Chloroplast Genome Rearrangements and the Evolution of Giant Lobelias from Herbaceous Ancestors[1]

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Phylogenetic relationships among 16 species of *Lobelia* and single representatives of *Monopsis* and *Sclerotheca* (Lobeliaceae) were assessed by mapping restriction sites and major structural rearrangements (deletions and inversions) in the large single-copy region of the chloroplast genome. Eleven inversions and five different gene arrangements were found. A deletion involving ORF512 is associated with many of the inversions, and all inversion endpoints are located in intergenic spacer regions. Analysis of 132 phylogenetically informative restriction sites produced three equally parsimonious trees of 219 steps, with a consistency index of 0.60. The restriction-site and inversion data yield congruent trees, indicating that the giant lobelias from around the world are derived from diploid herbaceous ancestors. The giant lobelias consist of a Chilean hexaploid group and a pantropical tetraploid group. The woody genus *Sclerotheca* is clearly derived from a giant *Lobelia* ancestor, while the herbaceous *Monopsis* is probably derived from herbaceous lobelias. The giant lobelias from eastern Africa are weakly supported as monophyletic with the inclusion of the Brazilian *L. organensis*. Relationships among the Pacific and Asian giant lobelias are not fully resolved and await more detailed study.

Introduction

Lobelia, the largest genus within the family Lobeliaceae, comprises over 350 species that range from small, slender herbs to woody, giant-rosette plants. The spectacular evolutionary radiations of the giant-rosette species in the mountains of eastern Africa and the Hawaiian Islands have established these groups as premier botanical examples of adaptive radiation and have prompted much discussion concerning their evolutionary histories. Corner (1949, 1954) considered the giant-rosette growth form to be primitive, a position rejected by Carlquist (1962, 1980) but favored by Mabberley (1974*a,* 1974*b,* 1975, 1982). The giant lobelias have an almost pantropical distribution, with additional species found elsewhere in the Pacific basin and in Asia, west Africa, the Caribbean, Brazil, and Chile. The purpose of the present study was to assess the relationships among representatives of the giant lobelias worldwide, to determine whether those in eastern Africa constitute a monophyletic group, and to select the most appropriate outgroup for a detailed study of the evolution of the giant lobelias in eastern Africa.

Phylogenetic analysis of restriction-site variation in chloroplast genomes has been used to construct explicit and robust hypotheses of phylogenetic relationships in a number of plant groups (Palmer et al. 1988*a;* Soltis et al. 1992). Another approach

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to extracting phylogenetic information from chloroplast DNA (cpDNA) is by analyzing the distribution of major structural rearrangements. Evolution of cpDNA is typically conservative (Palmer 1985; Downie and Palmer 1992), and, as a consequence, the infrequent major structural rearrangements of the chloroplast genome (e.g., inversions and the insertion or deletion of genes and introns) usually provide strong evidence of monophyly. In several recent studies, inversions have proved to be useful characters for phylogenetic reconstruction at several levels of land-plant evolution (Palmer and Thompson 1982; Jansen and Palmer 1988; Bruneau et al. 1990; Raubeson and Jansen 1992).

The existence of multiple, poorly characterized rearrangements in Lobeliaceae (and Campanulaceae) cpDNA was inferred several years ago, on the basis of complex hybridization patterns obtained during restriction-enzyme surveys (Palmer 1985; Jansen and Palmer 1987). In the present paper, we report that the chloroplast genomes of *Lobelia* and related species differ considerably in structure from the vast majority of land plants examined, with 11 inversions and 3 major deletions apparent in the large single-copy region of the genome. This information, in conjunction with an analysis of restriction-site variation, is used to assess the congruence between these two classes of molecular data and to assess the phylogenetic relationships among representatives of *Lobelia*. In particular, we examine the hypothesis that the giant lobelias were derived from herbaceous ancestors.

Material and Methods

Plant material of 18 species representing three genera of Lobeliaceae was obtained from various sources (table 1), either as seed or as fresh or dried leaf material. The

Table 1
Species Examined for cpDNA Restriction-Site Variation and Structural Rearrangements

Species and Author	Source[a]	Geographic Origin
Lobelia boninensis Koidz.[b]	RBG	Bonin (Pacific)
L. bridgesii Hook & Am.[b]	Lammers	Chile
L. cardinalis L.[b]	Knox	North America
L. erinus L.[c]	Ayers	Africa
L. excelsa Bonpl.[b]	Lammers	Chile
L. fervens Thunb.[b]	Know	Africa
L. giberroa Hemsley[b]	Knox	Africa
L. gloria-montis Rock[b]	Sytsma et al.	Hawaii
L. holstii Engl.[b]	Knox	Africa
L. hypoleuca Hillebrand[b]	Sytsma et al.	Hawaii
L. nicotianifolia Heyne *ex* Roem. & Schult.[b]	RBG	China
L. organensis Gardner[b]	RBG	Brazil
L. petiolata Hauman[b]	Knox	Africa
L. polyphylla Hook & Arn.[b]	Lammers	Chile
L. stricklandiae Gilliland[b]	C and L	Africa
L. tupa L.[b]	RBG	Chile
Monopsis lutea (L.) Urban[b,c]	Ayers	Africa
Sclerotheca jayorum Rayn.[b,c]	Sytsma	Tahiti (Pacific)

[a] RBG = Royal Botanic Gardens, Kew; Lammers = Thomas G. Lammers; Knox = Eric Knox; Ayers = Tina J. Ayers; Sytsma et al. = Kenneth J. Sytsma, Thomas J. Givnish, and James F. Smith; C and L = Colin Congdon and Jon Lovett; and Sytsma = Kenneth J. Sytsma.

[b] DNAs digested with 12 enzymes and probed with 22 lettuce subclones.

[c] DNAs digested with four enzymes and probed with 109 tobacco subclones.

isolation of cpDNA or total cellular DNA from leaf material was accomplished by using either the sucrose gradient technique of Palmer (1986) or the modified CTAB procedure of Doyle and Doyle (1987), respectively. In most cases the DNAs were purified by centrifugation in cesium chloride/ethidium bromide gradients.

DNA aliquots from 17 of the species listed in table 1 were digested with each of 12 restriction enzymes: *Ase*I, *Bam*HI, *Ban*II, *Bcl*I, *Bgl*II, *Bst*NI, *Cla*I, *Eco*O109, *Eco*RI, *Eco*RV, *Hin*dIII, and *Nci*I. Twenty-two clones of a lettuce cpDNA library (Jansen and Palmer 1987, 1988) were used as probes in filter-hybridization experiments to map restriction sites in these cpDNAs. Two subclones from tobacco (SolClones 2 and 3 in Olmstead and Palmer 1992) were used as probes to represent the one uncloned portion of the lettuce genome. DNA aliquots from *Monopsis lutea, Sclerotheca jayorum,* and an additional species, *Lobelia erinus,* were digested with each of four enzymes (*Bam*HI, *Bgl*II, *Eco*RV, and *Hin*dIII) and were probed with clones of a tobacco cpDNA library derived from an initial set of large, cloned restriction fragments (Sugiura et al. 1986). These 109 probes have a size range of 0.2–3.5 kb, average ~1.2 kb, and together constitute the entire tobacco chloroplast genome. These probes are considerably smaller than the lettuce probes and permit the detection of small rearrangements that often are undetected when larger probes are used.

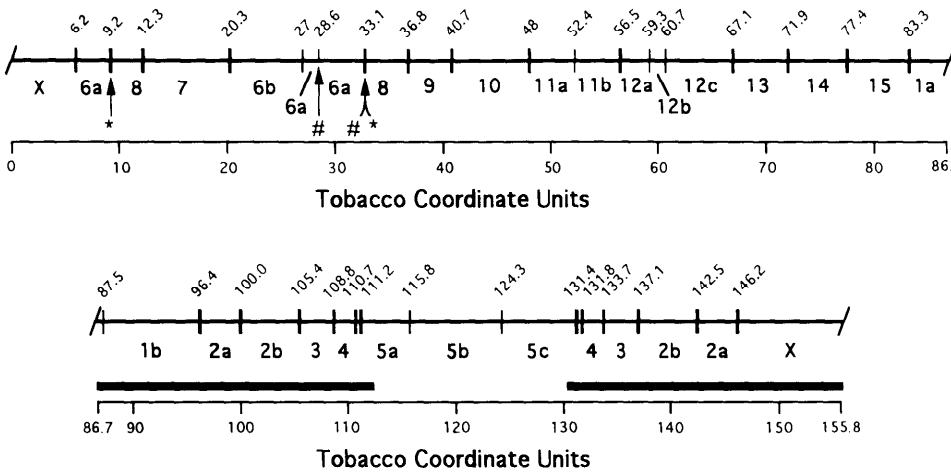
The small tobacco probes provide differential hybridization of the 5′ and 3′ ends of many chloroplast genes, and this allowed us to determine the direction of transcription for these genes. In other cases where the genes were too small or where 5′ and 3′ probes were not available, the orientation of genes was based on their cotranscription with genes for which we did have both 5′ and 3′ gene probes. Knowledge of the endpoint coordinates of these probes (Shinozaki et al. 1986), in conjunction with their small sizes, permits accurate estimates to be made of the locations of rearrangement events in the genomes of Lobeliaceae, by reference to the known genome of tobacco. The estimates in this reference system are presented in kilobase increments, which are herein referred to as "tobacco coordinate units" (TCU). Requests regarding probe descriptions and availability should be directed to J. D. Palmer.

Restriction-enzyme digestions, agarose-gel electrophoresis, bidirectional transfer of DNA fragments from agarose gels to nylon filters (Zetabind™; AMF Cuno), labeling of recombinant plasmids with ^{32}P by nick-translation, filter hybridizations, and autoradiography were performed according to methods described by Palmer (1986) and Palmer et al. (1988*a*). Filters were washed twice for 5 min at room temperature in 2 × saline sodium citrate and 0.5% sodium dodecyl sulfate, followed by three 60-min washes at 65°C prior to autoradiography.

The inclusion of lettuce in this study (digested with *Ban*II, an enzyme that cuts at the *Sac*I sites that define many of the lettuce clones) allowed estimates to be made of the tobacco sequence coordinates corresponding to the endpoints of the probes in the lettuce clone bank (fig. 1). We feel that our estimates are generally within 100 bp of the actual endpoints.

Detailed restriction-site maps for four enzymes were constructed for each of the three species investigated, by using the set of 109 cloned restriction fragments of tobacco cpDNA. These maps were compared with those constructed for 12 restriction enzymes, for each of the 17 species, by using the 22 lettuce probes. Restriction-site variation across taxa and across enzymes was used in conjunction with mapping, to distinguish length mutations from restriction-site mutations and to estimate the coordinates of inversion endpoints. For the lettuce-probed species, the map coordinates were then

A



B

Probe	Size	Probe	Size	Probe	Size	Probe	Size
1	12.3	5	18.8	7	7.0	12	10.6
1a	3.8	5a	3.6	8	6.7	12a	2.6
1b	8.3	5b	9.1	9	3.8	12b	1.2
2	9.9	5c	5.5	10	6.9	12c	6.5
2a	3.7	6	14.7	11	7.7	13	4.6
2b	6.2	6a	7.5	11a	4.3	14	5.4
3	3.5	6b	7.2	11b	3.4	15	6.3
4	1.8						

X - Uncloned 15.4 kb fragment

FIG. 1.—A, Endpoint estimates and map of the lettuce probes. The scale is in tobacco coordinate units. Endpoint estimates are presented above the map. The significant figures denote perceived accuracy of the estimates. The lettuce probes are labeled with numbers, and subclones are labeled with letters below the map. The 22-kb inversion is indicated by the asterisks, and the previously unreported 4.5-kb inversion is indicated by pound signs. B, Lettuce probe sizes (after Jansen et al. 1992).

used to select appropriate tobacco subclone probes to confirm the deletions and to refine the endpoint coordinate estimates.

The phylogenetic analysis of restriction sites excluded all variation that was ambiguous with respect to assessment of homology, including small length variants, sites at or near inversion endpoints, and clusters of small fragments. The analyses were conducted by using PAUP (Swofford 1990) on a Macintosh II computer. Both Wagner parsimony, which weights all restriction-site changes equally, and the scheme of Albert et al. (1992), which weights site gains over site losses, were used. Bootstrap analysis (Felsenstein 1985) and decay analysis (Donoghue et al. 1992) were used to examine the support for monophyletic groups identified by the most parsimonious tree.

Lobelia erinus was not included in the restriction-site analysis because data were available for only 4 of the 12 enzymes. This species is a readily available ornamental, and we have therefore not omitted it from the remainder of the study.

Results

cpDNA Organization

Deletions

Three major deletions from the chloroplast genome were suggested by the failure of three tobacco probes to hybridize to some or all of the 18 DNAs investigated in this study under conditions in which most angiosperm cpDNAs hybridized strongly. All examined Lobeliaceae show a deletion corresponding to the region of the genome containing a 512-codon open reading frame (ORF512; deletion A in table 2) of poorly known function (Smith et al. 1991). Two adjacent tobacco probes, a 1.349-kb *Bam*HI fragment containing 5′ ORF512 (59.305–60.654 TCU) and a 1.413-kb *Bam*HI-*Kpn*I fragment containing 3′ ORF512 (60.654–62.067 TCU), failed to hybridize to filter-bound DNAs of all examined species. This gene is also missing from the chloroplast genomes of rice (Hiratsuka et al. 1989), Campanulaceae, and a few other angiosperms (Downie and Palmer 1992). Although the nonhybridizing probes suggest a deletion of 2.8 kb, our map estimates indicate the presence of ~1.2 kb of nonhybridizing DNA in this region. This suggests an actual deletion of 1.6 kb, with sufficient sequence divergence of the flanking regions to prevent hybridization. Because this deletion site has served as an endpoint for subsequent inversions, it is difficult to estimate precisely the position of the deletion. We have presented the estimated midpoints for all three deletions (table 2), and we have used these values as endpoint estimates for inversions that have involved the deletion sites.

Filter hybridization, restriction-site mapping, and polymerase chain reaction (PCR) analysis revealed a 0.9-kb deletion in *Lobelia holstii* (deletion B′ in table 2).

Table 2
Characterization of Chloroplast Genome Rearrangement Patterns

	Size (kb)	Midpoint (TCU)	Endpoint 1 (TCU)	Endpoint 2 (TCU)	Rearrangement Pattern[a] I	II	III	IV	V
Deletions:									
A	1.6	60.7	NA	NA	+	+	+	+	+
B′	0.9	73.3	NA	NA	+				
B″	1.6	73.7	NA	NA		+			
Inversions:									
1	59.0	NA	1.7	60.7		+	+	+	+
2	51.9	NA	1.7/60.7	53.6			+	+	+
3	25.0	NA	28.6	53.6/60.7			+		
4	43.1	NA	10.5	28.6/60.7			+		
5	23.2	NA	10.5/60.7	48.5			+		
6	5.1	NA	48.5	53.6/60.7				+	
7	13.2	NA	47.5	60.7	+				
8	15.0	NA	60.7/47.5	65.3	+				
9	33.9	NA	28.6	65.3/60.7	+				
10	51.5	NA	9.2	1.7/60.7		+			
11	13.0	NA	60.7/1.7	73.7		+			

[a] I = *Lobelia holstii;* II = *Monopsis lutea;* III = *L. fervens* and *L. erinus;* IV = *L. cardinalis;* and V = *L. boninensis, L. giberroa, L. gloria-montis, L. hypoleuca, L. nicotianifolia, L. organensis, L. petiolata, L. stricklandiae, L. bridgesii, L. excelsa, L. polyphylla, L. tupa,* and *Sclerotheca jayorum.* A plus sign (+) indicates presence of a rearrangement, and a blank indicates absence of a rearrangement.

A 0.841-kb *Bcl*I-*Xba*I fragment from tobacco (72.895–73.736 TCU)—corresponding to a *clp*P gene internal region that contains 110 bp of intron 1, all of exon 2 (291 bp), and 440 bp of intron 2—failed to hybridize to *L. holstii* cpDNA, whereas probes containing the 5′ and 3′ ends of *clp*P hybridized strongly to *L. holstii* cpDNA. Restriction-site mapping also identified a 0.9-kb deletion in this region. Finally, primers located in *rps*12 and *psb*B, a separation of 2.9 kb in tobacco, instead yielded a 2.0-kb PCR product in *L. holstii* (E. B. Knox, unpublished data). The other *Lobelia* species examined (and *Sclerotheca jayorum*) all show hybridization patterns, map distances, and PCR products consistent with retention of *clp*P.

Monopsis lutea also showed no hybridization with the internal *clp*P probe described above and showed strong hybridization to the adjacent probes. In this case, however, restriction-site mapping and PCR results indicate that the deletion is significantly larger, possibly as large as 1.6 kb (deletion B″ in table 2), although the results are complicated by the rearrangements in the region (fig. 2). The size differences between these *clp*P deletions complicates the assessment of homology. Either a shared

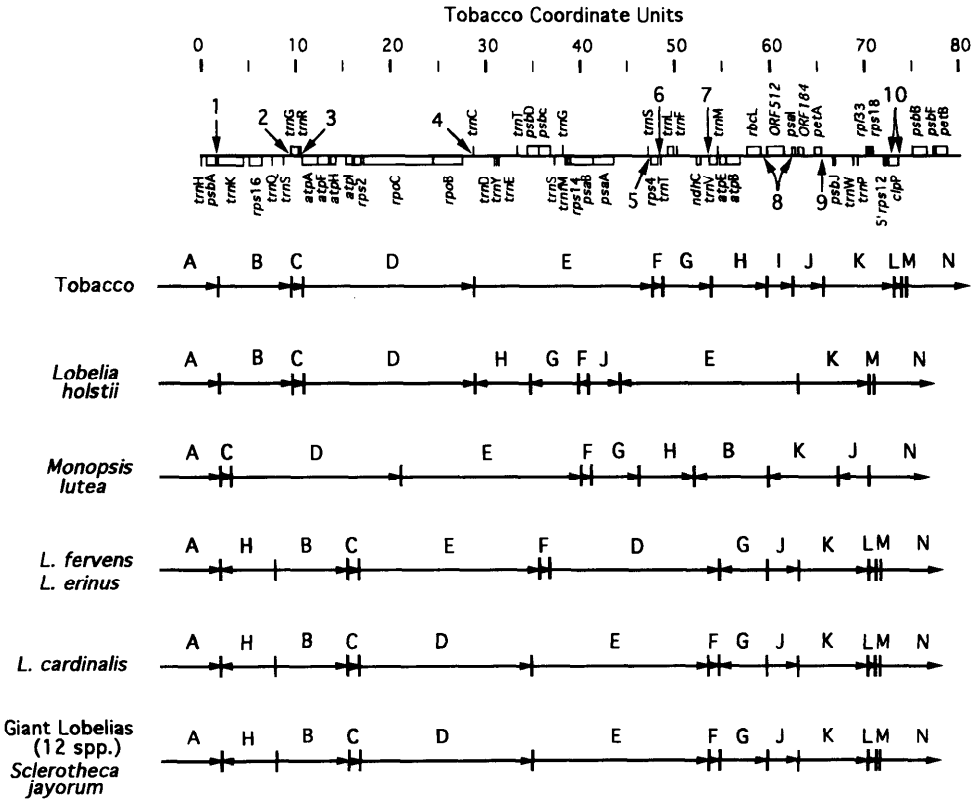


FIG. 2.—Arrangement of homologous fragment blocks in Lobeliaceae and tobacco cpDNAs. The gene map illustrates most genes within most of the large single-copy region of the chloroplast genome of tobacco (modified from Shinozaki et al. 1986). The scale is in tobacco coordinate units. Precise locations of the endpoints of inversions (indicated by the small arrows numbered 1–10) and deletions (arrows numbered 8 and 10) are presented in table 2 and are described in the text. The pattern of gene order in tobacco is compared with the five rearrangement patterns observed in Lobeliaceae. Homologous fragment blocks involved in the hypothesized evolutionary rearrangements are identified by the same letter. The orientation of fragment blocks is indicated by the horizontal arrows.

0.9-kb deletion was followed by a 0.7-kb deletion in *M. lutea,* or the *clp*P deletions represent independent losses. A complete understanding of this situation must await sequencing of the appropriate regions, but the independent loss of portions of *clp*P would seem unsurprising, in light of similar *clp*P losses observed in other widely divergent lineages of angiosperms (Downie and Palmer 1992).

Inversions

Eleven inversions were identified in the chloroplast genomes of Lobeliaceae (table 2). The locations of the endpoints of these inversions are as follows (fig. 2): endpoint 1 maps in the 215-bp spacer region between the genes *psb*A and *trn*K; endpoint 2 maps in the 780-bp spacer between *trn*S and *trn*G; endpoint 3 maps in the 124-bp spacer between *trn*R and *atp*A; endpoint 4 maps close to *trn*C in the 1,282-bp spacer between *rpo*B and *trn*C; endpoint 5 maps in the 331-bp spacer between *trn*S and *rps*4; endpoint 6 maps in the 372-bp spacer between *rps*4 and *trn*T; endpoint 7 maps close to *trn*V in the 1,088-bp spacer between *ndh*C and *trn*V; endpoint 8 maps to the spacer between *rbc*L and *psa*I, which includes the ORF512 deletion; endpoint 9 maps in the 1,066-bp spacer between *pet*A and *psb*J; and endpoint 10 maps to the *clp*P deletion.

Nine of 10 inversion endpoints are located in intergenic spacer regions, and 7 lie close to tRNA genes. The 10th inversion endpoint lies in or near the disrupted *clp*P gene, in what could now be considered spacer region. In each of the five inversion patterns discussed below, the ORF512 deletion site served as an endpoint for at least one inversion, and in three of the patterns this deletion site potentially served as an endpoint for all of the inversions. Three inversions have not used this deletion site directly as an endpoint, but in each of these cases it is possible to construct parsimonious scenarios in which the deletion site is used indirectly as an endpoint from a previous inversion. The *clp*P deletion site served as an inversion endpoint only once, in *M. lutea.* In none of the inversions are both endpoints unique.

In total, five inversion patterns (fig. 2) are apparent for 18 species of Lobeliaceae. Knowledge of the precise number and positions of rearrangement endpoints allows us to present models for the evolution of these genomes from a tobacco-like ancestral genome. If one allows for only deletions and inversions, three events (one deletion and two inversions) suffice to transform the gene order present in tobacco into that found in *S. jayorum* and the 12 giant lobelias. One additional inversion is necessary to create the gene arrangement found in *L. cardinalis,* whereas three additional inversions are necessary to account for the gene arrangement exhibited by *L. fervens* and *L. erinus.* (Restriction-site data from *L. erinus* are available for only 4 of 12 enzymes and so were not included in the restriction-site analysis. Inspection of the restriction-site maps for *L. fervens* and *L. erinus* indicates that these two species are closely related.) For each of the rearrangement patterns exhibited by *M. lutea* and *L. holstii* (fig. 2), two deletion and three inversion events have occurred, with no inversion being the same between these two species.

Comparison of the rearrangement patterns suggests that a progression of inversions has occurred within the Lobeliaceae. With the exception of *L. holstii,* all lobelias (and *S. jayorum*) have two inversions in common (inversions 1 and 2 in table 2). *Monopsis lutea* shares the first of these but not the second, suggesting that the lineage leading to *Monopsis* diverged from the main *Lobelia* lineage prior to the second inversion. The additional inversions observed in herbaceous *L. cardinalis* and *L. fervens* (and *L. erinus*) would have had to occur after inversions 1 and 2 and divergence from the lineage leading to the giant lobelias. The three inversions in *L. holstii* are unique and

clearly not derived from any of the other four patterns. The synapomorphic rearrangements can be used to construct a phylogenetic hypothesis (fig. 3), which suggests that the giant lobelias are derived from herbaceous ancestors.

The hypothesized sequence of rearrangement events during the evolution of the *L. fervens* (and *L. erinus*) chloroplast genome from a tobacco-like ancestral genome is presented in figure 4. Phylogenetic analysis constrains the order of events involving deletion A and inversions 1 and 2 but does not reveal the order of inversions 3–5. The sequence presented assumes that the ORF512 deletion site continued to serve as an endpoint for the final three inversions; however, either the actual temporal order of these inversions or the actual evolutionary changes may have been more complex.

Comparison of the genome arrangements between *L. fervens* (and *L. erinus*) and *L. cardinalis* (fig. 2) indicates a similar position and orientation of fragment G. Treating inversion 6 (fig. 3) as a synapomorphy that unites *L. cardinalis* with *L. fervens* (and *L. erinus*) does not provide a more parsimonious hypothesis. The initial savings of one step, which is due to the shared inversion of fragment G, is offset by an additional step that would be required to transform the *L. cardinalis* genome into the arrangement observed in *L. fervens* (and *L. erinus*). This alternative hypothesis is equally parsimonious at a global level—but not at a local level, because it requires six instead of five inversions (i.e., one more than the minimum) in the lineage leading to *L. fervens* and *L. erinus*. The results of the restriction-site analysis presented below support the hypothesis presented in figure 3.

Phylogenetic Analysis of Restriction-Site Mutations

In total, 132 restriction sites were identified as phylogenetically informative among the 17 species examined (data matrix available on request from E. B. Knox). The phylogenetic analysis conducted by using Wagner parsimony and by implementing the branch-and-bound option (Hendy and Penny 1987) resulted in three equally parsimonious cladograms with a length of 219 steps and a consistency index (CI) of 0.60 (Kluge and Farris 1969). The strict consensus tree is presented in figure 5.

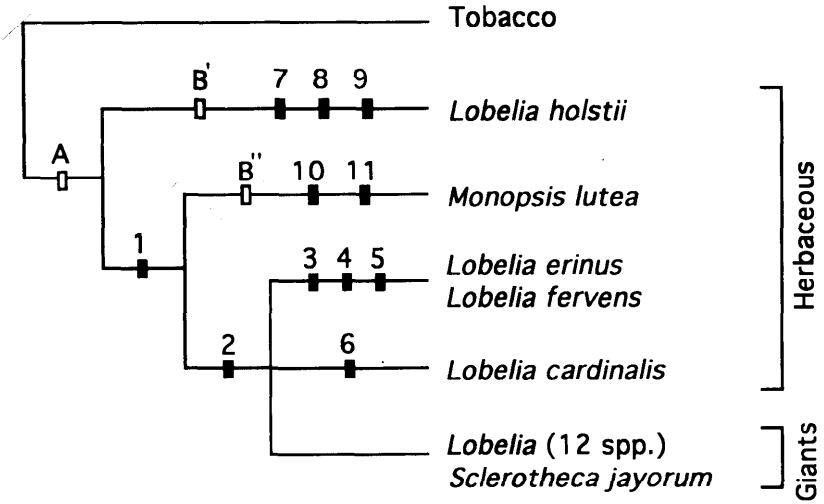


FIG. 3.—Hypothesized phylogeny of Lobeliaceae, on the basis of major structural rearrangements. The 3 deletions (unblackened blocks) and 11 inversions (blackened blocks) are characterized in table 2. The sequence of inversions along each lineage has not been determined and may differ from that presented.

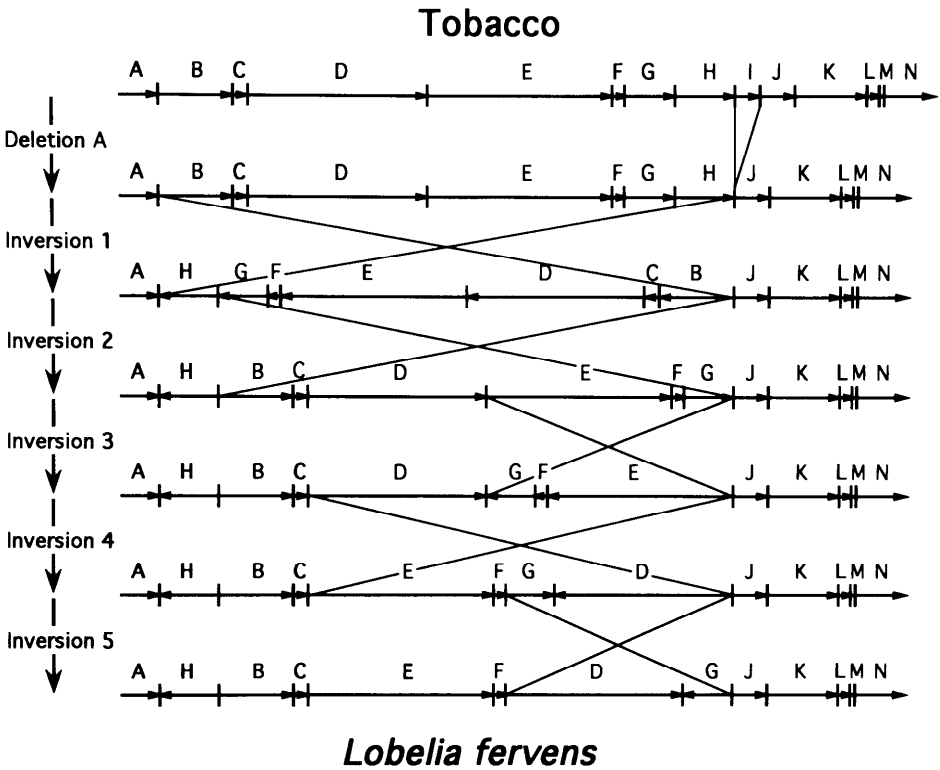


FIG. 4.—Hypothesized sequence of rearrangement events during evolution of the *Lobelia fervens* chloroplast genome from a tobacco-like ancestral genome. Step 1 is the ORF512 deletion from a tobacco-like ancestral genome. Steps 2–6 are 59.0-, 51.9-, 25.0-, 43.1-, and 23.2-kb inversions, respectively. The rearrangement pattern exemplified after the second inversion in the sequence is that of *Sclerotheca* and 12 of the 17 species of *Lobelia* examined. Both the order of deletion A and those of inversions 1 and 2 are revealed by phylogenetic analysis. The order of the remaining inversions may be different from that presented. Lettered arrows indicate homologous fragment blocks and correspond to those presented in fig. 3.

Lobelia holstii was used to root the cladograms because it lacks the synapomorphic inversion (inversion 1 in table 2) found in all other Lobeliaceae included in this study. This study originally included a *Campanula* species to serve as the ultimate outgroup, but unfortunately the *Campanula* genome was so extensively rearranged that homologies in restriction sites could not be confidently determined. Although the *clp*P deletions in *L. fervens* and *M. lutea* are different sizes, we have labeled them B′ and B″ to indicate a possible synapomorphic origin and that inversion 1 might be homoplasious. In the absence of an ultimate outgroup, we used the known sequence of tobacco (Shinozaki et al. 1986) to assess the likelihood that the basal lineage is *L. holstii, M. lutea,* or a clade comprising both species. This admittedly anecdotal approach provides weak support for *L. holstii* as the basal lineage. It is important, however, that varying the outgroup from *L. holstii* to *M. lutea* to both species had no effect on either the remaining topology or the branch lengths beyond the node connecting *L. cardinalis.* Although some uncertainty remains concerning the basal relations, the remainder of the phylogeny appears robust.

A bootstrap analysis (Felsenstein 1985) was conducted with 500 replicates, to measure support for the monophyletic groups identified in the cladogram. A decay

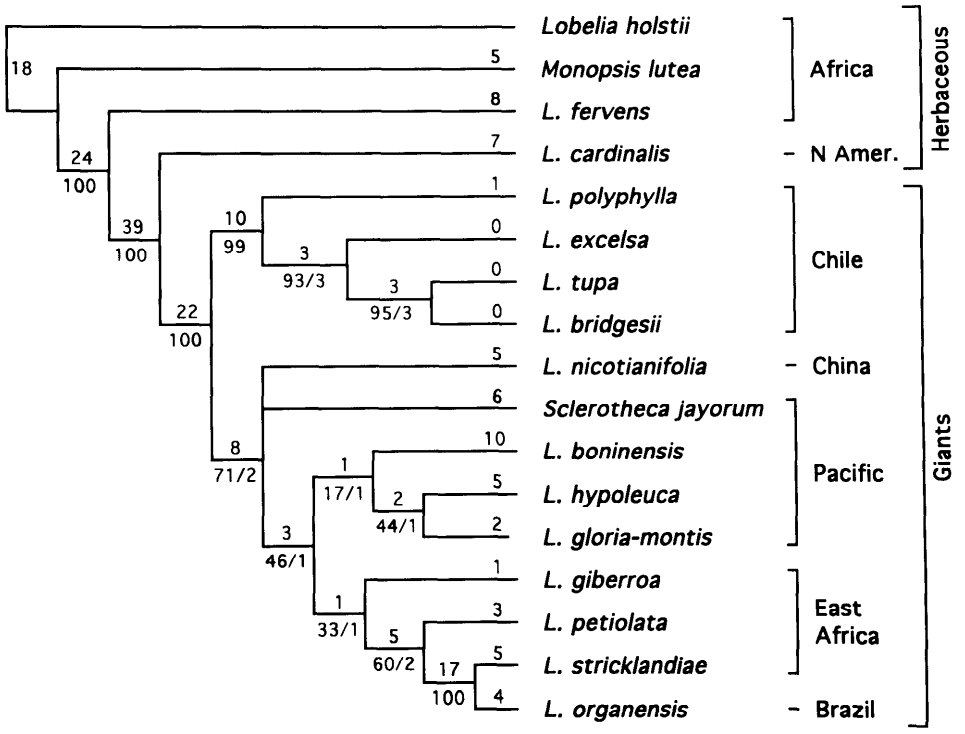


FIG. 5.—Wagner parsimony tree for 17 species of Lobeliaceae, using 132 informative cpDNA restriction-site mutations. This is a strict consensus of three equally parsimonious trees generated by the branch-and-bound option of PAUP. The tree has 219 steps and a CI of 0.60. The numbers above the line at each node and along each lineage indicate the number of site fixations. The number of site fixations preceding the trichotomy is the minimum branch length among the three equally parsimonious trees. Because autapomorphies are not included, these values should not be used to compute genetic distances. The numbers below the lines preceding each slash are the percentage of occurrences of a monophyletic group in 500 bootstrap samples. The numbers after each slash indicate the increased number of steps needed for a clade to lose resolution. Decay values higher than 5 are not included.

analysis (Donoghue et al. 1992) was conducted as an alternative measure of support. The 220-step consensus tree retained clades with bootstrap values at ≥60%; the 221-step consensus tree retained clades at ≥93%; and the 222-step consensus tree retained clades at ≥99%; and these clades were still supported in the 223- and 224-step consensus trees. The bootstrap and decay values are presented in figure 5.

The cladogram is congruent with, but much more highly resolved than, the phylogenetic hypothesis based on cpDNA rearrangements (fig. 3). The relationships among the four herbaceous species are all supported, as are the groupings of the four hexaploid Chilean species and the remaining tetraploid giant lobelias (including *S. jayorum*). With the exception of the clade comprising *L. petiolata, L. stricklandiae,* and *L. organensis,* the topology within the giant lobelia clade receives weak bootstrap support, because of the small number of synapomorphies unaffected by homoplasy.

The stability of the resolution within the giant lobelia clade was investigated by using unequal character-state weighted parsimony analysis and functional ingroup analysis. In virtually all cases the eastern African/Brazilian clade was topologically unaffected despite the moderate bootstrap values for the lower nodes. Relationships among the giant lobelias from eastern Africa are the subject of a dissertation (by

E. B. Knox) and will be reported separately. However, the inclusion of the Brazilian *L. organensis* in the eastern African clade is surprising, in light of the robustness of its association (100% bootstrap value) with *L. stricklandiae* from eastern Africa. The relationships among the remaining species are unstable and must be considered unresolved, although some insight into these relationships is available at this time.

User-defined weighting of restriction-site gain:loss (Albert et al. 1992) over a range of 1:1–1.8:1.0 always yielded one of the three equally parsimonious trees found by using Wagner parsimony. This tree unites *L. nicotianifolia* and *S. jayorum* as the basal clade of the tetraploid giant lobelias. Functional ingroup analysis offers an alternative approach to assessing the influence of homoplasy. Removing the four herbaceous species and using the Chilean clade as an outgroup yields two equally parsimonious trees that vary with respect to the basal taxon (*L. nicotianifolia* vs. *S. jayorum*). These two trees have the same topology as do the two remaining trees found by using Wagner parsimony. The data matrix is sufficiently structured by the Chilean and the eastern African/Brazilian clades that removal of either clade results in a loss of resolution of the remaining giant species. With selective removal of some of the Pacific species it is possible to dissociate *L. giberroa* from the remainder of the eastern African/Brazilian clade. The eastern African/Brazilian clade is diagnosed in the most parsimonious cladograms by a nonhomoplasious gain of a *Hin*dIII restriction site at 81.4 TCU, but the monophyly of this clade is only weakly supported by these data.

Discussion

The chloroplast genomes of Lobeliaceae are rearranged in comparison with the genomes of most other land plants that have been studied, and they provide a model system for studying the evolution of chloroplast genomic structure. Analysis of each of the five genome arrangements (fig. 2) enabled us to generate hypotheses of the most parsimonious transformations from a tobacco-like genome arrangement, by using only inversions and deletions. These locally parsimonious reconstructions were compared for possible synapomorphic events, in order to construct a globally parsimonious hypothesis of plant relationships (fig. 3).

Global parsimony favors our hypothesis of independent *clp*P deletions in *Lobelia holstii* and *Monopsis lutea.* A synapomorphic 0.9-kb deletion would require both an additional 0.7-kb deletion and the independent evolution of inversion 1 in *M. lutea.* The independent loss of *clp*P is more parsimonious, because it, also, requires two deletions, but inversion 1 is synapomorphic. However, deletions, if piecemeal, will be more common than the underlying inactivation of a gene, and these two classes of events should be accorded different weight if the events can be disentangled. Inversions do not suffer from the potentially homoplasious effects of gene transfer, but inversion endpoints are not unique, and a total of three homoplasious inversions have been discovered within the Ranunculaceae (Hoot 1991) and the Caryophyllales (Downie and Palmer, accepted). An inversion of fragment G resulted in the position and orientation observed in *L. cardinalis.* Use of the same endpoints in a more complicated series of inversions left fragment G in the same position and orientation in *L. fervens* (and *L. erinus*). Reconsideration of local parsimony, together with support from the restriction-site data, allowed the differences in process to be disentangled from the similarity in pattern.

Despite the congruence between our phylogenetic trees based on rearrangements and restriction sites, these data sets cannot be compared for some individual hypotheses.

Because *Campanula* was not useful as an ultimate outgroup, the restriction-site data cannot be used to test the hypothesis that (*a*) inversion 1 is synapomorphic and (*b*) the *clp*P deletions are homoplasious. The alternative hypothesis—that the *clp*P deletion is synapomorphic—precludes testing the synapomorphic status of inversion 2. Some facets of our rearrangement-based hypothesis cannot be rigorously tested with our available restriction-site data, and this hypothesis must still be regarded as weak. However, there is no strong evidence to the contrary. A final understanding of the early evolutionary events in the Lobeliaceae, both molecular and morphological, must, however, await a more detailed study (which includes sequencing the rearrangement regions) of the herbaceous members of the family.

The occurrence of autapomorphic inversions in *L. holstii, M. lutea, L. cardinalis, L. fervens,* and *L. erinus* provides the starting point for a screening program of herbaceous lobelias (and related genera), in order to determine relationships within the family. Ideally, further sampling will uncover species with intermediate genome arrangements and thereby permit the sequence of inversion events to be determined by direct interpretation. Chromosomal inversions have been used previously to document the historical pattern of species formation among populations of *Drosophila* within the Hawaiian Islands (Carson 1983). The tempo of cpDNA structural evolution in Lobeliaceae is of interest because it coincides with the extensive morphological evolution in this plant family and provides a separate class of data for use in evolutionary reconstruction.

Nature of cpDNA Rearrangements in the Lobeliaceae

The gene order exemplified by tobacco is similar to the ancestral gene order of nonlycopsid vascular plants (Raubeson and Jansen 1992), because it is found, with few exceptions, in all other examined angiosperms, ferns, and the gymnosperm *Ginkgo biloba* (Palmer 1985; Palmer and Stein 1986; Downie and Palmer 1992). Of the Asteridae examined (>34 families), most are colinear with tobacco; only a few families possess inversions, and these inversions are positionally distinct from those described here (S. R. Downie and J. D. Palmer, unpublished data).

In most altered genomes, the order of genes can be derived from the ancestral form by either one inversion or, as in the case of grasses, three inversions (Quigley and Weil 1985; Howe et al. 1988; Hiratsuka et al. 1989). In only four groups of land plants (peas, subclover, conifers, and geranium) have complex cases of structural rearrangements been described (Palmer et al. 1987, 1988*b;* Strauss et al. 1988; Milligan et al. 1989). In conifers and the two legume groups, sequence rearrangement may have been accelerated because of the absence of the inverted repeat (IR), a structure thought to contribute an element of stability to the chloroplast genome (Palmer and Thompson 1982; Strauss et al. 1988). The presence of multiple inversions in the IR-retaining chloroplast genomes of Lobeliaceae illustrates an unusually rapid rate of evolutionary change in this family, relative to that of most other land plants examined. The factors that may have been responsible for this are discussed below.

The repeated reuse of a few inversion endpoints within Lobeliaceae, as well as the presence of inversion endpoints in similar regions of the genomes of widely divergent plant lineages (discussed below), suggests a possible mechanistic basis for these inversions. Inversions may not be random events but, instead, may rely on some underlying molecular factor responsible for recombinational hot spots (Kung et al. 1982). The occurrence of short repeat elements in highly rearranged cpDNAs (Quigley and Weil 1985; Bowman and Dyer 1986; Palmer et al. 1987; Bowman et al. 1988; Moon

et al. 1988; Strauss et al. 1988; Wolfe 1988; Milligan et al. 1989) has been implicated as a major factor in determining the prevalence of inversions. Homologous recombination between inversely oriented repeat sequences will produce an inversion of the intervening chromosomal segment; however, the exact physical relationship between inversion endpoints and repeated sequence in these rearranged cpDNAs has been little investigated. Also, the mechanistic factors or selective forces by which these repeats are created, dispersed throughout the genome, and putatively involved in generating the inversions are unknown. Inversion endpoints have also been observed to be associated with short, repeated sequences near or within tRNA genes (Howe et al. 1988; Hiratsuka et al. 1989; Shimada and Sugiura 1989), but, again, evidence showing how they might cause recombination is unclear. In Lobeliaceae, tRNA genes are located near the endpoints of most rearrangement fragment blocks (fig. 2). The elucidation of (*a*) the roles of short repeated elements and (*b*) their putative associations with tRNA genes in rearranging chloroplast genomes in Lobeliaceae must await DNA sequencing.

Many of the inversions described in Lobeliaceae have endpoints in the same or adjacent intergenic spacer regions of the genome as do other well-characterized inversions in other taxa. A common endpoint for many inversions occurs in intergenic spacer regions near *trn*G and *trn*R (9.2–10.5 TCU; fig. 2). *Monopsis lutea* and *L. fervens* (and *L. erinus*) have inversion endpoints in this same cluster of tRNA genes, as do pea, mung bean, *Oenothera,* grasses, Douglas fir, and virtually all members of the Asteraceae (Palmer and Thompson 1982; Herrmann et al. 1983; Palmer et al. 1985, 1987; Quigley and Weil 1985; Jansen and Palmer 1987; Strauss et al. 1988; Hiratsuka et al. 1989). A second shared inversion endpoint is located between *rpo*B and *trn*C (28.6 TCU; fig. 2) in *L. fervens* (and *L. erinus*) and *L. holstii.* This endpoint is also used in a small, previously undescribed inversion in lettuce and other members of the Asteraceae that possess the larger, 22-kb inversion (S. R. Downie, E. B. Knox, R. K. Jansen, and J. D. Palmer, unpublished data). The endpoints on either side of *rps*4 (47.5 and 48.5 TCU; fig. 2) are also used in Ranunculaceae (Hoot 1991), and the endpoint between *ndh*C and *trn*V (53.6 TCU; fig. 2) is used in Cactaceae and Chenopodiaceae (Downie and Palmer, accepted).

Many of the genes in angiosperm cpDNA are cotranscribed as part of operons (Palmer 1991). With the exception of two events involving legumes (Palmer et al. 1988*b;* Milligan et al. 1989), all gene rearrangements described in land plants have their boundaries between operons, rather than within them. None of the 11 inversions described in Lobeliaceae disrupts groups of genes that are transcriptionally linked in angiosperm cpDNA. In the case of *M. lutea,* where an inversion maps into *clp*P (table 2), this gene appears to have already been rendered nonfunctional by the prior deletion.

The Lobeliaceae offer a model system for molecular evolutionary studies, because the abundant structural evolution observed in the chloroplast genome has been contemporaneous with prolific speciation and significant morphological evolution. Species with intermediate genome arrangements could be used to characterize the rearrangement events, to evaluate sequence evolution since these events, and to discover the molecular basis of these rearrangements, so common in Lobeliaceae yet so uncommon in most other angiosperms examined.

Phylogenetic Implications

The phylogenetic analyses of cpDNA restriction sites and inversions indicate that the herbaceous habit, as represented by the species examined, constitutes the ancestral condition in Lobeliaceae. Carlquist (1962) used wood anatomy to suggest that the

giant lobelias originated paedomorphically from herbaceous ancestors. This view was challenged by Mabberley (1974*a*, 1974*b*, 1975), who suggested that the herbaceous species of *Lobelia* have been derived from large, thick-stemmed ("pachycaul") ancestors, the blue-flowered species originating from section *Rhynchopetalum* and the red-flowered species originating from section *Tylomium*. Rebuttals from both parties ensued (Carlquist 1980; Mabberley 1982). The molecular data (figs. 3 and 5) support Carlquist's claim of herbaceous ancestry for the giant lobelias and refute Mabberley's claim to the contrary. The monophyly of the giant lobelias is in accordance with the work of Wimmer (1953, pp. 607–677), who placed all our giant species in subgenus *Tupa*.

Mabberley (1975) presented a biogeographic hypothesis for the giant lobelias. This involved a South American origin from fleshy-fruited ancestors, followed by dispersal eastward via Africa and Asia, eventually reaching Hawaii to form section *Revolutella* (represented in this study by *L. hypoleuca*). Dispersal westward from South America was postulated to have given rise to a second Hawaiian section, *Galeatella* (represented by *L. gloria-montis*).

The taxonomic scope of our data does not permit evaluation of all facets of Mabberley's biogeographic hypothesis. In general, the available evidence contradicts his interpretations, although there is one point of agreement. The Chilean species form a distinct basal clade within the giant lobelias, which is not surprising in light of a knowledge of chromosome numbers. The herbaceous core lineage of *Lobelia* is evidently diploid ($n = 7$), whereas the Chilean species are hexaploids ($n = 21$), and the remaining giant lobelias are tetraploids ($n = 14$; E. B. Knox and R. R. Kowal, unpublished data).

Our data suggest that the South American species, not the Hawaiian species, are a mixed assemblage, possibly involving pantropical dispersal. The Hawaiian species (*L. hypoleuca* and *L. gloria-montis*) are weakly supported as a monophyletic group, although some topologies suggest that *L. boninensis* may have been derived from Hawaiian ancestry. The nested position of *L. organensis* in the eastern African clade suggests dispersal from Africa to Brazil, but morphological considerations raise the possibility of more than one transatlantic dispersal event. A definitive statement concerning biogeographic relationships must await examination of other species from Brazil, Cameroon, and Angola.

Carlquist (1969) was correct in surmising that *Sclerotheca* was derived from a *Lobelia*-like ancestor, but our results allow us to make the stronger claim that the genus *Sclerotheca* (as represented by *S. jayorum*) was derived from the genus *Lobelia*. We can make a similar—but currently weaker—claim concerning the derived status of *Monopsis*. A stronger statement requires more definitive resolution of the basal relationships in the family.

Our data weakly support the monophyly of the eastern African giant lobelias inclusive of *L. organensis*. Any of the remaining species included in this study could satisfactorily be used as an outgroup representative for polarizing data on the evolution of the giant lobelias in eastern Africa. However, the lack of resolution among the other giant lobelias makes the hexaploid Chilean species the best choice for use as an outgroup.

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Note added in proof.—ORF512, the site of whose deletion is associated with many of the inversions in Lobeliaceae cpDNAs, has recently been named "accD," in accord with its putative function as encoding the plastid homologue of the β subunit of the carboxyltransferase component of *Escherichia coli* acetyl-CoA carboxylase (Li and Cronan 1992).

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