bation of the vesicles and cytosol with GTP-γ-S before the addition of Gβγ produced a less marked effect (Fig. 3B), consistent with the reduced interaction of Gβγ with Gα subunits bound to GTP-γ-S. Like mastoparan, Gβγ partially inhibited fusion in the absence of GTP-γ-S (*21*).

The function of G proteins in signal transduction has been well characterized. However, recent studies implicate this class of GTP-binding proteins in vesicular transport. $G\alpha_{i\text{-}3}$ regulates transport of proteoglycans through the Golgi (*24*). Aluminum fluoride, which activates G proteins (*11*), blocks in vitro transport along the secretory (*12*) and endocytic pathways (*5, 13*). However, aluminum fluoride effects alone are difficult to interpret because fluoride is also known to inhibit many cellular phosphatases. Our observations that mastoparan reverses both the activation of fusion by GTP-γ-S at low cytosol concentrations and the inhibition of endosome fusion by GTP-γ-S at high cytosol concentrations suggests that a G protein is indeed involved in endosome fusion. Furthermore, our in vitro assay was specifically inhibited by the addition of Gβγ subunits. Our data do not allow us to decipher whether one or more G proteins may be regulating fusion. The effects of mastoparan and Gβγ may seem paradoxical, because mastoparan is expected to increase nucleotide exchange of G proteins whereas Gβγ has the opposite effect. However, a speculative model that includes two G proteins can resolve this paradox. In this model the activation of one G protein by mastoparan induces the dissociation of its Gβγ subunit. The resultant increase in the concentration of free Gβγ would favor binding of Gβγ to the second (stimulatory) G protein and block the function of that G protein. A similar subunit exchange model has been proposed to explain the roles of $G_i$ and $G_s$ in the regulation of adenylyl cyclase (*16*). Factors that participate in the vesicular transport among Golgi stacks interact with membranes by means of GTP-binding proteins. The release of the coat protein β-COP by brefeldin A is inhibited by GTP-γ-S and aluminum fluoride (*25, 26*). GTP-γ-S induces the binding of β-COP and ARF, a small GTP-binding protein, to membranes, and Gβγ also blocks these binding events (*26*). These data indicate that factors related to vesicular transport associate with membranes by means of GTP-binding proteins, some of which seem to be G proteins. We have suggested that GTP-γ-S mediates the irreversible binding of factors to endosomal membranes necessary for priming before fusion (*6*). Because the effect of mastoparan is competed by excess cytosol, it appears that one or more G proteins may influence the binding of cytosolic factors to endosomal membranes.

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# Chloroplast DNA Evidence on the Ancient Evolutionary Split in Vascular Land Plants


LINDA A. RAUBESON* AND ROBERT K. JANSEN†



**Two groups of extant plants, lycopsids and psilopsids, alternatively have been suggested to be the living representatives of the earliest diverging lineage in vascular plant evolution. The chloroplast DNA (cpDNA) gene order is known to contain an inversion in bryophytes and tracheophytes relative to one another. Characterization of tracheophyte cpDNAs shows that lycopsids share the gene order with bryophytes, whereas all other vascular plants share the inverted gene order. The distribution of this character provides strong support for the fundamental nature of the phylogenetic separation of lycopsids and marks the ancient evolutionary split in early vascular land plants.**


PHYLOGENETIC RELATIONSHIPS among the major extant lineages of vascular land plants are poorly resolved. Most recent systematic treatments consider each group a division and recognize no hierarchical relationships between these taxa (*1*). Explicit phylogenies have been produced by only a few workers (*2–5*). When a basal lineage has been hypothesized, it has varied between the psilopsids and lycopsids. Psilopsids (*6*) have been suggested as the earliest diverging clade primarily by neontologists (*2, 5*). These plants consist of dichotomizing aerial axes arising from prostrate rhizomes and appear similar, at least superficially, to some of the earliest appearing fossil vascular plants. Psilopsids lack roots even in the embryo, and the shoots bear emergences that may not be homologous to leaves. Alternatively, paleobotanists have suggested the basal placement of the lycopsids (*7*) on the basis of the stratigraphic occurrence of lycopsids in the fossil record and the


L. A. Raubeson, Department of Biology, Yale University, New Haven, CT 06511–7444.
R. K. Jansen, Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269–3042.

*To whom correspondence should be addressed, at the Department of Biological Sciences, Mount Holyoke College, South Hadley, MA 01075.
†Present address: Department of Botany, University of Texas, Austin, TX 78713.

**Fig. 1.** Representation of the strategy used to detect the orientation of the 30-kb inversion. The liverwort (top) and tobacco (bottom) genomes are represented through the region of the 30-kb inversion, arrows mark the position of the inversion endpoints. The positions of genes located adjacent to the inversion endpoints are indicated. Regions indicated by 31, 2, 10, and 12 correspond to tobacco cloned fragments used in the application of the strategy as described (*22*). The region marked 31 is in the inverted repeat of tobacco and in the large, single-copy region, adjacent to the inverted repeat, in the liverwort. When restriction fragments are detected that are homologous to both 31 and 10 (fragments spanning the region marked A) and others are detected that are homologous to both 2 and 12 (fragments spanning the region marked B), the genome shares the orientation with liverwort. Alternatively, when restriction fragments are detected spanning the region marked C (homologous to both 31 and 2 but not to 10) and the region marked D (homologous to 10 and 12 but not to 2), the genome is colinear with tobacco. The cloned tobacco fragments were chosen to be as close as possible to the endpoints, but this choice was constrained by the heterologous nature of the comparisons. Regions of tobacco had to contain sequences of high conservation among all the chloroplast genomes of vascular plants to achieve hybridization signal. Thus, the "endpoint" probes vary in distance from the endpoints with a proportionate difference in minimum size of possible spanning fragments. To apply the strategy we isolated total genomic DNA from fresh leaf tissue of representatives (Table 1) of the major vascular plant groups with the technique of Doyle and Doyle (*23*) with the addition of 2% w/v polyvinyl pyrrolidone (PVP) to the extraction buffer. The DNA was digested with numerous restriction enzymes (in single-enzyme digests) that cut infrequently in vascular plant cpDNA (6-bp, G,C–rich recognition site). The digested DNA was separated by electrophoresis in 0.75% agarose gels (run 15 to 20 cm) and transferred to a nylon membrane (Zetabind) according to the manufacturer's (Cuno, Meriden, Connecticut) instructions. The filters were hybridized sequentially to the four tobacco probes. [Probes were radioactively labeled with nick-translation; there was a 40-hour incubation at 55°C and washes in 2× saline sodium citrate (SSC) at 55°C.] The resulting films were then examined for patterns of fragments shared between probes. Results are given in Table 1.

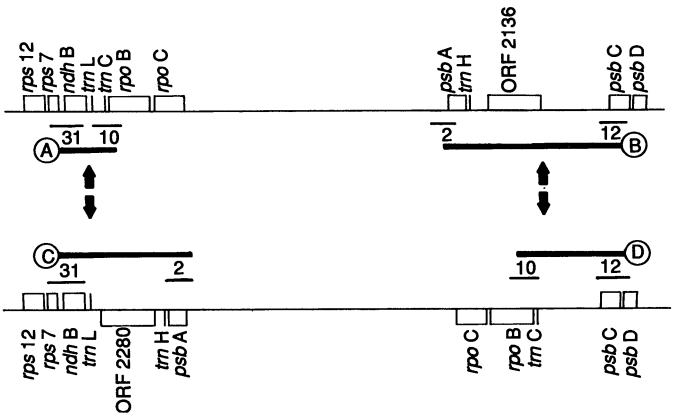


**Table 1.** Restriction fragments spanning the inversion endpoints. A, B, C, and D refer to regions spanning inversion endpoints in the two orientations of the 30-kb inversion as indicated in Fig. 1. The numbers 31, 10, 12, and 2 denote the cloned tobacco fragments used to detect fragments by filter hybridization. Sizes of the detected fragments are given in kilobase pairs. Restriction enzymes are V, Pvu II; H, Hind III; K, Kpn I; N, Nsi I; B, Bam HI; S, Sac I; and P, Pst I.

| Taxon | Orientation | |
|---|---|---|
| | *Liverwort* | |
| | A | B |
| | 31/10 | 2/12 |
| *Lycopodium* | 22 V | 15 K |
| | 23 H | |
| | 6.5 N | |
| *Selaginella** | 12 K | |
| *Isoetes** | 12 B | 7.2 B |
| | 12.3 H | 4.5 H |
| | *Tobacco* | |
| | C | D |
| | 31/2 | 10/12 |
| *Equisetum* | 30+ S | 8.8 B |
| | 20 V | 30+ V |
| | 8.0 H | 7.7 H |
| *Psilotum* | 10 N | 6.7 B |
| | 16 V | 6.6 H |
| *Botrychium** | | |
| *Marattia* | | 30+ B |
| | | 8.5 H |
| *Osmunda* | 15 V | 7.0 V |
| | 12 N | 9.0 H |
| | 30+ K | 6.2 K |
| *Podocarpus* | 15 P | 16 P |
| | | 12 S |
| *Ginkgo* | 2 N | 13 H |
| | 12 S | |
| | 9 V | |
| *Cycas* | 16 K | 10 H |
| | 12 V | |
| *Ephedra* | 13 B | 15 B |
| | 7 H | 16 K |
| | 25 K | 9 S |

*Presence of additional inversions in the region reduces the likelihood of detecting spanning fragments.

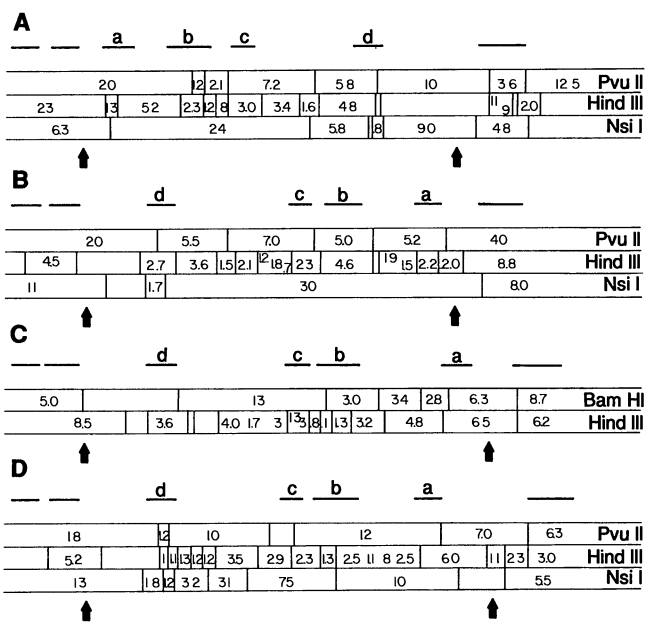


**Fig. 2.** Physical maps for the region of the 30-kb inversion. Each row of boxes represents a separate enzyme digest. Restriction sites of enzymes (indicated by vertical lines) are mapped relative to one another on the basis of information from double digests. Sizes of restriction fragments are indicated in kilobase pairs. Arrows below the maps represent the approximate extent of the 30-kb region. Above the maps, positions of individual genes are indicated. The two bars to the far left represent genes (*rps* 7, *rps* 12, and *ndh* B) outside of the rearrangement, adjacent to the inverted repeat. The unlabeled bar to the far right represents the position of *psb* D and *psb* C. Genes shown within the inversion are a = *rpo* C, b = *rpo* B, c = *atp* I, and d = *psb* A. Maps were generated by overlap hybridization (*24*) with cloned tobacco fragments provided by J. D. Palmer under conditions described in Fig. 1. Each of the taxa listed in Table 1 was mapped through the region of the inversion for at least two enzymes and the double digest. Shown are (**A**) *Lycopodium*, which shares the inversion orientation with *Marchantia*, and (**B**) *Equisetum*, (**C**) *Psilotum*, and (**D**) *Osmunda*, which share the 30-kb inversion with tobacco.

proposed phylogenetic relationships of early (Devonian) vascular land plants (*3, 4, 8–10*).

In a quest for novel, independent characters applicable to this question, we examined structural aspects of the chloroplast genome in representatives of each of the major extant lineages of vascular land plants. Structural mutations have proven useful in clarifying angiosperm phylogenetic relationships (*11, 12*), but nonangiospermous vascular plant cpDNAs have not been as fully investigated. In vascular land plants, structural mutations are rare, and the basic structure of the chloroplast genome appears to be highly conserved (*13, 14*). The fern *Osmunda cinnamomea* and the gymnosperm *Ginkgo biloba* share the gene order with the angiosperms tobacco and spinach. These tracheophyte cpDNAs differ from the chloroplast genome of the bryophyte, *Marchantia polymorpha*, by one 30-kb (kilobase pair) inversion (*15*). The one moss that has been examined has a

gene order similar to that of liverwort (*16*). It has been assumed that bryophytes and tracheophytes are each constant for one of these two inversion orientations (*12, 16, 17*). However, extensive sampling within either group (and especially among lower vascular plants) has not been conducted. In this study, representatives of all extant vascular plant groups were examined to determine the distribution of the inversion.

To determine the gene order in the region of the 30-kb inversion, we adopted two strategies: (i) a simple method of assessment to detect restriction fragments spanning the inversion endpoints (Fig. 1) and (ii) construction of physical maps in the region of the inversion. Detection of fragments (Table 1) and physical maps for the region of the 30-kb inversion (Fig. 2) provided data on the gene order of the region for representatives of each major extant lineage of vascular plant.

Our data show that the three lycopsids (*Lycopodium*, *Selaginella*, and *Isoetes*) share the orientation of the 30-kb region with *Marchantia*. The remaining vascular land plants share the tobacco orientation. Recent phylogenetic analyses all support a basal, paraphyletic position of the bryophytes (*2, 18*). Therefore, using *Marchantia* to polarize the inversion, we consider the *Marchantia*-moss-lycopsid condition to be ancestral (*19*), whereas the remaining (nonlycopsid) vascular land plants have the derived condition. The distribution of this character identifies the lycopsid group as the basal lineage in the vascular land plants. On the basis of the distribution of this character, *Psilotum*, sometimes considered primitive, is a derived member of the tracheophytes. Although psilopsids retain seemingly primitive features, they do have derived morphological characters, such as relatively complex vasculature and fused sporangia, in addition to the derived cpDNA inversion character. The basal position of the lycopsids has been supported by two lines of fossil evidence: (i) the early occurrence (Upper Silurian) of *Baragwanathia*, which has derived lycopsid features (*20*) and (ii) the proposed relationships of early vascular plants—rhyniophytes, zosterophylls, and trimerophytes—and their modern derivatives (*21*).

Our results offer additional support to the hypothesis that the lycopsids are the only modern descendants of one lineage resulting from the first evolutionary split of the ancestral stock of vascular land plants. We consider the distribution of the cpDNA inversion character to complement the independent paleobotanical evidence and to reinforce the isolated position of lycopsids among the vascular plants.

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7. Lycopsid = Lycophyta/Lycopsida, extant taxa Lycopodiales, "club mosses" (*Lycopodium* sensu lato, *Phylloglossum*), Selaginellales, "spike mosses" (*Selaginella*), and Isoetales, "quill worts" (*Isoetes*, *Stylites*). There are fossil representatives throughout the record over the past 400 million years.
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15. Palmer and Stein (*13*) generated cpDNA physical maps of *Osmunda* and *Ginkgo* and determined the positions of 20 genes. The positions of the 20 genes in the fern and gymnosperm were identical to the published gene positions of spinach. Since then the *Marchantia*, tobacco, and rice genomes have been completely sequenced [K. Ohyama *et al.*, *Nature* **322**, 572 (1986); K. Shinozaki *et al.*, *EMBO J.* **5**, 2043 (1986); J. Hiratsuka *et al.*, *Mol. Gen. Genet.* **217**, 185 (1989), respectively], and physical maps have been generated for many additional angiosperms (*12, 14*), some conifers [S. H. Strauss, J. D. Palmer, G. T. Howe, A. H. Doerksen, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3893 (1987); E. E. White, *Theor. Appl. Genet.* **79**, 119 (1990); L. A. Raubeson and R. K. Jansen, *Biochem. Syst. Ecol.* **20**, 17 (1991)], and additional leptosporangiate ferns [M. Hasebe and K. Iwatsuki, *Curr. Genet.* **17**, 359 (1990); D. B. Stein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press]. Tobacco and *Marchantia* share 70 of 71 genes in the large single-copy region in identical order except for the 30-kb inversion. In those tracheophytes examined to date, although some contain other rearrangements, all share the tobacco orientation of this inversion.
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20. Lycopsids are defined by synapomorphies (derived features) such as exarch xylem maturation (shared with zosterophylls), microphyllous leaves, and abaxial, foliar-borne sporangia, which are found in the fossil *Baragwanathia* from the Upper Silurian–Lower Devonian of Australia [(*10*); M. J. Garratt, J. D. Tims, R. B. Rickards, T. C. Chambers, J. G. Douglas, *J. Linn. Soc. London Bot.* **89**, 355 (1978); H. P. Banks, in *Biostratigraphy of Fossil Plants*, D. L. Dilcher and T. N. Taylor, Eds. (Dowden, Hutchinson, and Ross, Strausbourg, PA, 1980), pp. 1–24]. *Baragwanathia* is morphologically very similar to the modern lycopsid *Lycopodium lucidulum*. Thus, lycopsids are the first derived tracheophyte group to appear in the fossil record (possible sphenopsids, "horsetails," appear in the uppermost Devonian almost 50 million years later) and occur earlier than the generalized trimerophyte morphology compared with (but lacking derived features of) psilopsids (*9, 10*).
21. Early vascular plants consisted of simple, small, leafless, rootless dichotomizing axes (stems) bearing sporangia [reviewed in (*10*)]. Rhyniophytes appeared first and have small centrach xylem strands and terminal sporangia. Rhyniophytes are postulated to have given rise to zosterophylls and trimerophytes. Zosterophylls have exarch xylem and lateral sporangia and are thought to be ancestral to lycopsids [for example, K. Niklas and H. P. Banks, *Am. J. Bot.* 77, 274 (1990)]. Trimerophytes, somewhat larger and more complex than rhyniophytes, also have centrach xylem maturation and terminal sporangia. Trimerophytes are putative ancestors of the remaining (nonlycopsid) vascular plant groups (*4*).
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