engler (1930) defined the Saxifragaceae very broadly as comprising 15 subfamilies, represented by 80 genera and about 1,200 species. Subsequent treatments differ substantially, not only from the traditional Englerian interpretation, but from one another as well (Cronquist, 1981; Dahlgren, 1983; Thorne, 1983; Takhtajan,

1987), with little agreement regarding the circumscription, taxonomic rank, or phylogenetic relationships among many of the taxa. Comprehensive reviews of the differing taxonomic treatments are provided elsewhere (Spongberg, 1972; Cronquist, 1981; Soltis et al., 1990).

Our hybridization results indicate that the *rpl*2 intron is absent in 24 herbaceous genera (50 spp.) of Saxifragaceae (Appendix 1). The distribution of this missing intron corresponds precisely to Takhtajan's (1987) narrow circumscription of the Saxifragaceae (which he limits to 30 herbaceous genera), and implies that the intron loss occurred in the common ancestor of this restricted group. Comparative *rbc*L sequence data (Soltis et al., 1990) and cpDNA restriction site data (Soltis et al., unpubl. data) are congruent with the distribution of the *rpl*2 intron and provide further support for Takhtajan's hypothesis. The intron is present in all woody taxa examined from Engler's Saxifragaceae (*Brexia madagascariensis, Escallonia* sp., *Itea virginica,* two species of *Ribes,* and five genera of the Hydrangeaceae), and in the herbaceous *Francoa sonchifolia, Parnassia fimbriata,* and *Penthorum sedoides.* Cronquist (1981) includes these latter three genera, all of which possess the intron, in his interpretation of the Saxifragaceae. Two genera within the Crassulaceae (*Kalanchoe* and *Sedum*), generally regarded as being closely related to the Saxifragaceae (Takhtajan, 1980; Cronquist, 1981, 1988), also have the intron. The absence of the *rpl*2 intron agrees with other molecular data and with conventional taxonomic treatments in supporting the monophyly of the Saxifragaceae *s.s.* However, as in the case of the Caryophyllales and putatively related orders, the intron data are neutral with respect to the controversy regarding relationships between Saxifragaceae *s.s.* and other taxa placed in the Saxifragaceae by different authorities.

Convolvulaceae and Cuscuta.—The Convolvulaceae comprise about 50 genera and 1,500 species, largely of tropical and subtropical distribution, and are often thought to be allied most closely to the Solanaceae (Takhtajan, 1980; Cronquist, 1981, 1988). *Cuscuta,* a cosmopolitan genus of about 150 species of twining stem-parasites, often is allied with the Convolvulaceae either as a member of that family (Bentham and Hooker, 1896; Peter, 1897; Wilson, 1960; Thorne, 1983) or treated as a separate family, the Cuscutaceae (Takhtajan, 1980; Cronquist, 1981, 1988; Dahlgren, 1983). *Cuscuta* possesses a number of characters that clearly differentiate it from the Convolvulaceae, such as its parasitic, aphyllous nature and distinct embryological features. Nevertheless, it is generally accepted that *Cuscuta* is derived from the Convolvulaceae. The absence of the *rpl*2 intron in *Cuscuta* and the four genera of the Convolvulaceae examined suggests that they share a common ancestor and provides further evidence attesting to the close relationship between these two groups.

Menyanthaceae.—The Menyanthaceae comprise a small group of aquatic or semiaquatic herbs of cosmopolitan distribution. The family consists of five genera of uncertain relationship (Ornduff, 1973), three of which are monotypic. The absence of the *rpl*2 intron in all four examined genera (*Fauria, Menyanthes, Nymphoides,* and *Villarsia*) suggest that it was lost in the common ancestor of the family. Once relegated to infrafamilial status within the Gentianaceae, the family now is considered distinct but is often treated as closely related to the Gentianaceae. The four species of Gentianaceae that have been examined all possess the intron.

Discordance between diagnostic anatomical (Lindsey, 1938) and chemical characters (Hegnauer, 1969) precludes a consensus on the ordinal placement of the Menyanthaceae. Whereas other workers have included the Menyanthaceae within the Gentianales (Dahlgren, 1983; Thorne, 1983; Takhtajan, 1987), Cronquist (1981, 1988) favors its position within the Solanales. The shared absence of the *rpl*2 intron between the Convolvulaceae and Menyanthaceae, both placed in the Solanales by Cronquist (1981), could suggest a close relationship between these two families. However, phylogenetic analysis of *rbc*L sequence data (R. Olmstead, H. Michaels and J. Palmer, unpubl. data) and IR restriction site data (S. Downie and J. Palmer, unpubl. data) shows that Menyanthaceae should be placed in the Asterales and not in the Gentianales or So-

lanales. We therefore conclude that the *rpl*2 intron was lost independently in the common ancestors of the Menyanthaceae and Convolvulaceae.

Monsonia and Sarcocaulon (Geraniaceae).—The Geraniaceae include *Geranium, Erodium, Pelargonium, Monsonia* and *Sarcocaulon* (Hutchinson, 1969). Six other genera (*Biebersteinia, Rhynchotheca, Dirachma, Balbisia, Wendtia,* and *Viviania*), often segregated into four distinct families, are sometimes included in the Geraniaceae (Takhtajan, 1981; Cronquist, 1981). However, the taxonomic affinities of these genera to the Geraniaceae *s.s.* are not well established (for reviews see Cronquist, 1981, and Robertson, 1972) and material of these six genera was not available for this investigation. The five species of *Geranium,* two species of *Erodium* and 35 species of *Pelargonium* examined all possessed the intron, whereas the two species each of *Monsonia* and *Sarcocaulon* examined did not. Other characters also support a common ancestry of these two genera. *Monsonia* and *Sarcocaulon* are identical palynologically (Robertson, 1972), and both are distinguished by the possession of 15 stamens versus the 10 or fewer in the other three genera (Van der Walt, 1977). Only vegetative characters differentiate these genera (Venter, 1979). The Geraniaceae are morphologically similar to the Oxalidaceae, Tropaeolaceae, and possibly the Balsaminaceae (Cronquist, 1981, 1988); representatives from each of these three outgroups all possess the intron.

Drosera filiformis (Droseraceae).—The Droseraceae are a group of insectivorous plants consisting of four genera: *Aldrovanda, Dionaea, Drosera,* and *Drosophyllum. Drosera,* the only genus that is not monotypic, is widespread in both temperate and tropical regions. Although Cronquist (1981) treats the Droseraceae along with the Sarraceniaceae and Nepenthaceae in the Nepenthales, there is little evidence supporting a close relationship among these families (DeBuhr, 1975). Moreover, the latter two families possess the *rpl*2 intron. DeBuhr (1975) and Thorne (1983) placed the Droseraceae near the Saxifragaceae *s.l.,* whereas Takhtajan (1980) was more explicit by placing it close to *Parnassia* (his Parnassiaceae). Considering the loss of the *rpl*2 intron as a synapomorphy between the Droseraceae and the Saxifragaceae *s.s.* might be possible, but is discordant with *rbc*L sequence data which do not support a close relationship between *Parnassia* and the Saxifragaceae *s.s.* (Soltis et al., 1990). Sequencing of the *rbc*L gene in *Drosera* and comparing its sequence to those available from the Saxifragaceae should elucidate whether *Drosera* should be placed within, near, or excluded from, the Saxifragaceae.

Conclusions

This is the most extensive survey yet to be completed for the systematic distribution of a cpDNA rearrangement and demonstrates the potential of such a mutation for highlighting phylogenetic relationships at higher taxonomic levels. The loss of the *rpl*2 intron has occurred independently a minimum of six times in the dicotyledons. In spite of this level of homoplasy, the loss of the intron is still a powerful indicator of relationships within the different lineages in which it has occurred. Our results corroborate traditional and recent treatments attesting to the monophyly of the Caryophyllales, the Saxifragaceae (*s.s.*), the Convolvulaceae (including *Cuscuta*), and the Menyanthaceae. In the Geraniaceae, the absence of the *rpl*2 intron in *Monsonia* and *Sarcocaulon* suggests that these genera are more closely related to each other than either is to the other three genera.

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Corresponding Editor: M. J. Donoghue

APPENDIX 1. Angiosperms surveyed for the presence or absence of the *rpl*2 intron. Those species for which the *rpl*2 intron is absent are indicated by boldface. Those species for which the intron absence (or presence) has been confirmed by DNA sequencing are indicated by an asterisk (*). All other absences are postulated only on the basis of filter hybridizations. Numbers in parentheses indicate number of species examined. System of classification follows that of Cronquist (1981).

Dicotyledons
Asteridae
Acanthaceae
Graptophyllum pictum
Justicia carnea
Pachystachys lutea

APPENDIX 1. Continued.

Apocynaceae
Acokanthera oblongifolia
Amsonia tabernaemontana
Apocynum cannabinum
Ochrosia eliptica
Prestonia acutifolia
Vinca minor
Asclepiadaceae
Asclepias spp. (2)
Periploca sepium
Asteraceae
Barnadesia caryophylla
Lactuca sativa
Bignoniaceae
Campsis radicans
Catalpa bignonioides
Clytostoma callistegioides
Boraginaceae
Borago officinalis
Cyanoglossum grande
Heliotropium arborescens
Mertensia virginica
Buddlejaceae
Buddleja spp. (2)
Callitrichiaceae
Callitriche heterophylla
Calyceraceae
Boopis graminea
Gamocarpha poeppigii
Campanulaceae
Campanula spp. (2)
Jasione montana
Platycodon grandiflorus
Caprifoliaceae
Kolkwitzia amabilis
Lonicera subsessilis
Sambucus spp. (2)
Symphoricarpos albus
Viburnum acerifolium
Weigela hortensis
Convolvulaceae
Calonyction aculeatum
Calystegia sepia
***Convolvulus* spp. (2)**
Ipomoea pes-caprae
Cuscutaceae
***Cuscuta* sp.**
Dipsacaceae
Cephalaria leucantha
Dipsacus spp. (2)
Scabiosa ochroleuca
Gentianaceae
Exacum affine
Gentiana dahurica
Lisianthus skinneri
Obolaria virginica
Gesneriaceae
Alsobia dianthiflora
Nematanthus hirsutus
Streptocarpus holstii
Globulariaceae
Globularia salicinus

APPENDIX 1. Continued.

Goodeniaceae
Goodenia ovata
Scaveola taccada
Hydrophyllaceae
Eriodictyon californica
Hydrolea ovata
Hydrophyllum virginiana
Lamiaceae
Comanthosphace stellipila
Melissa officinalis
Pogostemon patchulii
Prasium majus
Prostanthera nivea
Salvia divinorum
Scutellaria bolanderi
Stachys officinalis
Teucrium canadense
Lentibulariaceae
Pinguicula caerulea
Lobeliaceae[a]
Hippobroma longiflora
Lobelia spp. (4)
Monopsis lutea
Sclerotheca jayorum
Loganiaceae
Fagraea zeylanica
Gelsemium sempervirens
Spigelia marilandica
Strychnos spinosa
Menyanthaceae
Fauria crista-galli
Menyanthes trifoliata
***Nymphoides* spp. (2)**
Villarsia calthifolia
Myoporaceae
Bontia daphnoides
Eremophila maculata
Myoporum spp. (2)
Nolanaceae
Nolana spathulata
Oleaceae
Forsythia ovata
Jasminum spp. (2)
Ligustrum spp. (2)
Syringa vulgaris
Orobanchaceae
Conopholis americana
*Epifagus virginiana**
Pedaliaceae
Proboscidea louisianica
Sesamum indicum
Plantaginaceae
Plantago spp. (2)
Polemoniaceae
Ipomopsis spp. (2)
Phlox 'Pinafore Pink'
Polemonium reptans
Rubiaceae
Coffea arabica
Galium boreale
Palicourea crocea
Pentas spp. (2)

APPENDIX 1. Continued.

Scrophulariaceae
Antirrhinum majus
Digitalis parviflorum
Paulownia tomentosa
Striga asiatica
Verbascum thapsus
Solanaceae
Iochroma cyaneum
Nicotiana spp. (2)
Schizanthus pinnatus
Solandra grandiflora
Valerianaceae
Valeriana sp.
Verbenaceae
Callicarpa dichotoma
Caryopteris clandonensis
Clerodendrum spp. (2)
Phyrma leptostachya
Phyla scaberrima
Premna japonica
Verbena bovariensis

Caryophyllidae
Caryophyllales
Aizoaceae
Delosperma tradescantioides
Trianthema portulacastrum
Amaranthaceae
Alternanthera dentata
Amaranthus* spp. (2)
Basellaceae
Anredera cordifolia
Cactaceae
Pereskia grandifolia
***Pereskiopsis* sp.**
Caryophyllaceae
Cerastium arvense*
Chenopodiaceae
Atriplex triangularis
Beta vulgaris*
Chenopodium murale*
Kochia americana*
Spinacia oleracea*
Didiereaceae
Alluaudia procera
Nyctaginaceae
Bougainvillea glabra
Phytolaccaceae
Phytolacca heterotepela
Portulacaceae
Claytonia caroliniana
Portulaca oleracea

Plumbaginales
Plumbaginaceae
Limonium gmelinii

Polygonales
Polygonaceae
Polygonum sp.
Rheum rhaponticum
Rumex sp.*

APPENDIX 1. Continued.

Dilleniidae
Begoniaceae
Begonia sp.
Bombacaceae
Quararibea aterolepis
Brassicaceae
Arabidopsis thaliana
Brassica juncea
Crambe abyssinica
Droseraceae
Drosera filiformis
Fouquieriaceae
Fouquieria splendens
Loasaceae
Eucnide hirta
Malvaceae
Gossypium hirsutum
Nepenthaceae
Nepenthes alata
Paeoniaceae
Paeonia lactiflora
Passifloraceae
Passiflora helleri
Primulaceae
Anagallis arvensis
Salicaceae
Populus spp. (2)
Salix spp. (8)
Sarraceniaceae
Sarracenia flava
Violaceae
Viola sororia

Hamamelidae
Cercidiphyllaceae
Cercidiphyllum sp.
Juglandaceae
Alfaroa williamsii
Ulmaceae
Aphananthe sp.
Celtis sp.
Hemiptelea sp.
Ulmus spp. (2)
Urticaceae
Pilea microphylla

Magnoliidae
Aristolochiaceae
Aristolochia durior
Berberidaceae
Podophyllum peltatum
Calycanthaceae
Calycanthus floridus
Lauraceae
Persea americana
Magnoliaceae
Liriodendron tulipifera
Papaveraceae
Eschscholzia californica
Piperaceae
Piper nigrum

APPENDIX 1. Continued.

Ranunculaceae
Caltha palustris
Saururaceae
Saururus cernuus
Winteraceae
Drimys winteri

Rosidae
Aceraceae
Acer negundo
Anacardiaceae
Rhus typhina
Apiaceae
Coriandrum sativum
Daucus carota
Araliaceae
Hedera helix
Trevesia sundaica
Balsaminaceae
Impatiens biflora
Brexiaceae[b]
Brexia madagascariensis
Cornaceae
Aucuba japonica
Cornus spp. (2)
Crassulaceae
Kalanchoe fedtschenkoi
Sedum laxum
Escalloniaceae[b]
Escallonia sp.
Euphorbiaceae
Euphorbia polychroma
Fabaceae
Cicer arietinum
Coursetia hypoleuca
Glycine max
Hebestigma cubense
Lathyrus odoratus
Medicago spp. (6)
Piscidia piscipula
Pisum sativum
Sesbania sesban
Trifolium subterraneum
Vicia faba
Vigna radiata
Francoaceae[b]
Francoa sonchifolia
Geraniaceae
Erodium spp. (2)
Geranium spp. (5)
Pelargonium spp. (35)
***Monsonia* spp. (2)**
***Sarcocaulon* spp. (2)**
Grossulariaceae
Ribes spp. (2)
Hippocastanaceae
Aesculus hippocastanum
Hydrangeaceae
Cardiandra alternifolia
Carpenteria californica
Hydrangea sp.
Kirengeshoma palmata
Philadelphus lewisii
Schizophragma hydrangeoides
Iteaceae[b]
Itea virginica
Krameriaceae
Krameria sp.
Linaceae
Linum grandiflorum
Onagraceae
Boisduvalia densiflora
Clarkia bottae
Epilobium angustifolium
Fuchsia hybrida
Gaura biennis
Hauya heydeana
Lopezia reisenbachii
Ludwigia ravenii
Oenothera missouriensis
Oxaliaceae
Oxalis oregana
Parnassiaceae[b]
Parnassia fimbriata
Penthoraceae[b]
Penthorum sedoides
Polygalaceae
Securidaca diversifolia
Rosaceae
Amelanchier canadensis
Malus sylvestris
Spiraea nipponica
Sapindaceae
Ungnadia speciosa
Saxifragaceae
Astilbe taquetii
Astilboides tabularis
Bensoniella oregana
Bergenia cordifolia
Bolandra californica
***Boykinia* spp. (6)**
Chrysosplenium americanum
Conimitella williamsii
Darmera peltata
Elmera racemosa
***Heuchera* spp. (6)**
***Lithophragma* spp. (5)**
***Mitella* spp. (5)**
Mukdenia rosii
Peltoboykinia tellimoides
***Rodgersia* spp. (4)**
***Saxifraga* spp. (5)**
Suksdorfia violacea
Sullivantia oregana
Tanakaea radicans
Telesonix jamesii
Tellima grandiflora
***Tiarella* spp. (2)**
Tolmiea menziesii
Tropaeolaceae
Tropaeolum majus

Monocotyledons
Alismatidae

APPENDIX 1. Continued.

Alismataceae
Sagittaria latifolia
Arecidae
Araceae
Symplocarpus foetidus
Arecaceae
Pritchardia eriostachys
Commelinidae
Commelinaceae
Commelina sp.
Poaceae
Bambusa sp.
Oryza sativa
Zea mays
Liliidae
Iridaceae
Lapeirousia cruenta
Liliaceae
Allium cepa
Calochortus uniflorus
Chlorophytum sp.
Tricyrtis spp. (2)
Orchidaceae
Cochleanthes discolor

APPENDIX 1. Continued.

Corallorhiza odontorhiza
Pontederiaceae
Eichhornia crassipes
Velloziaceae
Barbacenia carnata
Vellozia sp.
Zingiberidae
Bromeliaceae
Aechmea nudicaulis
Brocchinia micrantha
Cryptanthus sp.
Dyckia ragonesei
Glomeropitcairnia penduliflora
Guzmania coriostachys
Hechtia macdougalii
Orthophytum fosterianum
Pitcairnia schiedeana
Puya lilloi
Tillandsia spp. (3)
Vriesea sp.
Wittrockia campus-portoi
Strelitziaceae
Strelitzia reginae

[a] Lobeliaceae is considered here distinct from the Campanulaceae based on Bremer (1987).
[b] Takhtajan's (1987) classification system is followed based on data presented herein and Soltis et al. (1990).