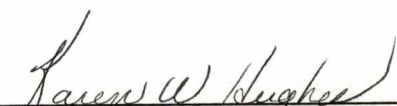
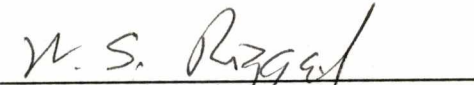
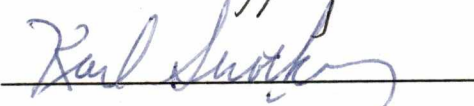
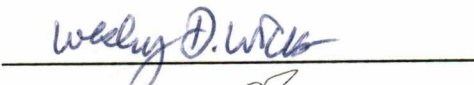


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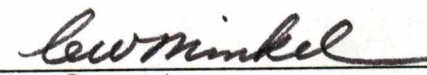
I am submitting herewith a dissertation written by Patrick Joseph Calie entitled "A Molecular Characterization of the Chloroplast Genome of the Bryophyte Physcomitrella patens (Hedw.) BSG." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.


Karen W. Hughes, Major Professor

We have read this dissertation
and recommend its acceptance:


Accepted for the Council:


Vice Provost
and Dean of the Graduate School

A MOLECULAR CHARACTERIZATION OF THE CHLOROPLAST GENOME
OF THE BRYOPHYTE PHYSCOMITRELLA PATENS
(HEDW.) BSG

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Patrick Joseph Calie

December 1986

DEDICATION

To those special people in my life
who gave me the strength to believe in myself
and the courage to reach for the stars.

ACKNOWLEDGMENTS

I thank Dr. Karen W. Hughes for serving as my major professor. Appreciation is also extended to Dr. B. C. Mullin for her efforts expended on my behalf; to Dr. E. E. Schilling for his discussions and insights concerning plant evolution and phylogeny; and to Drs. K. Sirotkin, W. S. Riggsby, and W. D. Wicks for their reviews and evaluations of this research.

I also thank Dr. P. L. Walne for her personal interest in my development as a scientist; Dr. W. D. Wicks for providing me with the opportunity to conduct research in molecular biology; Drs. Doris Schlichter and John Wills for teaching me the fundamentals of molecular biology manipulations and techniques; and Dr. W. S. Riggsby for encouraging me to think and question in an analytical and logical manner.

For technical assistance in the laboratory I am grateful to Mr. J. Thom Dermody, Ms. Kym Clevenger, Ms. Nancy Fredrick, and Mr. Pat Kimmons. Their many hours spent in the cold room provided me with welcome relief from my plastid purgatory.

In addition to all my associates in the Department of Botany, I am very grateful to Mr. K. D. McFarland for his friendship, encouragement, and discussions of bryology; and to Ms. Nancy Fredrick, for being the friend and person that she is.

Appreciation is extended to the following individuals for their useful discussions, technical support, and other interactions:

Dr. Gary Sayler, Dr. Malcolm Shields, Mr. Alec Breen, Dr. Jeff Becker, Mr. Michael Island, Dr. Herb Smith, Dr. Brent Lasker, Mr. Andy Shaw, Mr. Mark Doolittle, and Dr. John Koontz.

And finally, I thank my family and my wife, Mary Reeves, for their continuous patience, support, and encouragement throughout this project.

ABSTRACT

A restriction endonuclease cleavage site map for the enzymes Cla I and Bgl II has been constructed, by filter hybridizations using purified Cla I restriction fragments as hybridization probes, for the chloroplast genome of a bryophyte, the moss Physcomitrella patens (Hedw.) BSG. The plastid chromosome contains approximately 122 kb organized into a small (17 kb) and a large (81 kb) single copy region separated by two copies of a repeat sequence (12 kb) oriented in an inverted arrangement. The following genes have been mapped using heterologous probes from corn, spinach, pea, and petunia: the 16S and 23S rRNA genes; the ribosomal protein genes rpl 2 and rps 19; the ATP synthase subunit genes atp A, B, E, H, and F; the Photosystem I P700 chlorophyll apoprotein genes psa A1 and psa A2; the cytochrome f (pet A), cytochrome b6 (pet B), and subunit 4 of the cytochrome b/f complex (pet D) genes; the gene encoding the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (rbc L); and the genes for the Photosystem II 32 kD herbicide binding protein (psb A), the 51 kD and the 44 kD herbicide binding proteins (psb A), the 51 kD and the 44 kD chlorophyll a binding proteins (psb B and psb C), the "D-2" protein (psb D), and cytochrome f (psb E). With the exception of a 20 kb inversion bordered internally by psb A and atp H, the general order and arrangement of the moss chloroplast genes are very similar to the consensus angiosperm chloroplast genome

typified by that of spinach. In the moss chloroplast genome, as in the majority of the angiosperm chloroplast genomes, there is a correlation between the presence of an inverted repeat structure and a highly conserved gene order.

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CHAPTER I

INTRODUCTION

Size and Conformation of Chloroplast DNA

In the majority of plants surveyed, the chloroplast genome is represented by a single, closed circular molecule (Bedbrook and Kolodner, 1979; Wallace, 1982; Whitfeld and Bottomley, 1983; Palmer, 1985), the sole exception being two species of brown algae which contain multipartite chloroplast genomes (Dalmon et al., 1983). The chloroplast contains approximately 20-60 copies of plastid DNA molecule (Whitfeld and Bottomley, 1983).

In algae, the size for chloroplast genomes ranges from 85 kb in Codium fragile (Chlorophyta) (Hedberg et al., 1981) to over 2000 kb in Acetabularia mediterranea (Chlorophyta) (Green et al., 1977). In terrestrial plants the size range is much narrower. Over 200 species representing 40 families have been surveyed, and range from 120-160 kb (Palmer, 1985). Three exceptions are the chloroplast genomes of Nicotiana acuminata (Solanaceae) (170 kb) (Shen et al., 1982), Spirodela oligorhiza (Lemnaceae) (180 kb) (van Ee et al., 1982), and Pelargonium hortorum (Geraniaceae) (217 kb) (J. Palmer, J. Nugent, and L. Herbon, unpubl.).

Repeat Sequences

There are three distinct types of repeat sequences present in chloroplast genomes: tandem, dispersed, and inverted repeats.

Acetabularia mediterranea contains 5 copies of a 10 kb tandem repeat (Tymms and Schweiger, 1985). The photosynthetic protist, Euglena gracilis (Euglenophyta), contains from 1-5 copies of a 6 kb tandem repeat, which encode a complete set of ribosomal RNA (rRNA) genes (5S, 16S, and 23S) (Köllner and Delius, 1982; Köllner et al., 1984; Wurtz and Buetow, 1981).

Dispersed repeats are found in both algal and angiosperm taxa. Chlamydomonas reinhardtii and C. smithii (Chlorophyta) contain 25-40 copies of a 100-300 bp repeat scattered throughout the chloroplast genome (Rochaix, 1978; Rochaix and Malnoe, 1978; Gelvin and Howell, 1979; Palmer et al., 1985). Trifolium subterraneum (Leguminosae) contains 5 copies of a 200-1000 bp repeat scattered throughout 1/4 of the genome (J. Palmer and W. Thompson, unpubl.).

The most common type of repeat found in chloroplast genomes is the inverted repeat. With the exception of the taxa mentioned above, it is found in virtually all chloroplast genomes surveyed. It ranges from 10-76 kb, and divides the chromosome into a small (20 kb) and a large (80 kb) single copy region (Palmer, 1985).

A number of postulates have been put forth concerning the functional significance of the inverted repeat structure. Adelberg and Bergquist (1972) proposed that the structure prevents loss via deletions of sequences contained within the repeat by allowing for intramolecular recombination between the repeats. Karrer and Gall (1976) hypothesized that inverted repeats, which contain the

extra-chromosomal rRNA genes in the protozoan Tetrahymena pyriformis, could allow for a mechanism (e.g., intramolecular recombination) which could maintain sequence homogeneity within the repeats. Conversely, Barker and Dayhoff (1980) proposed that the inverted repeat, due to the presence of duplicate sequences, could allow for increased evolutionary flexibility through sequence divergence, leading to the creation of functionally different gene products.

Another hypothesis is that the inverted repeat in chloroplasts functions to stabilize the genome against major sequence rearrangements (Fluhr and Edelman, 1981a; Palmer and Thompson, 1981, 1982; Palmer, 1985). There are three lines of evidence which support this proposal: 1) global cross-hybridization, 2) gene-mapping, and 3) comparative restriction-mapping investigations.

1) In global cross-hybridization experiments, chloroplast DNA restriction fragments, representing an entire genome, are used individually as hybridization probes against restriction endonuclease digests of chloroplast DNA from a different taxon to locate and map regions of homology. Thus, the extent of sequence rearrangement can be determined. For example, Nicotiana tabacum (Solanaceae) restriction fragments were used as probes against chloroplast genomic digests of Petunia parodii (Solanaceae), Atropa belladonna (Solanaceae), and Spinacia oleracea (Chenopodiaceae). All four genomes were essentially colinear with respect to their sequence arrangement, the sole exception being a small single-copy region near the end of one of the inverted repeats in Spinacia oleracea (Fluhr and Edelman,

1981a). In a separate set of experiments clone banks of Vigna radiata (Leguminosae) and Pisum sativum (Leguminosae) were hybridized to chloroplast genomic digests of Pisum sativum, Vigna radiata, Spinacia oleracea, Petunia hybrida, Vicia faba (Leguminosae), Cucumis sativa (Cucurbitaceae), and Zea mays (Gramineae). Both Pisum sativum and Vicia faba lack the inverted repeat; the other taxa have it. The arrangement of sequences in the genomes with the inverted repeats is highly conserved, while genomes lacking the inverted repeat exhibited an "extremely scrambled" arrangement of sequences. There are only two rearrangements among the 5 genomes with the repeat structure. In one case, an inversion of 50 kb differentiates Spinacia oleracea, Petunia hybrida, and Cucumis sativa from Vigna radiata. In another instance, Zea mays differs from Spinacia oleracea, Petunia hybrida, and Cucumis sativa by a second event--a small inversion within the larger 50 kb inversion. In contrast, numerous rearrangements are seen when either Vigna radiata or Pisum sativum are compared to Vicia faba. Thus, there are more rearrangements between the chloroplast genomes of two members of the same tribe of the Leguminosae (Viciae) than among members of different families or orders where the inverted repeat is present.

2) Gene-mapping studies have been used to assay the positional conservation of specific genes among more distantly related chloroplast genomes. Representative taxa from 24 different angiosperm families contain a gene order similar to that of Spinacia oleracea (Palmer, 1985) (Table 1--gene symbols are given in Table 2). Representative

Table 1. Chloroplast genomes for which the locations of specific genes have been determined.

Taxon	Genes ¹	References
BRYOPHYTA		
<u>Marchantia polymorpha</u>	16S, 23S rRNA rbc L	Ohyama et al., 1983
PTERIDOPHYTA		
<u>Osmunda cinnamomea</u>	16S, 23S rRNA rbc L atp A,B psb A	Palmer and Stein, 1982
DICOTYLEDONES		
<u>Atriplex triangularis</u>	16S, 23S rRNA rbc L psb A	Palmer, 1982
<u>Beta vulgaris</u>	16S rRNA rbc L psb A	Brears et al., 1986
<u>Brassica</u> spp.	16S, 23S rRNA rbc L psb A atp A,B,E,H pet A	Palmer et al., 1983 Vedel and Mathieu, 1983 Xiao et al., 1986
<u>Carthamus tinctorius</u>	16S, 23S rRNA rbc L psb A atp A	Ma and Smith, 1985
<u>Cucumis sativa</u>	16S, 23S rRNA rbc L psb A	Palmer, 1982
<u>Glycine max</u>	16S, 23S rRNA rps 19 rbc L psb A atp A,B,E,H pet A	Palmer et al., 1983 Spielmann et al., 1983 Spielmann and Stutz, 1983 Singh et al., 1985

Table 1 (continued)

Taxon	Genes ¹	References
<u>Lycopersicon</u> spp.	16S, 23S rRNA rbc L	Palmer and Zamir, 1982
<u>Lycopersicon</u> <u>esculentum</u>	16S, 23S rRNA rbc L psb A,B,C,D,E atp A,B,E,F,H pet A,B,D	Phillips, 1985
<u>Medicago</u> <u>sativa</u>	16S, 23S rRNA rbc L psb A atp A,B,E	Palmer et al., 1985a
<u>Nicotiana</u> <u>otophora</u>	16S, 23S rRNA rbc L	Zhu et al., 1982
<u>N. tabacum</u>	16S, 23S rRNA rbc L rpl 2, rps 19 psb A atp A,B,E,H pet A,C,D	Lin and Kung, 1984 Sugita et al., 1984 Fluhr et al., 1983
<u>Oenothera</u> <u>hookeri</u>	16S, 23S rRNA rbc L psb A,E atp A pet A	Tyagi and Herrmann, 1986
<u>Petunia</u> <u>hybrida</u>	16S, 23S rRNA rbc L atp A,B,E,H	Bovenberg et al., 1984a, 1984b
<u>Phaseolus</u> <u>vulgaris</u>	16S, 23S rRNA rbc L psb A	Palmer et al., 1983b
<u>Pisum</u> <u>sativum</u>	16S, 23S rRNA rbc L psa A1,A2 psb A,B,C,D,E atp A,B,E,H pet A,B	Bookjans et al., 1986 Berends et al., 1986 Courtice et al., 1985 Huttly and Gray, 1984 Smith and Gray, 1984 Willey et al., 1983

Table 1 (continued)

Taxon	Genes ¹	References
<u>Sinapis alba</u>	16S, 23S rRNA rbc L psb A	Link, 1981 Link and Langridge, 1984
<u>Solanum</u> spp.	16S, 23S rRNA rbc L psb A	Palmer and Zamir, 1982
<u>Spinacia oleracea</u>	16S, 23S rRNA rbc L rpl 2, rps 19 psa A1,A2 psb A,B,C,D,E atp A,B,E,H pet A,B,D	Erion et al., 1981 Alt et al., 1983a, 1983b Westhoff et al., 1983 Holschuh et al., 1984 Westhoff et al., 1985a, 1985b
<u>Vicia faba</u>	16S, 23S rRNA rbc L psb A atp A,B,E,H pet A	Palmer and Thompson, 1982 Ko et al., 1983
<u>Vigna radiata</u>	16S, 23S rRNA rbc L psb A	Palmer et al., 1983b
MONOCOTYLEDONES		
<u>Pennisetum americanum</u>	16S, 23S rRNA rbc L psb A atp B	Thomas et al., 1984
<u>Oryza sativa</u>	16S, 23S rRNA rbc L psb A	Hirai et al., 1985
<u>Spirodela oligorhiza</u>	16S, 23S rRNA rbc L atp A,B,E psb A	van Ee et al., 1982 deHeij et al., 1983

Table 1 (continued)

Taxon	Genes ¹	References
<u>Triticum aestivum</u>	16S, 23S rRNA rbc L psb A,B,C,D,E atp A,B,E,H pet A	Bowman et al., 1981 Howe et al., 1982 Howe et al., 1983 Willey et al., 1984b Courtice et al., 1985
<u>Zea mays</u>	16S, 23S rRNA rbc L psb A atp B,E	Larrinua et al., 1983 Schwarz and Kossel, 1980

¹Gene symbols are identified in Table 2.

Table 2. Symbols and identities of gene probes.

Symbol	Gene-Encoded Product
	<u>ATP Synthase:</u>
atp A	Coupling Factor _I -
atp B	the alpha,
atp E	beta, and
	epsilon subunits
atp F	Coupling Factor ₀ -
atp H	subunit I and
	the proton-translocating subunit
	<u>Photosystem I:</u>
psa A1	the two P700 chlorophyll a apoproteins
psa A2	
	<u>Cytochrome b/f Complex:</u>
pet A	cytochrome f,
pet B	cytochrome b6, and
pet D	subunit 4
	<u>Photosystem II:</u>
psb A	the "32kD" herbicide binding protein,
psb B	the 51 kD and
psb C	the 44 kD chlorophyll a binding proteins,
psb D	the "D-2" protein, and
psb E	cytochrome b559
	<u>Ribulose-1,5-bisphosphate Carboxylase/Oxygenase:</u>
rbc L	the large subunit
	<u>Ribosome Constituents:</u>
16S rDNA	the 16S rRNA and
23S rDNA	the 23S rRNA
rpl 2	The chloroplast ribosomal proteins exhibiting
rps 19	homology to the <u>E. coli</u> ribosomal proteins
	L2 and S19, respectively

taxa from 6 different angiosperm families have gene order differences which can be explained on the basis of 1 or 2 simple inversions (Palmer, 1985). Those taxa which lack the inverted repeat (Pisum sativum and Vicia faba) have a gene arrangement which is very different from that of Spinacia oleracea, and can be explained on the basis of massive sequence rearrangements within the chloroplast genome (Palmer, 1985).

3) In many cases the level of sequence conservation extends to restriction endonuclease cleavage sites. Studies involving closely related taxa, e.g., Triticum spp. and Aegilops spp. (Bowman et al., 1983), Nicotiana tabacum and Spinacia oleracea (Fluhr and Edelman, 1981a), 5 Oenothera species (Gordon et al., 1982), 12 Lycopersicon species (Palmer and Zamir, 1982), 11 Nicotiana species (Salts et al., 1984), and Atriplex triangularis (Chenopodiaceae) and Spinacia oleracea (Palmer, 1982) have shown that the restriction maps between species of the same genus and, in some cases, of different genera but of the same family are highly conserved. No such conservation is seen between Vicia faba and Pisum sativum (Palmer, 1985).

Gene Organization

Most chloroplast genomes which contain an inverted repeat exhibit a highly conserved gene order and arrangement, typified by that of Spinacia oleracea (Figure 1A). Several deviations from this consensus, due to inversions, have been documented--e.g., in Vigna radiata (Palmer et al., 1983b), Oenothera hookeri (Tyagi and Herrmann,

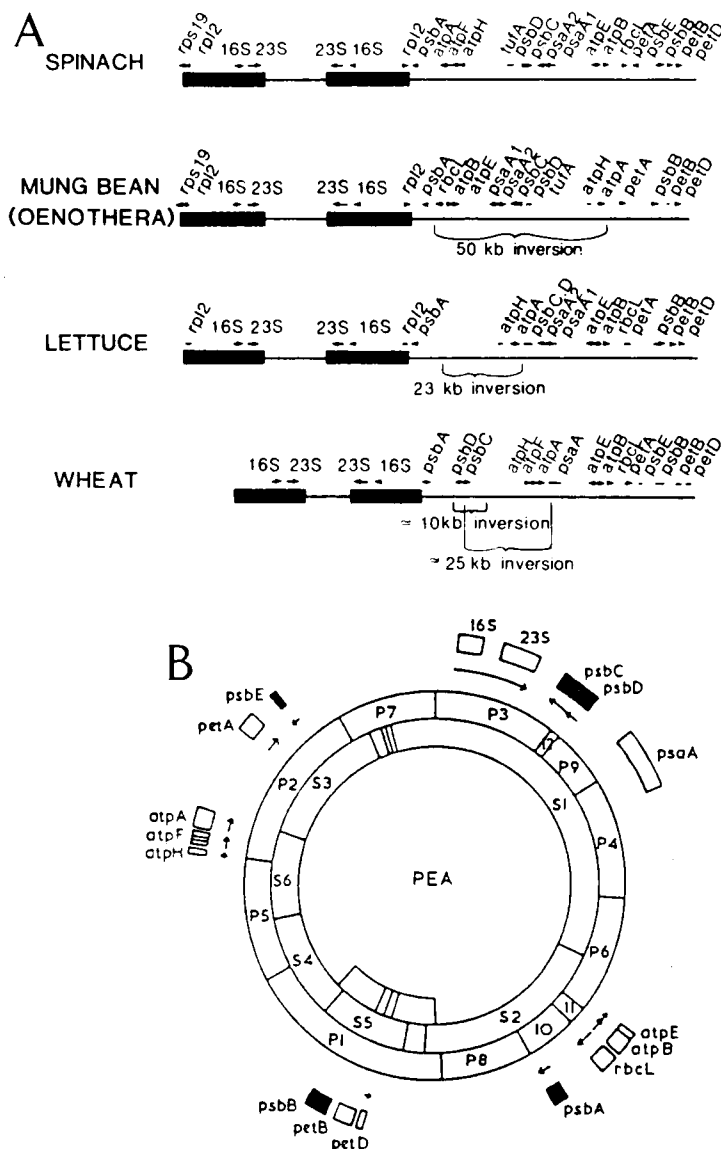


Figure 1. Gene maps of several angiosperm chloroplast genomes.

- A. Maps of the consensus angiosperm chloroplast gene order (typified by *Spinacia oleracea*), and other angiosperm ct genomes which have undergone rearrangements due to inversion events. The dark boxes represent the inverted repeats (from Palmer, 1985, with permission).
- B. Map of the *Pisum sativum* chloroplast genome (from Courtice et al., 1985, with permission).

1986), Lactuca sativa (Palmer et al., 1985), and Triticum aestivum (Courtice et al. 1985) (Figure 1A). In both Pisum sativum (Figure 1B) and Vicia faba the gene order is by comparison highly rearranged, although certain gene clusters do appear to be highly conserved (Palmer et al., 1983b; Huttly and Gray, 1984; Ko et al., 1983a). In the following discussion the consensus locations of specific genes typified by spinach (Figure 1A) are first outlined. Variations on the consensus are then detailed. Research which forms the basis for this discussion is referenced in Table 1; gene symbols are as in Table 2.

16S, 23S rRNA. The ribosomal RNA genes are located within the inverted repeats, in the order 16S-23S-5S. The 23S gene and the 5S gene are separated by 300 bp; a spacer region of 2-3 kb separates the 16S and 23S genes (Wallace, 1982; Whitfield and Bottomley, 1983; Palmer, 1985). In Pisum sativum and Vicia faba a single copy of each rRNA gene is found in the order listed above (Palmer and Thompson, 1982; Palmer et al., 1983b).

rpl 2, rps 19. In Spinacia oleracea, Nicotiana debneyi, and Glycine max, the rps 19 gene spans the junction between the inverted repeat and the large single-copy spacer region (Zurawski et al., 1984; Spielmann and Stutz, 1983). In Nicotiana tabacum the gene lies entirely within the large single-copy region, and in Zea mays it is within the inverted repeat (Sugita and Sugiura, 1983; Schwarz and Kossel, 1980). Thus the border between the inverted repeat and the large single-copy region is variable (i.e., it may entirely include,

partially include, or exclude the gene coding for the plastid S19 protein) (Zurawski et al., 1984). Rpl 2 is located within the inverted repeat in both Spinacia oleracea and Nicotiana debneyi, adjacent to rps 19 (Zurawski et al., 1984).

rbc L. Rbc L is located 30-35 kb from the proximal 16S rRNA flanked by psb A, and 65-75 kb from the distal 16S rRNA gene. In Glycine max, Vigna radiata, Pisum sativum, and Vicia faba rbc L is only several kb away from psb A (Palmer et al., 1983b; Spielmann et al., 1983; Singh et al., 1985; Tyagi and Herrmann, 1986; Ko et al., 1983).

Pet A, B, and D. Pet A is 3-5 kb from rbc L, and 12-15 kb from pet B. In Pisum sativum pet A is 30 kb from pet B, 18 kb from the 16S rRNA gene, and 60 kb from rbc L. In Vicia faba pet A is located 48 kb from the 16S rRNA gene (Berends et al., 1986; Ko et al., 1983). In Oenothera hookeri pet A maps 2 kb from psb E. However, instead of mapping adjacent to rbc L, pet A maps adjacent to atp A, due to a 45 kb inversion (Tyagi and Herrmann, 1986) (Figure 1A). In Glycine max, Vicia faba and Pisum sativum pet A maps close to atp A (Singh et al., 1985). Pet B maps to within 5 kb of pet D, which in turn lies within 7 kb of the copy of the inverted repeat which is opposite the other copy flanked by psb A.

psa A1, A2. These two genes are very close, separated by as little as 25 bp. The gene cluster is located near the center

of the large single-copy region, *psa A2* separated from *psb C* by 5 kb. In *Pisum sativum* *psa A1* is located 1 kb from *pet A* and 3 kb from *atp A* (Smith and Gray, 1984).

atp A,B,E,F,H. *Atp B* and *atp E* overlap by 4 bp and are located 1 kb from *rbc L*. *Atp A* and *atp H* are separated by 2-5 kb. This gene cluster is separated from *atpB/atpE* by 40-50 kb. In *Triticum aestivum* 20 kb separates *atpB/atpE* from *atpA*; this can be explained on the basis of a 28 kb inversion (Figure 1) (Howe et al., 1983). Significantly, in *Pisum sativum* and *Vicia faba* the organization and spatial arrangement of *atp A,B,E*, and *H* are similar to those genomes which contain the inverted repeats (Huttly and Gray, 1984; Ko et al., 1983). The *Glycine max* *atp A* gene maps distal to *psb A*, and *atpB/atpE* and *rbc L* map proximal to *psb A*, the result of a large inversion (Singh et al., 1985). *Atp F* is 500 bp from *atp A*, and 600 bp from *atp H*.

psb A,B,C,D,E. *Psb A* maps to within 5 kb of one of the inverted repeats, and 50 kb from *rbc L*. In *Glycine max* and *Vigna radiata* *psb A* and *rbc L* are within several kb of each other (Palmer et al., 1983b; Spielmann et al., 1983). This arrangement is also seen in *Pisum sativum* and *Vicia faba*, where the gene cluster (*psb A* and *rbc L*) maps 35 kb from the 23S rRNA gene (Palmer et al., 1983b; Ko et al., 1983). In *Oryza sativa* *psb A* is 20 kb from the inverted repeat, the result of a large inversion (Hirai et al., 1985). *Psb B* is several kb from *pet B*, and maps approximately 180° (on a circular

map) from psb A. Psb C is 40 kb from psb B, and 2-3 kb from psa A2. The 3' end of psb D overlaps the first 50 bp of the 5' end of psb C. Psb E is 7 kb from psb B, and psb E is 1 kb from pet A. In Pisum sativum and Triticum aestivum psb D and psb C are 55 kb from psb B and within close proximity to psa A1 and psa A2 (Berends et al., 1986). In Triticum aestivum two inversions, one of 5 kb and one of 28 kb, have resulted in the relocation of atp A next to psa A1, and psb D adjacent to psb A (Courtice et al., 1985) (Figure 1A).

Definition of the Project

There has been a bias toward angiosperms in chloroplast genome studies. Over 230 angiosperm taxa, representing 33 families, have been characterized to some extent (Palmer, 1985; Crouse et al., 1985). Conversely, among non-angiosperm land plants only two hepatics (Sphaerocarpos donellii and Marchantia polymorpha), three pteridophytes (Pteris vittata, Asplenium nidus, and Osmunda cinnamomea), and one gymnosperm (Ginkgo biloba) have been examined; detailed restriction maps are available only for Osmunda cinnamomea and Marchantia polymorpha (Herrmann et al., 1980; Palmer and Thompson, 1982; Palmer and Stein, 1982; Ohyama et al., 1983). Clearly, further information is needed on non-angiosperm plant chloroplast genomes if one is to accurately discern patterns and trends of chloroplast genome evolution (Palmer and Thompson, 1982; Palmer, 1985).

The objectives of this study were as follows: 1) To determine whether repeat elements, i.e., tandem, dispersed or inverted, are

present in the moss chloroplast genome. 2) To determine the extent of sequence rearrangement and/or conservation by the comparison of the genetic map of the moss chloroplast genome with that of the consensus angiosperm gene order, typified by Spinacia oleracea (Figure 1A, page 11).

Description of Gene Probes

The use of heterologous sources for gene probes in constructing genetic maps of chloroplast genomes is well established. For example, an internal region of the gene coding for ribulose-1,5-bisphosphate carboxylase/oxygenase obtained from the monocot Zea mays has been successfully used to locate the homologue in such evolutionarily diverse taxa as the protist Euglena gracilis (Stiegler et al., 1982), the hepatic Marchantia polymorpha (Ohya et al., 1983), the pteridophyte Osmunda cinnamomea (Palmer and Stein, 1982), and the dicots Spinacia oleracea (Whitfield and Bottomley, 1980), Atriplex triangularis and Cucumis sativa (Palmer, 1982). The atp β and ϵ gene probes from Spinacia oleracea were used to map the corresponding genes in Osmunda cinnamomea (Palmer and Stein, 1982). In a more extreme case, the tuf A gene (encoding the protein synthesis polypeptide elongation factor) from E. coli successfully hybridized to the corresponding gene in C. reinhardtii (Watson and Surzycki, 1982). Table 2, page 9, lists the symbols for the gene probes utilized in this study; Table 3 describes the probe constructs. The polypeptides encoded by the genes are described below.

Table 3. Descriptions of gene probes.

Designation	Gene(s)	Plant Source	Insert	Reference(s)
1	atp B	<u>Spinacia oleracea</u>	<u>Eco RI-Eco RI</u> 1980 bp	Zurawski et al., 1982
2	atp E	<u>Spinacia oleracea</u>	<u>Eco RI-Xba I</u> 420 bp	Ibid.
3	3' region of atp H	<u>Pisum sativum</u>	<u>Pst I-Bam HI</u> 800 bp	Huttley and Gray, 1984
4	atp F, 5' region of atp A	<u>Spinacia oleracea</u>	<u>Sal I-Hind III</u> 1.5 kbp	Westhoff et al., 1985a
5	atp F, atp A	<u>Spinacia oleracea</u>	<u>Sal I-Sal I</u> 2.4 kbp	Ibid.
6	psaA1, 5' region of psa A2	<u>Spinacia oleracea</u>	<u>Bam HI-Bam HI</u> 2.4 kbp	Alt et al., 1984
7	3' region of psa A2	<u>Spinacia oleracea</u>	<u>Bam HI-Bam HI</u> 1.6 kbp	Ibid.
8	5' proximal 200 bp of psb D	<u>Pisum sativum</u>	<u>Bam HI-Pst I</u> 720 bp	Rasmussen et al., 1984
9	3' proximal 830 bp of psb D	<u>Pisum sativum</u>	<u>Pst I-Pst I</u> 1.1 kbp	Ibid.

Table 3 (continued)

Designation	Gene(s)	Plant Source	Insert	Reference(s)
10	3' region of psb C	<u>Spinacia oleracea</u>	Bam <u>HI-Pst</u> I 350 bp	Alt et al., 1984
11	5' region of pet A	<u>Pisum sativum</u>	Bam <u>HI-Bam</u> HI 3.2 kbp	Willey et al., 1984
12	3' region of pet A	<u>Pisum sativum</u>	Bam <u>HI-Bam</u> HI 1.1 kbp	Ibid.
13	5' region of psb E	<u>Spinacia oleracea</u>	Eco <u>RI-Eco</u> RI 650 bp	Herrmann et al., 1984
14	3' region of psb E	<u>Spinacia oleracea</u>	Eco <u>RI-Eco</u> RI 500 bp	Ibid.
15	5' region of psb B	<u>Spinacia oleracea</u>	Bam <u>HI-Bam</u> HI 375 bp	Heinemeyer et al., 1984
16	3' region of psb B	<u>Spinacia oleracea</u>	Bam <u>HI-Sal</u> I 1.5 kbp	Ibid.
17	pet B	<u>Spinacia oleracea</u>	Sal <u>I-Bam</u> HI 2.4 kbp	Ibid.
18	5' region of pet D	<u>Spinacia oleracea</u>	Bam <u>HI-Bam</u> HI 300 bp	Ibid.

Table 3 (continued)

Designation	Gene(s)	Plant Source	Insert	Reference(s)
19	3' region of pet D	<u>Spinacia oleracea</u>	<u>Bam HI-Sal I</u> 2.0 kbp	Ibid.
20	rpl 2 intron + 3' region; rps 19	<u>Petunia hybrida</u>	<u>Pst I-Pst I</u> 1.5 kbp	Sugita and Sugiyura, 1983; J. D. Palmer, pers. comm.
pZmc 100	16S rRNA 23S rRNA	<u>Zea mays</u>	<u>Bam HI-Bam HI</u> 3.2 kbp <u>Hind III-Hind III</u> 1.2 kbp	Bedbrook et al., 1977
pZmc 461	rbc L	<u>Zea mays</u>	<u>Pst I-Pst I</u> 567 kbp	McIntosh et al., 1980
pZr 12	psa A1, psa A2	<u>Zea mays</u>	<u>Eco RI-Eco RI</u> 6.3 kbp	Fish et al., 1985
pZr 48	atp B, atp E	<u>Zea mays</u>	<u>Eco RI-Eco RI</u> 2.8 kbp	Krebbers et al., 1982
pZmc 427	psb A	<u>Zea mays</u>	<u>Bam HI-Eco RI</u> 2.2 kbp	Bogorad et al., 1980

Ribosomal RNAs. Chloroplast ribosomes contain three major RNA species, with sedimentation coefficients of 23S, 16S, and 5S. The 23S and 5S rRNAs are components of the 50S ribosomal subunit; the 16S rRNA is a component of the 30S subunit (Lyttleton, 1962).

Large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase. The 3-phospho-D-glycerate carboxylase (dimerizing) catalyzes two reactions in the chloroplast stroma: 1) the fixation of atmospheric CO₂ in the initial step of the Calvin reductive pentose phosphate cycle, resulting in the conversion of D-ribulose-1,5-bisphosphate to D-phosphoglyceric acid; and 2) the oxygenation of ribulose-1,5-bisphosphate yielding 3-phosphoglyceric acid and phosphoglycolic acid. The holoenzyme (560 kD) is composed of 8 identical large subunits (51-58 kD) (encoded by the chloroplast genome) and 8 identical small subunits (12-18 kD) (encoded by the nuclear genome) (Miziorko and Lorimer, 1983).

Photosystem I P700 apoproteins. Photosystem I contains a reaction center chlorophyll, designated as P700, which is complexed with two polypeptides: psa A1 (66-69 kD) and psa A2 (65-67 kD) (Metz et al., 1983). The precise function of these polypeptides has not been established (Fish et al., 1985).

Photosystem II polypeptides. There are 5 polypeptides associated with Photosystem II. Psb A (32 kD), a polypeptide added to the thylakoid membrane during photoinduced development (Bedbrook

et al., 1977) is responsible for binding the herbicides 3-(3,4-dichlorodiphenyl)-1,1 dimethylurea and atrazine (Steinback et al., 1981) and may act as a plastoquinone reductase (Alt et al., 1984). Psb B (54 kD) is directly complexed with the P680 chlorophyll a molecule (Camm and Green, 1983). A 44 kD protein (psb C) may constitute an internal light collecting antenna in addition to the chlorophyll a/b antenna complex (Staehelin and Arntzen, 1983). The precise functions of psb D (30-34 kD) and psb E (cytochrome b₅₅₉) are presently unknown (Rasmussen et al., 1984; Herrmann et al., 1984).

Cytochrome b/f complex. The cytochrome b/f complex, localized in the thylakoid membrane, is involved in proton translocation across the membrane in the photosynthetic electron transport chain. Four proteins constitute the complex. One, the Rieske FeS protein (19 kD) is encoded by the nuclear genome. The other three--cytochromes f (34 kD) and b₆ (23 kD) and subunit 4 (17 kD) are encoded by the chloroplast genome (Alt et al., 1983a).

ATP synthase coupling factor. The chloroplast membrane contains a protein complex capable of coupling ATP synthesis to proton translocation across the membrane. The complex is composed of 2 multisubunit domains: a membrane sector, CF₀, composed of at least 3 polypeptides designated I, II, and III, and an extrinsic sector, CF₁, with 5 components designated α , β , δ , γ , and ϵ . The membrane sector is responsible for proton translocation, and the peripheral sector catalyzes the phosphorylation of ADP (Nelson, 1981). The

α , β , and ϵ subunits of CF_I , and subunits I and II of CF_0 are encoded by the chloroplast genome (Westhoff et al., 1981; Westhoff et al., 1985a; Howe et al., 1983).

Ribosomal proteins. Nucleotide sequence analysis has revealed two chloroplast genes, *rpl 2* and *rps 19*, which bear homology to the *E. coli* ribosomal proteins L2 and S19, respectively (Zurawski et al., 1984; Sugita and Sugiura, 1983).

CHAPTER II

PROCEDURES

Moss Gametophyte Tissue Culture

Several different parameters were varied in an attempt to optimize the growth conditions. Among the factors tested were light intensity, CO₂ concentration, media composition, and culture volume. The following protocol was found to be the most efficient.

Approximately 1 gm (fresh weight) of gametophyte tissue was subcultured into 1.5 liters of Knop's medium, as outlined by Collier and Hughes (1980). Cultures were incubated at 20° C under a 2% CO₂-enriched atmosphere with constant illumination (wavelength = 330-750 nm, fluence = 36-40 Joules/cm²). After 3 weeks of growth the tissue was maintained in the dark for 48 hours at 20° C to destarch the chloroplasts (starch grains can rupture the chloroplasts during the isolation process). The tissue was then harvested, washed several times in 0.1% SDS, rinsed several minutes in deionized water, and stored at 4° C until needed. Typical yields of tissue were approximately 100 gm/1 gm initial inoculum.

Chloroplast Isolation/DNA Purification

Gametophyte tissue (200 gm fresh weight) was frozen for 15 minutes in liquid nitrogen, ground for 5 seconds in a Micro-Mill (Bel-Art Products, Pequannock, N.J.), then dispersed into 1 liter

of extraction buffer (0.35M sorbitol, 50mM Tris HCl pH 9.0, 10mM Na₂EDTA, 5mM DTT, 0.1% BSA, 5mM spermine, 5mM spermidine). The homogenate was ground in a Waring blender (2x5 second bursts), filtered through 4 layers of cheese cloth and 2 layers of Nitex screen (Tekto Industries, Elmsford, NY) (100 μ mesh), and centrifuged at 4Kxg for 15 minutes. The resultant pellet was resuspended in 6 ml wash buffer (0.35M sorbitol, 50mM Tris·HCl·pH 9.0, 25mM Na₂EDTA, 5mM spermine, 5mM spermidine), layered over a discontinuous sucrose gradient (25-35-45-55% w/v in wash buffer) and centrifuged at 20Kxg for 45 minutes. Chloroplasts were removed from the 35%-45% interface, diluted with a ten-fold excess of wash buffer, and centrifuged at 4Kxg for 15 minutes. All of the above steps were carried out at 4° C.

Chloroplasts were lysed in the presence of 2% SDS and 200 μ g/ml self-digested Proteinase K at 37° C for 1 hour. The chloroplast lysate was adjusted to a refractive index of 1.3550-1.3600 with a 50mM Tris·HCl pH 8.0 20mM Na₂EDTA solution saturated with cesium chloride, incubated on ice for 1 hour, and centrifuged at 12Kxg for 30 minutes at 4°C. The resulting supernatant was adjusted to a refractive index of 1.3700-1.3720 with the saturated cesium chloride solution, underlaid by a cushion consisting of cesium chloride ($n=1.3910$) and ethidium bromide (300 μ g/ml) in 50mM Tris·HCl pH 8.0 20 mM Na₂EDTA and centrifuged at 175Kxg for 36 hours at 20° C.

Chloroplast DNA bands were visualized with a longwave (360 nm) UV lamp, removed from the centrifuge tubes with an 18 gauge

needle, extracted with NaCl-saturated 2-propanol, dialyzed against 3 changes of 1 liter TE buffer (10mM Tris·HCl pH 8.0 1mM Na₂EDTA) over 48 hours, and precipitated in the presence of 1/10 volume 3M Na acetate pH 5.2 and 2 volumes 95% ethanol at -20° C overnight. After centrifugation at 12Kxg for 30 minutes at -20° C the DNA pellet was dried in vacuo and resuspended in TE buffer. Typical yields were 15-20 µg DNA/200 gm tissue (fresh weight).

Chloroplast DNA Manipulations

Restriction endonuclease digestion/gel electrophoresis. Approximately 0.5-1 µg chloroplast DNA was digested with an excess (10-20 units) of the appropriate restriction endonuclease under the conditions recommended by the supplier (New England Biolabs, Beverly, Mass.). Digests were electrophoresed through a 0.7% horizontal agarose gel (20x25x0.4 cm) at 50 volts 30 milliamps (constant current) for 16 hours in TBE buffer (Maniatis et al., 1982). Gels were stained in ethidium bromide and photographed as described (Davis et al., 1980).

Transfer to nitrocellulose. DNA fragments within the gels were depurinated by washing the gels in 0.25M HCl for 7.5 minutes. The gels were rinsed thoroughly in deionized water; the DNA fragments were then denatured by washing twice in 1.5M NaCl 0.5M NaOH for 15 minutes. After a second rinse in water, the gels were neutralized by washing twice in 2M NaCl 0.5M Tris·HCl pH 7.5 for 15 minutes. The DNA fragments were then transferred in a bidirectional manner from the gel to nitrocellulose filters (Southern, 1975; Smith and Summers,

1980; Meinkoth and Wahl, 1984). After allowing transfer to proceed for 1 hour, the nitrocellulose filters were washed in 6X SSC for 5 minutes, air-dried, then dried at 80° C in vacuo for 20 minutes. Filters were stored at room temperature in the dark until needed (Southern, 1975; Smith and Summers, 1981; Meinkoth and Wahl, 1984). To assay for the efficiency of transfer, blotted gels were re-stained in ethidium bromide and photographed as outlined previously. In a typical experiment no DNA could be visually detected on the gel after transfer to nitrocellulose.

Prehybridization of filters. Nitrocellulose filters were incubated with 75 µl hybridization mixture (5X SSPE, 0.1% SDS, 5X Denhardt's solution, and 100 µg/ml denatured and sheared salmon sperm DNA)/cm² filter area for 1 hour at 68° C (Meinkoth and Wahl, 1984).

Restriction Fragment Mapping

Probe fragment labeling. Approximately 1 µg of chloroplast DNA was digested to completion with Cla I. The reaction mixture was incubated at 65° C for 5 minutes (to dissociate any proteins bound to the fragment termini, then placed on ice. The following were then added: 15 pmol ³²P-dCTP (Sp. Act. = 3000 Ci/mmol), 15 pmol d ³²P-dGTP (Sp. Act. = 3000 Ci/mmol), reaction buffer (final concentration = 50mM Tris·HCl pH 7.4, 10mM MgCl₂, 1mM DTT, and 100 µg/ml BSA) (Drouin, 1980), and 2 units of the large fragment (Klenow) of DNA polymerase I (New England Biolabs). This reaction was incubated

at 20° C for 15 minutes, then terminated by the addition of Na₂EDTA to a final concentration of 30mM.

Probe fragment fractionation. Radiolabeled Cla I fragments #1-#4 (26.5 kb-14.3 kb) were fractionated on a 0.5% horizontal agarose gel (20x25x0.5 cm). Cla I fragments #5-#12 (9.4 kb-2.4 kb) were fractionated on a 1% gel (same dimensions as above). Individual restriction fragments were excised from the gels with razor blades, placed in 500 µl TE buffer, and incubated at 65° C (to weaken the gel matrix) for 1 hour. The probes were then denatured by boiling for 15 minutes, then immediately incubated with the appropriate nitro-cellulose filter at 68° C for 36 hours.

Gene Mapping

Plasmid isolation. E. coli strain HB101 cells, containing the plasmids pZmc 461, pZmc 100, pZr 48, and pZr 12 were obtained from Dr. L. Bogorad (Harvard University). Recombinant cells were selected, grown, and plasmids were amplified as outlined by Maniatis et al. (1982). Large-scale plasmid preparations were conducted as outlined by Birnboim and Doly (1979) and modified by Ish-Horowicz (Maniatis et al., 1982). Plasmids #1-#20 (Table 3, page 17) were obtained from Dr. J. Palmer (University of Michigan). E. coli strain HB101 cells were transformed with these 20 plasmids as outlined by Mandel and Higa (1970). Minipreparations for plasmid DNA isolations were as outlined by Birnboim, and Doly (1979) and modified by Ish-Horowicz (Maniatis et al., 1982). All plasmids were verified by

diagnostic restriction endonuclease digestions and electrophoresis with calibration standards.

Probe labeling and hybridization. Approximately 0.5 μg of plasmid DNA was labeled with ^{32}P -dCTP (Sp. Act. = 3000 Ci/mmol) with a nick-translation kit, following the protocol outlined by the supplier (Bethesda Research Labs, Gaithersburg, MD). Unincorporated nucleotides were removed from the labeled DNA by chromatography through a Sephadex G-50 column (Davis et al., 1980). Labeled probe DNA was first diluted into 500 μl TE buffer, denatured by boiling for 10 minutes, then rapidly cooled on ice. Approximately 10^5 - 10^6 cpm/ml hybridization mixture was added to the hybridization solution, which was incubated at 68°C for 36 hours.

Nitrocellulose Filter Washes/Autoradiography

After incubation at the appropriate conditions for hybridization the wet filters were subjected to the following series of washes: 5 minutes in 2X SSC 0.5% SDS at room temperature; 15 minutes in 2X SSC 0.1% SDS at room temperature; and two successive washes for 60 minutes in 0.1X SSC 0.5% SDS at 68°C (Maniatis et al., 1982). Dried filters were exposed to Kodak Xomat XAR-5 film with a single DuPont Cronex intensifying screen at -70°C for 3-14 days.

CHAPTER III

RESULTS

Size Determination of Moss Chloroplast DNA

The most frequent approach to determine the size of a chloroplast genome is by summation of chloroplast DNA restriction fragments co-electrophoresed with molecular weight standards. Size estimates obtained with this experimental approach are in excellent agreement with estimates obtained by electron microscopy (Behn and Herrmann, 1977; Köller and Delius, 1980; Herrmann et al., 1980; Seyer et al., 1981; Ohyama et al., 1983). In contrast, there are often large discrepancies between estimates obtained through reassociation kinetics and either restriction fragment size summations or electron microscopic measurements (Behn and Herrmann, 1977; Palmer and Thompson, 1981).

This study utilized a 0.7% horizontal agarose gel and co-electrophoresis with intact λ DNA (49.1 kb), λ DNA digested with Sal I (producing fragments of 33.3 kb and 15.2 kb), Bam HI (17.4 kb, 7.3 kb, 6.8 kb, 6.5 kb, and 5.5 kb), and Hind III (23.7 kb, 9.5 kb, 6.7 kb, 4.3 kb, 2.3 kb, and 2.0 kb) (Davis et al., 1980), and ϕ X 174 RF II DNA digested with Hae III (1.4 kb, 1.1 kb, 0.9 kb) (New England Biolabs) to determine the sizes of moss chloroplast DNA fragments produced by single digestions with Cla I, Sac I, and Bgl II, and double digestions with Cla I/Sac I and Cla I/Bgl II

(Figure 2, Table 4). These summations yield an average of 122 kb.

Mapping Restriction Endonuclease Cleavage Sites

One essential element in our protocol was the successful labeling of chloroplast DNA restriction fragments generated by Cla I for use as hybridization probes against filter-bound DNA. The use of a nick-translation reaction without DNase I (Maniatis et al., 1975) modified to radiolabel chloroplast DNA (Palmer, 1982) proved to be unsuccessful with the moss system. Chloroplast DNA labeled according to the conditions outlined (Palmer, 1982) was extremely degraded, rendering it unsuitable for our mapping purposes. This degradation could have been due to the presence of contaminating amounts of DNase I in commercially available preparations of DNA polymerase I (Davis et al., 1980), and an excessive number of nicks in the moss chloroplast DNA. Titrations with both DNA polymerase I and ^{32}P -dCTP failed to provide DNA that was both relatively undegraded and yet of sufficient specific activity to permit accurate restriction mapping. This problem was obviated by the use of the large fragment (Klenow) of DNA polymerase I in labeling the 3' termini of restriction fragments (Drouin, 1980; Maniatis et al., 1982). Digestion of DNA with Cla I yields fragments with recessed 3' termini and unpaired cytosine and guanine residues at the 5' termini. End-labeling with double isotopes (i.e., ^{32}P -dCTP and ^{32}P -dGTP) under reaction conditions designed to maximize the polymerization rate and minimize the 3' $\rightarrow$ 5' exonuclease activity by the Klenow fragment (D. Amorese, pers.

Figure 2. Single and double digestion products of Physcomitrella patens chloroplast DNA with the following restriction endonucleases: Lane A--Sac I; lane B--Sac I/Cla I; lane C--Cla I; lane D--Cla I/Bgl II; lane E--Bgl II. Fragment sizes were determined by co-electrophoresis with a range of phage λ DNA fragments produced by separate digestions with Hind III, Eco RI and Sal I, as well as undigested phage λ DNA. Molecular weight sizes are listed in kilobases along the left ordinate.

A B C D E

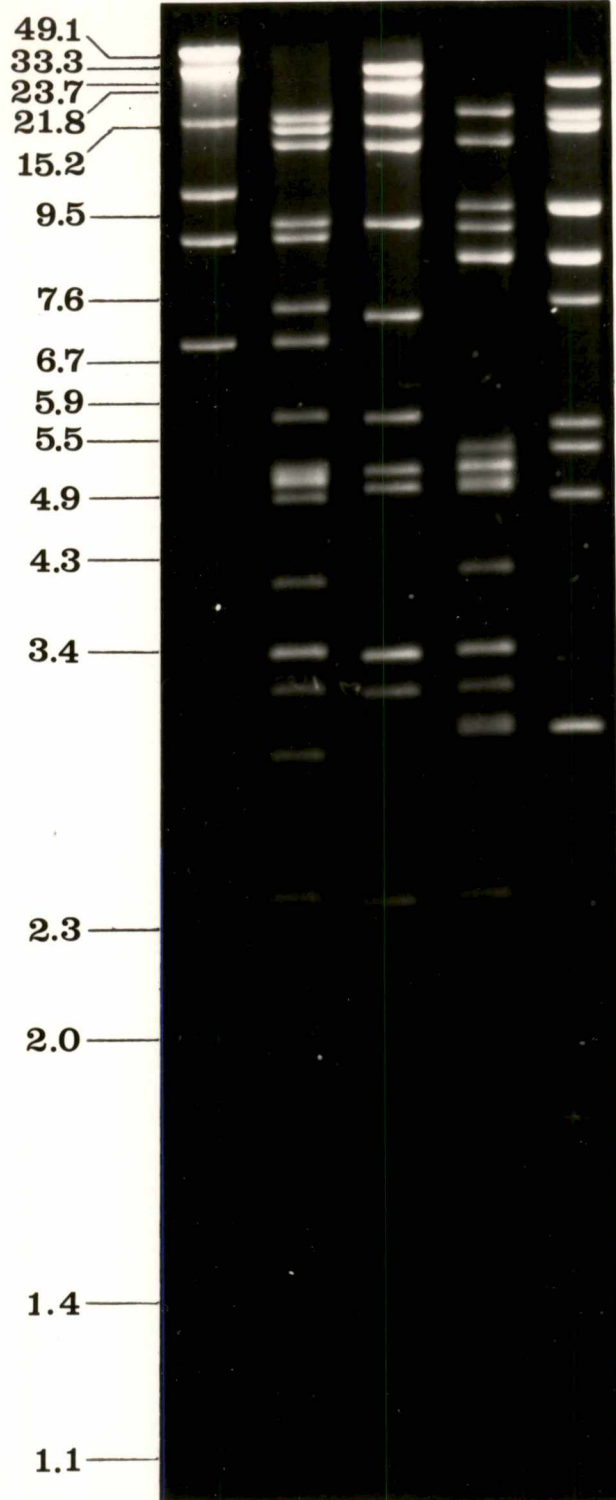


Table 4. Molecular sizes (in kilobases) of Physcomitrella patens chloroplast DNA fragments produced by digestion with restriction endonucleases.^a

Fragment Designations	Sac I	Sac I/Cla I	Cla I	Cla I/Bgl II	Bgl II
1	53 ^b	15.5	26.5	15.7	23.7
2	26	14.8	21.7	14.3	15.7
3	15.0	14.3	15.0	9.6	15.1
4	10.5	9.4	14.3	9.2	9.6 (x2)
5	8.8	8.8	9.4	8.4 (x2)	8.4 (x2)
6	6.9	7.8	7.4	5.4	7.6
7	2.0	6.9	5.8	5.2 (x2)	5.7
8		5.8	5.2	5.0 (x2)	5.4
9		5.2	5.0	4.2	4.9
10		5.1	3.4 (x2)	3.4 (x2)	2.9 (x2)
11		4.9	3.2	3.2	1.8
12		3.8	2.4	3.0	1.3
13		3.4 (x2)		2.9	
14		3.2		2.4	
15		2.9		1.8	
16		2.4		1.5	
17		2.0		1.3	
	<u>122.2</u>	<u>119.6</u>	<u>122.7</u>	<u>121.7</u>	<u>123.0</u>

^aFragments less than 1 kb in size were not mapped.

^bDetermined by summation of sizes of sub-fragments produced by digestion with a second restriction endonuclease.

comm.) yielded restriction fragments that were free of any detectable degradation (i.e., resulting from the labeling reaction) and labeled to a sufficient specific activity to allow for rapid autoradiographic visualization of hybridization signals (e.g., 3-7 days). End-labeling reactions, whether by the Klenow fragment (Drouin, 1980) or T4 DNA polymerase (O'Farrell, 1981) for 3' or flush termini, or by T4 polynucleotide kinase for 5' termini (Maxam and Gilbert, 1980) offer a suitable alternative to the nick-translation reaction for labeling chloroplast DNA restriction fragments for mapping purposes (however, the T4 system was not effective in our hands). As only the termini are labeled, the level of incorporation will be lower than with nick-translation; however, the reduction in DNA degradation and subsequent decrease in cross-contamination of fractionated probe fragments is a decided advantage.

Strategy. Restriction endonuclease cleavage sites were mapped by the following principles. A set of chloroplast DNA restriction fragments (i.e., generated by Cla I) were radiolabeled and then fractionated by gel electrophoresis. The fragments were subsequently used as hybridization probes against filter-bound chloroplast DNA genomic digests generated by single enzymes (i.e., Bgl II and Sac I), and double digests involving both the enzymes used to generate the single digests and those used to generate the probe fragments (i.e., Bgl II/Cla I, Sac I/Cla I). Cross-hybridizations to single digests generated overlaps between probe and filter-bound fragments.

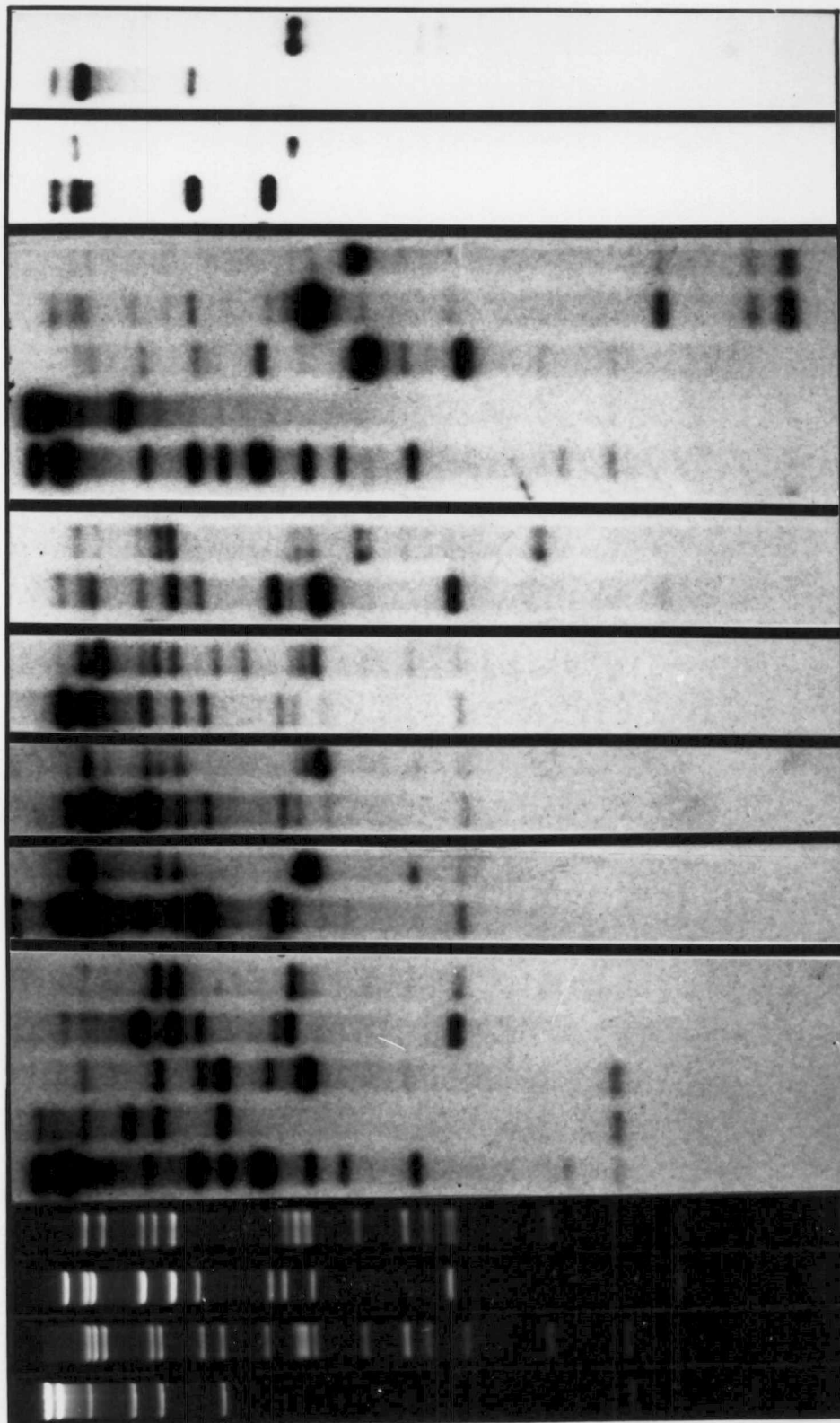
Similar hybridizations to the double digests yielded the precise locations of cleavage sites within the probe fragments (Palmer, 1982). Self-hybridizations of probe fragments to chloroplast genomic digests generated by the same enzyme (i.e., Cla I) revealed the presence of reiterated sequences within the probe fragments (Fluhr and Edelman, 1981b). Mapping ambiguities due either to cross-contamination of restriction fragment probes or the inability to order small restriction fragments located internally to larger fragments were resolved through hybridizations with defined gene probes (Spielmann et al., 1983).

Mapping logic. Preliminary mapping analysis of the moss chloroplast genome with the rRNA gene probes revealed the presence of inverted repeat structures. An efficient approach in constructing a restriction endonuclease cleavage site map for a genome with inverted repeat sequences is to begin mapping with the largest probe fragment in the large single-copy region, then extending the map into the repeat region with single-copy flanking sequences as reference points. In the following discussion Cla I probe fragment designations are as in Table 4 and the molecular weights of restriction fragments are listed parenthetically. The data are illustrated in Figure 3 and Appendix (Figure 8) and summarized in Table 5.

Artifactual signals could be the result of cross-contamination of gel-electrophoresed probe fragments due to degradation of larger probe fragments. When two probe fragments are very similar in their electrophoretic mobility (e.g., Cla 8 (5.2) and Cla 9 (5.0)) there

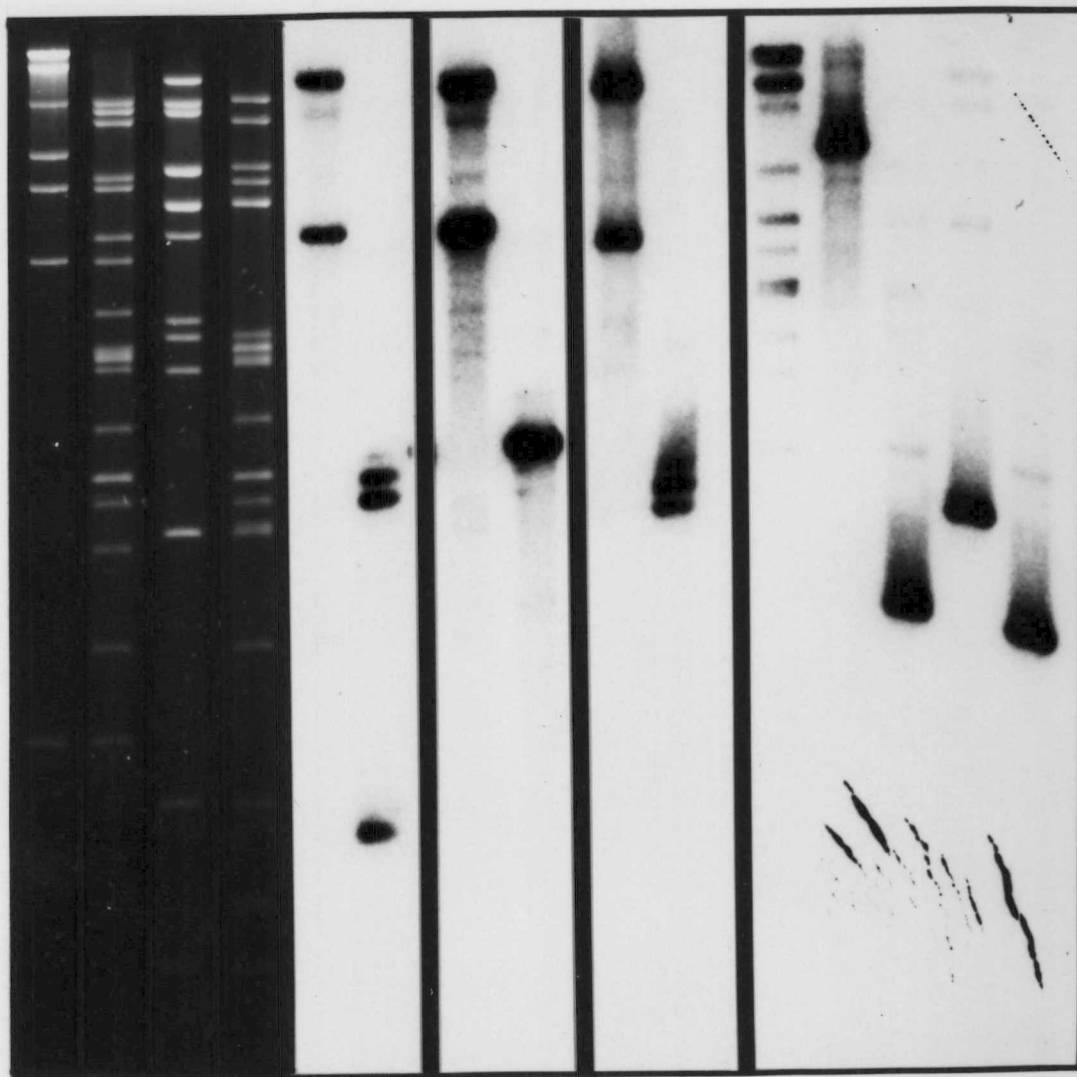
Figure 3. Results of hybridizations with Physcomitrella patens radio-labeled Cla I chloroplast DNA fragments to chloroplast DNA digested by Sac I (A), Sac I/Cla I (B), Bgl II (C), Bgl II/Cla I (D). Numbers beneath the lanes refer to the size of the specific Cla I fragment used as hybridization probes. Reference markers of phage λ DNA digested with Eco RI and Hind III, as well as intact phage λ DNA, are in lanes marked λ .

A B C D λ A B C D C D C D C D C D C D C D C D



1 2 3 4 5 6 7 8

A B C D C D C D C D λ A B C D



9

10

11

12

Table 5. Summary of restriction mapping hybridization data.^a

Cla I Probe Fragment	Filter-Bound Fragments			
	Bgl II ^b	Bgl II/Cla I ^b	Sac I	Sac I/Cla I
1 (26.5)	4 (9.6)	4 (9.2)	4 (10.5)	5 (8.8)
	5 (8.4)	5 (8.4)	5 (8.8)	7 (6.9)
	8 (5.4)	6 (5.4)	6 (6.9)	11 (4.9)
	10 (2.9)	13 (2.9)	7 (2.0)	17 (2.0)
2 (21.7)	1 (23.7)	1 (15.7)	--	--
	2 (15.7)	7 (5.2)		
	3 (15.1)	8 (5.0)		
3 (15.0)	3 (15.1)	3 (9.6)	--	--
	4 (9.6)	8 (5.0)		
4 (14.3)	1 (23.7)	2 (14.3)	--	--
5 (9.4)	5 (8.4)	5 (8.4)	--	--
	7 (5.7)			
	9 (4.9)			
6 (7.4)	9 (4.9)	9 (4.2)	4 (10.5)	12 (3.8)
	11 (1.8)	15 (1.8)		15 (2.9)
	12 (1.3)	17 (1.3)		
7 (5.8)	1 (23.7)	1 (15.7)	--	--
	2 (15.7)	7 (5.2)		
	6 (7.6)			
	7 (5.7)			
8 (5.2)	3 (15.1)	7 (5.2)	--	--
9 (5.0)	1 (23.7)	11 (3.2)	--	--
	6 (7.6)	12 (3.0)		
		16 (1.5)		
10 (3.4)	1 (23.7)	10 (3.4)	--	--
	6 (7.6)			
11 (3.2)	1 (23.7)	11 (3.2)	--	--
	6 (7.6)	12 (3.0)		
12 (2.4)	10 (2.9)	14 (2.4)	4 (10.5)	16 (2.4)

^aHybridization signals due to artifact or contamination are excluded (see text for explanation, pp. 35 and 40). Fragment designations are as in Table 4, page 33; molecular weights are listed parenthetically.

^bBased on data from replicate experiments (Appendix, Figure 8).

could be contamination of the larger probe fragment by the smaller (Palmer, 1982). Faint hybridization signals could also arise as a consequence of hybridization between small (100-1000 bp) dispersed repeat elements, such as those found in the C. reinhardtii and the I. subterraneum chloroplast genomes (op. cit.). True hybridization signals, representing an extensive amount of sequence homology between restriction fragments, were distinguished from artifactual signals resulting from either probe fragment contamination or dispersed repeat elements on the following basis. First, by assembling the autoradiograms in order of probe fragment size and comparing the intensity of autoradiographic signals, true hybridization signals were usually discernible above the background of artifactual bands. Second, hybridizations of specific gene probes to Cla I, Sac I, and Bgl II digests, as well as to Cla I/Bgl II double digests, permitted an unambiguous assignment of restriction fragments to a precise map location.

Cla 1 (26.5 kbp) hybridizes with Bgl 4 (9.6), 5 (8.4), 8 (5.4), and 10 (2.9). Bgl 5, 8, and 10 are internal to Cla 1, as evidenced by the double digestion fragments to which Cla 1 hybridizes to, i.e., Cla/Bgl 5 (8.4), 6 (5.4), and 13 (2.9). Bgl 4 (9.6) overlaps by 0.4 kb into the adjacent Cla fragment, 9.2 kb of Bgl 4 being contained by Cla 1 as evidenced by hybridization to Cla/Bgl 4 (9.2). Cla 3 (15.0) hybridizes with Bgl 3 (15.1), Bgl 4 (9.6) and Cla/Bgl 3 (9.6). There are therefore two Bgl 4 (9.6) fragments--one internal to Cla 3 (15.0), and one which overlaps with Cla 1 (26.5) and another Cla fragment. These data do not allow one to determine if Cla 3 is also

hybridizing to the Bgl 4 fragment extending into Cla 1, as the 0.6 kbp fragment was not retained by the gel. There are also no clear data indicating which fragment is juxtaposed to the opposite end of Cla 1. The mapping of a subset of Sac fragments will allow for this extension.

Cla 1 (26.5) hybridizes to Sac 5 (8.8), 6 (6.9), 7 (2.0), and 4 (10.5), and to Sac/Cla fragments 5 (8.8), 7 (6.9), 11 (4.9), and 17 (2.0). Sac 5, 6, and 7 are internal to Cla 1; Sac 4 overlaps into the contiguous Cla fragment(s). Cla 1 (26.5), Cla 12 (2.4) and Cla 6 (7.4) all hybridize to Sac 4 (10.5). Sac 4 (10.5) therefore extends 3.8 kb into Cla 6 (7.4), contains Cla 12 (2.4) as an internal fragment and extends 4 kb into Cla 1 (inferred from the total size of Sac 4 (10.5 kb) - Sac/Cla 12 (3.8 kb) - Sac/Cla 16 (2.4 kb) = 4 kb). The order of the three Cla fragments is therefore Cla 1--Cla 12--Cla 6. The order of the internal Sac and Bgl fragments cannot be determined from the restriction mapping data alone; the gene mapping data will allow for this ordering. Due to the limiting amounts of moss chloroplast DNA, the additional Sac fragments were not mapped. Because of their large size (i.e., Sac 1 (53 kb), Sac 2 (26 kb), and Sac 3 (15 kb)), little resolution would have been added to the map by the inclusion of these larger fragments.

Cla 12 (2.4) is internal to Bgl 10 (2.9) (Cla/Bgl 14 = 2.4). As Cla 6 (7.4) does not hybridize to Bgl 10 (2.9), this second copy of Bgl 10 must overlap by approximately 500 bp into Cla 1 (26.5).

This would explain the lack of unambiguous evidence for a conterminous fragment, as Cla 1 also contains an internal copy of Bgl 10. Essentially the same condition exists at each terminus--the Cla fragment adjacent to Cla 1 contains a second copy of a Bgl fragment internal to Cla 1. Cla 6 (7.4) contains Bgl 12 (1.3) and Bgl 11 (1.8) as internal fragments (Bgl/Cla 15 = 1.8, Bgl/Cla 17 = 1.3). Bgl 9 (4.9) extends 4.2 kb (Bgl/Cla 9) into Cla 6. The remainder of Bgl 9 extends 600 bp into Cla 5 (9.4 kb), which in turn contains Bgl 5 (8.4) as an internal fragment (Bgl/Cla 5 = 8.4), and extends 600 bp into Bgl 7 (5.7).

At this point the inverted repeat is encountered. Bgl 7 (5.7) extends 5.2 kb (Bgl/Cla 7) into Cla 7 (5.8), which in turn hybridizes to Bgl 1 (23.7), 2 (15.7), 6 (7.6), and 7 (5.7). Furthermore, cross-hybridization of probe fragments is observed between Cla 7 (5.8) and Cla 2 (21.7), and Cla 11 (3.2) and Cla 9 (5.0) (Appendix, Figure 7). Cla 7 and Cla 2, and Cla 11 and Cla 9 are therefore present in the different copies of the inverted repeat structure. In addition, Cla 10 (3.4) hybridizes to Bgl 1 (23.7) and Bgl 6 (7.6), and is internal to each (Bgl/Cla 10 = 3.4). Cla 10 is therefore present in two copies within the inverted repeat sequences. Approximately 800 bp of Cla 7 (5.8) extends into Bgl 6 (7.4), which in turn contains as an internal fragment Cla 10 (3.4), and 3 kb of Cla 9 (Bgl/Cla 12). The remainder of Cla 9 (Cla/Bgl 16 = 1.5) extends into Bgl 1. Cla 4 (14.3), which hybridized only to itself in control Cla 1--Cla 1 hybridizations (Appendix, Figure 7) is internal to Bgl 1 (Cla/Bgl 2 = 14.3).

Bgl 1 (23.7) must therefore extend through Cla 11 (3.2) and Cla 10 (3.4), both internal (Bgl/Cla 10 = 3.4, Bgl/Cla 11 = 3.2) to Bgl 1 (23.7), and 500 bp into Cla 2 (21.7). Bgl 2 (15.7) must be an internal fragment to Cla 2 (Bgl/Cla 1 = 15.7), which extends 5 kb (Bgl/Cla 8) into Bgl 3 (15.1). Both Cla 8 (5.2), which is internal to Bgl 3 (15.1) (Cla/Bgl 7 = 5.2) and Cla 3 (15) hybridize to Bgl 3 (15.1). Cla 3 extends 5 kb (Bgl/Cla 8 = 5.0) into Bgl 3 (15.1), contains Bgl 4 (9.6) as an internal fragment, and extends 400 bp into the second Bgl 4 fragment (9.6) contained within Cla 1 (26.5). The circularity of the map is thus established.

Gene Mapping

Results of hybridizations with defined chloroplast gene probes (Table 3, page 17) to moss chloroplast DNA digest are summarized in Table 6 and illustrated in Figure 4. The approximate locations of the genes on the moss chloroplast genome are illustrated diagrammatically in Figure 5. In the rDNA mapping data set there are a number of faint reciprocal hybridization signals present (Figure 4; Appendix, Figure 9). That is, Cla 10 (3.4) which contains the 23S rDNA, is responsible for producing a faint hybridization signal in the 16S rDNA mapping data set; Cla 2 (21.7) and Cla 7 (5.8), both which hybridize strongly to the 16S rDNA, hybridize very weakly to the 23 rDNA gene probe. Likewise, Bgl 2 (15.7) and Bgl 7 (5.7) hybridize very strongly to the 16S rDNA probe, and apparently very weakly to the 23S rDNA probe. The reverse is the case for Bgl 2 (15.7) and 6 (7.6). There are two possible explanations for this.

Table 6. Summary of gene mapping hybridization data.^a

Designation	Gene(s)	Filter-Bound Fragments		
		Sac I	Cla I	Bgl II
pZmc 100 (3.2 kbp Bam HI- Bam HI fragment)	16S rRNA	--	2 (21.7) 7 (5.8) 10 (3.4) ^b	1 (23.7) ^b 2 (15.7) 6 (7.6) ^b 7 (5.7)
pZmc 100 (1.2 kbp Hind III- Hind III fragment)	23S rRNA	--	2 (21.7) ^b 7 (5.8) ^b 10 (3.4)	1 (23.7) 2 (15.7) ^b 6 (7.6) 7 (5.7) ^b
20	rpl 2, rps 19	--	2 (21.7)	2 (15.7)
pZmc 461	rpc L	7 (2.0)	1 (26.5)	8 (5.4)
pZmc 427	psb A	--	3 (15.0)	4 (9.6)
15, 16 ^c	psb B	--	5 (9.4) 6 (7.4)	9 (4.9)
8, 9 ^c	psb D	--	1 (26.5)	4 (9.6)
10	psb C	6 (6.9)	1 (26.5)	4 (9.6)
13, 14 ^c	psb E	4 (10.5)	6 (7.4) 12 (2.4) ^d	10 (2.9) ^d 12 (1.3) ^d
6, 7 ^c pZr 12 ^e	psa A1, psa A2	6 (6.9)	1 (26.5)	4 (9.6) 5 (8.4)
4, 5 ^c	atp A atp F	--	3 (15.0) 8 (5.2)	3 (15.1)
1, 2 ^{c,f} pZr 48 ^e	atp B, atp E	5 (8.8) 7 (2.0)	1 (26.5)	8 (5.4) 10 (2.9)
3	atp H	--	8 (5.2)	3 (15.1)
11, 12 ^c	pet A	4 (10.5)	12 (2.4)	10 (2.9)
17	pet B	--	5 (9.4)	5 (8.4)
18, 19 ^c	pet D	--	5 (9.4) ^d	5 (8.4) ^d

^aProbe designations are as in Table 3, page 17; Sac I, Cla I and Bgl II restriction fragment designations are as in Table 4, page 33. Molecular weights in kilobases are listed parenthetically.

^bArtifactual signals most likely due to cross-contamination with neighboring gel-electrophoresed restriction fragments (see text, pages 40 and 49, for explanation).

^cBoth probes were combined in a single hybridization experiment.

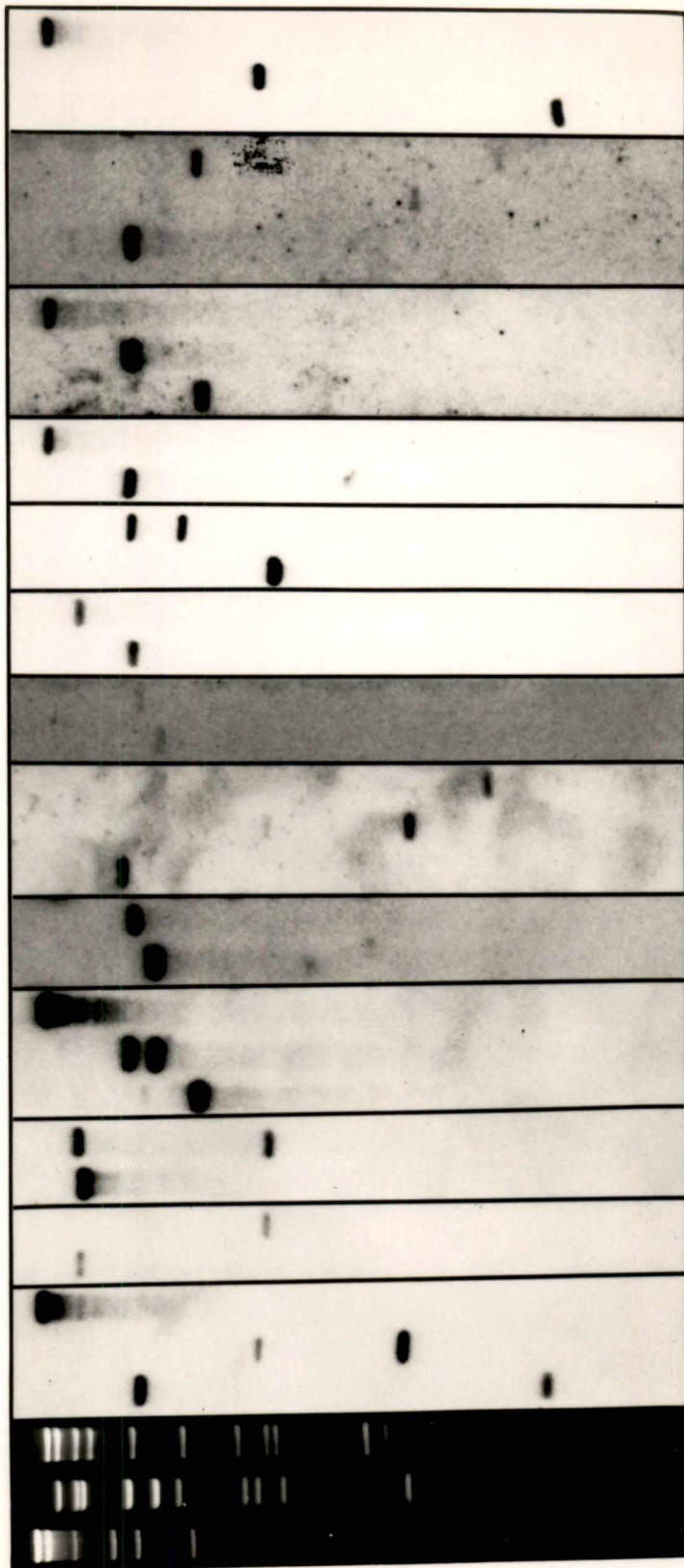
^dFaint signals visible on either the original or replicate autoradiographs.

^eIdentical hybridization results were obtained using defined probes from either spinach or corn (Appendix, Figure 10).

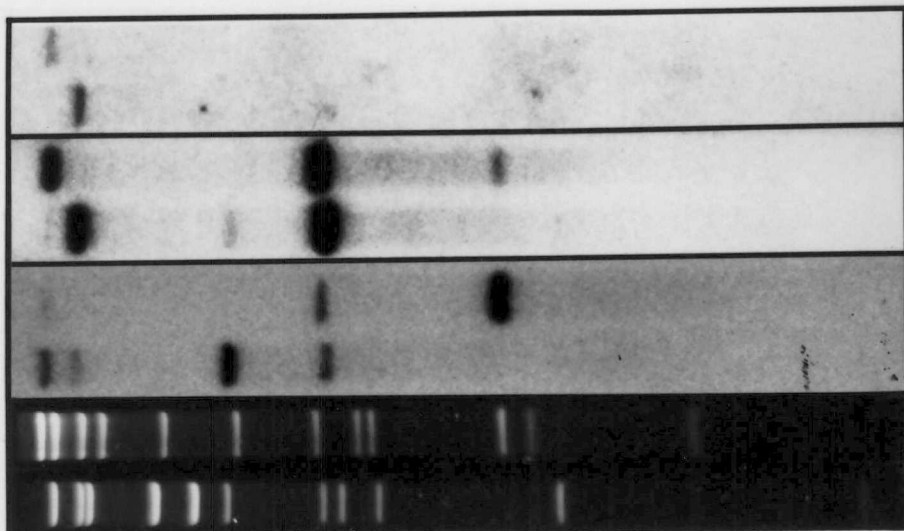
^fIndividual hybridizations were conducted with probes #1 and #2, yielding the final mapping data shown in Figure 5, page 47.

Figure 4. Results of hybridizations between Physcomitrella patens chloroplast DNA digested with Sac I (S), Cla I (C), and Bgl II (B) and defined chloroplast gene probes indicated by the symbol (Table 2, page 9) beneath the respective autoradiogram. Probes which encompassed different regions of the same gene were combined into a single hybridization experiment.

S B C S B C S B C S B C S B C S B C S B C S B C S B C S B C



B C B C B C B C



23S 16S rpl2
rps19

Figure 5. Physical map of the Physcomitrella patens chloroplast genome. Restriction fragment designations are as in Table 1, page 5; gene symbols as in Table 2, page 9. The approximate locations of the genes are indicated by the bracketed regions. The maximum extent of the inverted repeat is indicated by the external lines parallel to the circular map.

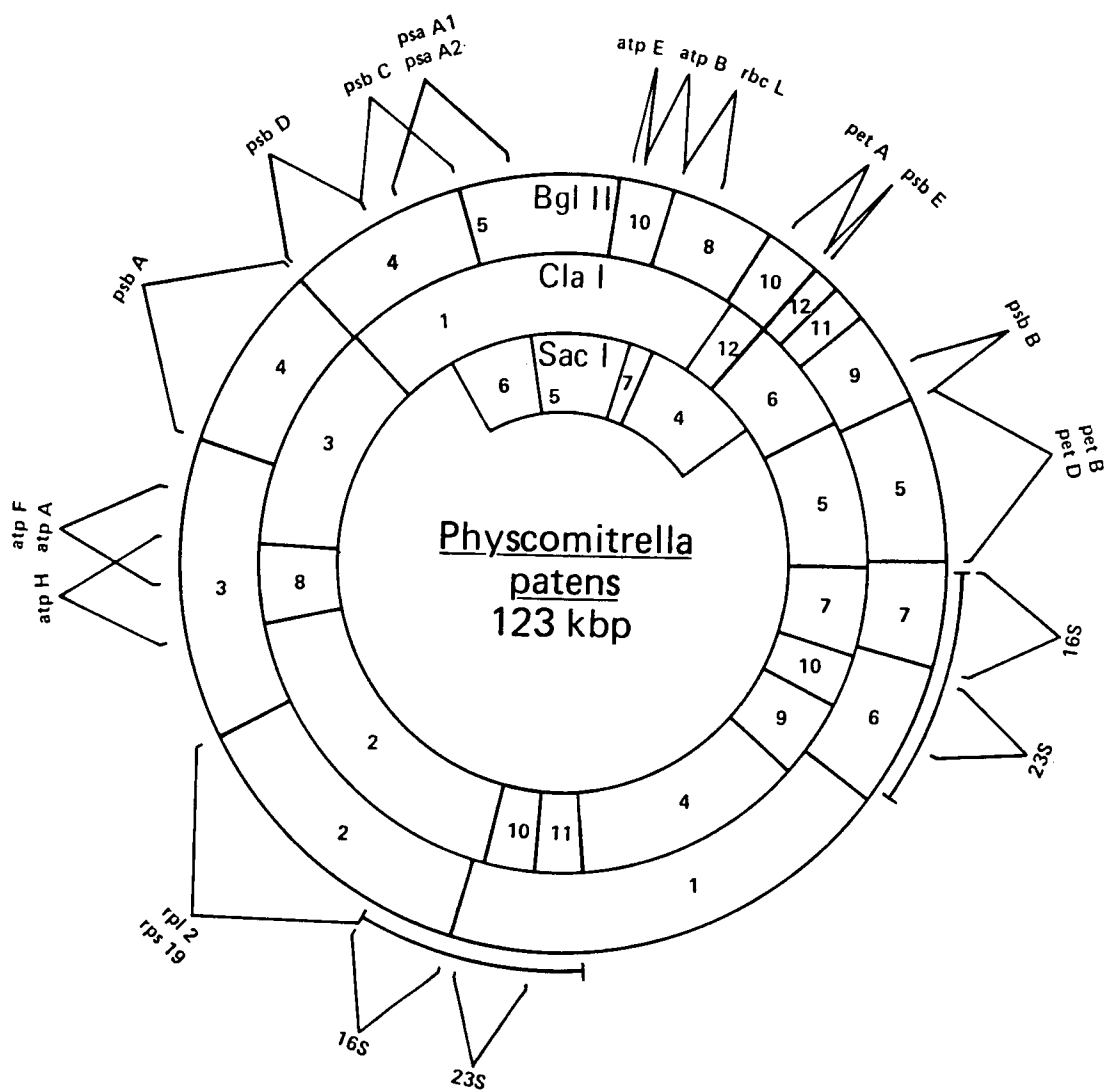


Figure 5

First, there could be a small amount of overlap of juxtaposed restriction fragments into a particular gene, resulting in weak hybridizations signals due to the small region of sequence homology between probe and filter-bound fragment. We tend to discount this explanation, as little difference can be seen in the intensity of autoradiographic signals from hybrids between radiolabeled chloroplast DNA restriction fragments and filter-bound fragments containing different lengths of sequence homology (Table 5, page 37; Figure 3, page 35). Second, the gel-fractionated Zea mays rDNA restriction fragments could be cross-contaminated by neighboring fragments (as is the case with some chloroplast DNA restriction fragment probes, i.e., Figure 3) resulting in artifactual signals. It has been reported that even after two cycles of electrophoretic purification a restriction fragment can still retain contamination from neighboring electrophoresed fragments (Barbacid, 1984).

Gene probes #2, 5, 10, pZmc 461, and the 1.2 kb BamHI subfragment from pZmc 100 contain only specific gene sequences; thus hybridization between these and chloroplast DNA restriction fragments must be due to actual homology between the angiosperm and moss chloroplast genes. Probes #1, 3, 4, 6-8, 9, 11-20, pZr 48, pZr 12, and pZmc 427 and the 3.2 kb Hind III subfragment from pZmc 100 contain, in addition to specific chloroplast genes, flanking sequences. Thus, we cannot exclude the possibility that hybridization with these probes are due to homologies which lie outside the regions containing the gene-specific sequences.

The use of heterologous gene probes in constructing maps of chloroplast DNA is well-established. Among the more extreme cases are the use of corn rbc L to locate the homologue in the protist Euglena gracilis (Stiegler et al., 1982), the hepatic Marchantia polymorpha (Ohyama et al., 1983) and the pteridophyte Osmunda cinnamomea (Palmer and Stein, 1982). E. coli has been the source of a probe to map the polypeptide elongation factor gene tuf A in the alga Chlamydomonas reinhardtii (Watson and Surzycki, 1982). Spinach provided probes for psb A, atp A and atp B which hybridized strongly to Osmunda cinnamomea chloroplast DNA (Palmer and Stein, 1982). Our results extend the number of chloroplast genes which appear to be conserved (to some extent) throughout the plant kingdom to a total of 19 (Table 2, page 9).

Restriction fragment Cla 1 (26.5) contains several internal Bgl and Sac restriction fragments which can now be ordered through the gene-mapping data. Probe pZr 48, containing atp B and atp E, hybridized to Bgl 8 (5.4), Bgl 10 (2.9), Sac 5 (8.8), and Sac 7 (2.0). Probe pZr 12, harboring psa A1 and psa A2, hybridized to Bgl 4 (9.6), Bgl 5 (8.4), and Sac 6 (6.9). These data, in conjunction with the gene restriction mapping data outlined previously, establish the following restriction fragment order: Bgl 4-5-10-8-10, and Sac 6-5-7-4.

The inverted repeat. The maximum size of the inverted repeat, derived by summation of the sizes of the restriction fragments contained within the repeat (Cla 10 (3.4) + Bgl/Cla 7 (5.2) + Bgl/Cla

12 (3.0)) is 11.6 kb. The universal arrangement of the rRNA genes within the inverted repeat, i.e., the 23S rDNA proximal to and the 16S rDNA distal to the small single copy region, is conserved in the moss chloroplast genome. The regions of *rpl* 2 and *rps* 19 encompassed by the gene probe (Table 3, page 17) lie outside the inverted repeat within the large single copy region, most likely near the border of between these two regions (although these data do not allow for a more precise positioning of *rpl* 2 and *rps* 19).

CHAPTER IV

DISCUSSION

Bryophyte (moss and liverwort) chloroplast DNAs approach the lower size limit for land plants. The hepatic Sphaerocarpos donellii contains the smallest genome (115.5 kb) (Herrmann et al., 1980), followed by the hepatic Marchantia polymorpha (121 kb) (Ohyama et al., 1983) and the moss Physcomitrella patens (122 kb). It is interesting to note that the pteridophytes Osmunda cinnamomea (144 kb), Asplenium nidus and Pteris vittata (134.4 kb), and the gymnosperm Ginkgo biloba (158 kb), contain genomes which approach the mid-range for angiosperm chloroplast genomes (Herrmann et al., 1980, Palmer and Stein, 1982; Palmer, 1985).

The inverted repeats of most angiosperms fall within the range of 22-26 kb (Palmer, 1985). Among the non-angiosperm land plants, the trend appears to be toward much smaller inverted repeat regions, e.g., Osmunda cinnamomea (8-13 kb) (Palmer and Stein, 1982), Marchantia polymorpha (11.7 kb) (Ohyama et al., 1983), and Physcomitrella patens (11.6 kb). Size-differences among angiosperm chloroplast DNAs can be accounted for by changes in the size of the inverted repeat, with the small and large single-copy regions remaining rather constant at 20 kb and 80 kb (Palmer, 1985). This also holds true for the bryophytes sampled, as both Marchantia polymorpha (Ohyama et al. 1983) and Physcomitrella patens contain single-copy regions of

approximately 16-17 and 80-82 kb. The pteridophyte Osmunda cinnamomea is an exception, in that it contains a small inverted repeat but a rather large single-copy region (approximately 98 kb) (Palmer and Stein, 1982). The presence of the inverted repeat in such diverse plants as algae (Palmer, 1985), bryophytes and pteridophytes, as well as angiosperms supports the supposition that the inverted repeat is most likely of ancient origin, having arisen relatively early during plant evolution (Palmer and Thompson, 1982).

The most significant finding of this investigation is that the moss genome contains a gene order which is identical to that of spinach (with the exception of a single large inversion). Thus, with the exceptions noted previously, the order and arrangement of chloroplast genes have been preserved since the divergence of the mosses from the lineage which give rise to the angiosperms in the Devonian (345 MYA) and perhaps as early as the Silurian (375 MYA) (Scagel et al., 1982; Schofield, 1985). A number of angiosperm genomes with the inverted repeat also contain large inversions. The legumes, mung bean (Vigna radiata), soybean (Glycine max) and common bean (Phaseolus vulgaris), contain a 50 kb inversion bordered internally by atp A and rbc L (Palmer et al., 1983b); a similar inversion is also found in Oenothera (Tyagi and Herrmann, 1986). An inversion in lettuce (Lactuca sativa) bordered externally by psb C/psb D and psb A contains 23 kb (Palmer, 1985). Inversions are also present in several monocot genomes. Corn (Zea mays) contains two inversions: one of 50 kb, and a smaller one within the larger (Palmer and Thompson,

1982). Wheat (Triticum aestivum) contains two inversions: one of 28 kb bordered internally by psb C and atp A, and a second of 5 kb, containing psb D and psb C (Courtice et al., 1985). In rice (Oryza sativa) an inversion has relocated psb A from its flanking position adjacent to a copy of the inverted repeat to a position 20 kb away (Hirai et al., 1985). A similar event appears to have occurred in the moss chloroplast genome. A 20 kb segment, bordered internally by psb A and atp H, and also containing atp A and atp F, has been inverted so that psb A is now approximately 6-35 kb away from a copy of the inverted repeat. It would be worthwhile to determine if certain sequence elements are present at the borders of these inverted regions which might play a role in mediating recombination events (Palmer, 1985).

Reassociation kinetic analysis has revealed a class of chloroplast DNA sequences which have been highly conserved throughout land plant evolution (Bisaro and Siegel, 1980; Lamppa and Bendich, 1979, 1981). Approximately 30% of the chloroplast genome of 5 dicots and 4 monocots (representatives of two evolutionary lines which diverged approximately 125-150 MYA) (Cronquist, 1985) is strongly conserved, while the remainder of the genome is comprised of sequences which are much more divergent. A portion of these conserved sequences (exhibiting no more than 5%-7% base change) are also present in 2 gymnosperms and 4 pteridophytes. Thus these sequences have been conserved through 400 million years of evolution (Lamppa and Bendich, 1979). The conserved sequences are distributed throughout 50% of

the chloroplast genome. However, as approximately 50% of the highly conserved sequences are less than 550 bp in length, and the median length of a heterologous region is 250 bp, it appears unlikely that any highly conserved sequence individually encodes an entire gene. Rather, as is the case with the murine α and β globin genes (Nishioka and Leder, 1979), it is most probably that different regions of chloroplast genes are evolving at different rates (Lamppa and Bendich, 1981). In our study, all 25 gene probes from 3 dicots and 1 monocot successfully hybridized to moss chloroplast DNA. It is apparent that regions of these genes fall into this class of highly conserved sequences.

Bryophyte phylogeny has long been a major interest to botanists, leading to a diversity of views concerning the relationships not only between bryophytes and tracheophytes (i.e., pteridophytes, gymnosperms, and angiosperms) but also among the bryophytes themselves (i.e., mosses, liverworts, and hornworts) (Schuster, 1979; Schofield, 1985) (One putative scheme is illustrated in Figure 6). Some groups (e.g., the genus Sphagnum) retain relatively few characters that can be used to elucidate their phylogenetic relationships with other organisms, due to extinction of intermediate types or evolutionary loss of characters (Mishler and Churchill, 1984). Characters derived from structural studies of chloroplast DNA (e.g., the type of repeat element present, evidence of recombination events, genome rearrangements) would provide an enlarged data base from which more accurate interpretations of bryophyte phylogeny could be derived. Indeed,

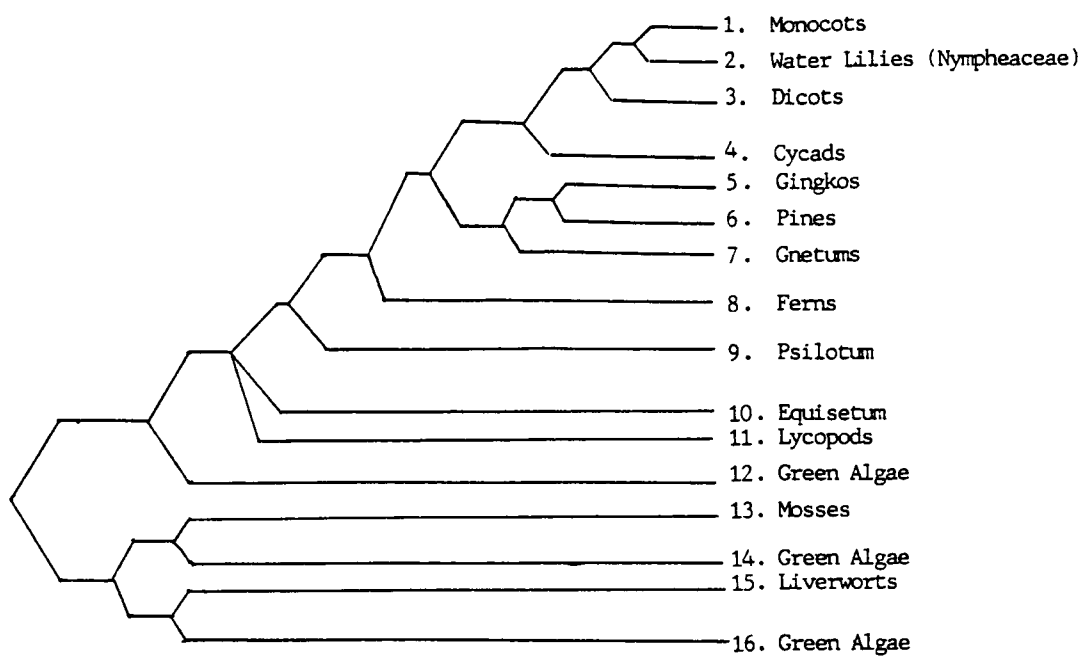


Figure 6. A phylogenetic tree showing putative relationships between major plant taxa (derived by E. E. Schilling, unpubl.)

a phylogenetic scheme for 7 angiosperms based solely on chloroplast DNA structural characteristics (Palmer and Thompson, 1982) is consistent with generally accepted concepts of plant phylogeny based on classical taxonomic criteria (Cronquist, 1985).

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APPENDIX

Figure 7. Results of hybridizations between Cla I digested moss chloroplast DNA and individual radiolabeled Cla I restriction fragment probes. Probe fragment designations, listed above each respective lane, are as in Table 4, page 33. Phage λ DNA molecular weight markers are in lanes designated as λ .

λ 1 2 3 4 5 6 7 8 9 10 11 12 λ

49.1—
23.7, 21.8—

9.5—

7.6—

6.7—

5.9—

5.5—

4.9—

4.3—

3.4—

2.3—

2.0—

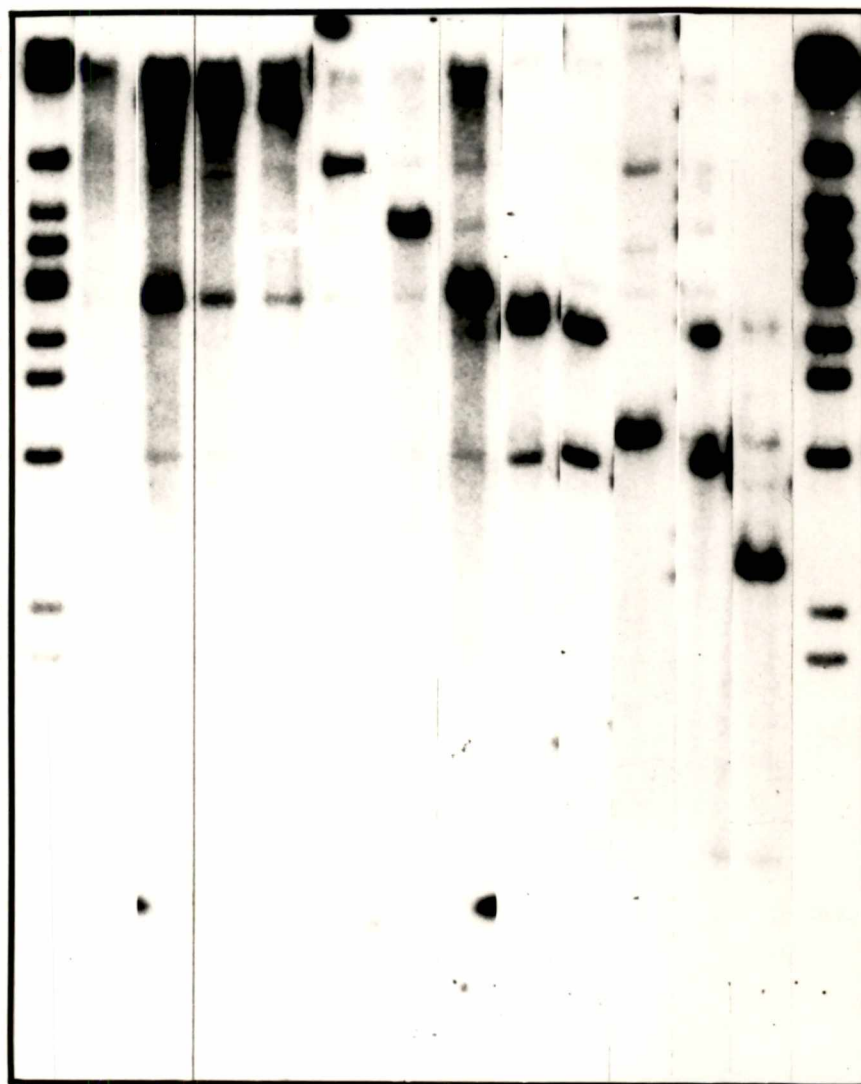
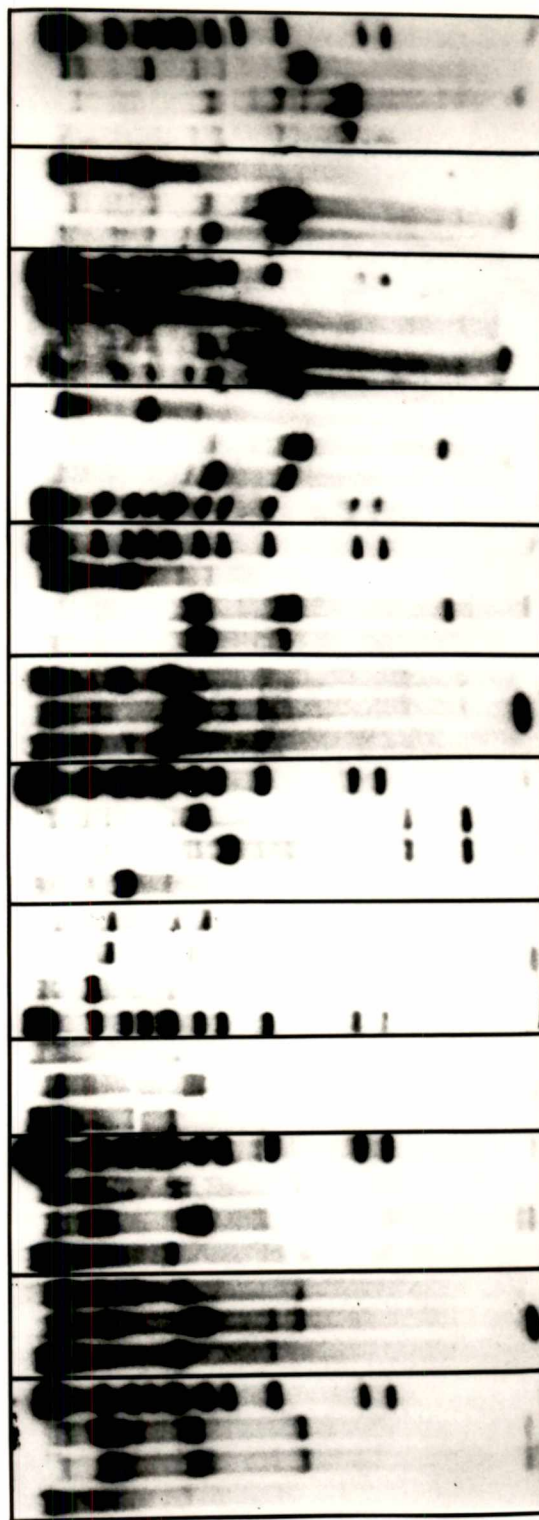


Figure 8. Results of hybridizations between radiolabeled moss chloroplast DNA Cla I restriction fragments and moss chloroplast DNA digested by Cla I (A), Bgl II/Cla I (B), and Bgl II (C). Molecular weights in kb are listed in the left ordinate; molecular weight markers are in lane d. Probe designations are as in Table 4, page 33.

a b c λ a b c a b c λ a b c a b c λ a b c a b c λ a b c a b c λ a b c a b c λ



491
218, 237
9.5
7.6
6.7
5.9
5.5
4.9
4.3
3.4
2.3
2.0

1 2 3 4 5 6 7 8 9 10 11 12

Figure 9. Gene mapping within the inverted repeat. Radiolabeled Zea mays 16S and 23S gene probes (Table 3, page 17) were hybridized to moss chloroplast DNA digested by Cla I (A), Cla I/Bgl II (B), and Bgl II (C). Molecular weights in kb are listed in the left ordinate.

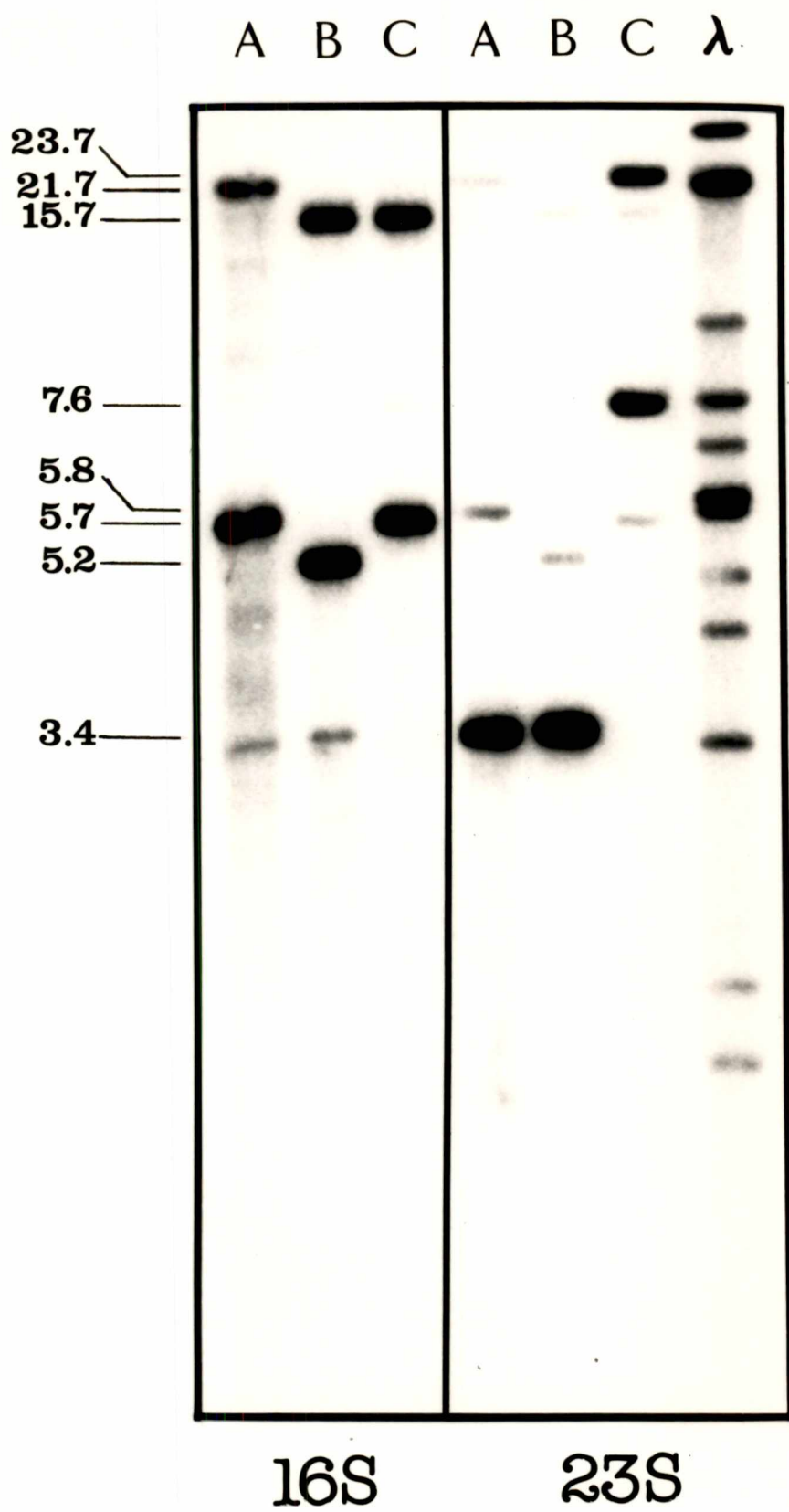


Figure 10. Results of hybridizations between heterologous probes from Spinacia oleracea and Zea mays to Physcomitrella patens chloroplast DNA. Gene symbols are as in Table 3, page 17.

VITA

Patrick Joseph Calie was born in Paterson, NJ on July 8, 1953. He attended elementary school at St. Catherine's School in Glen Rock, NJ, and secondary school at Paramus Catholic Boy's High School in Paramus, NJ. In 1971 he entered Rutgers University-Newark College of Arts and Sciences, from which he received the B.A. degree with a major in Botany in 1975. In 1977 he entered The Department of Botany at The University of Tennessee, Knoxville, from which he received the M.S. degree in 1980 with a concentration in plant systematics. From 1980-1981 he was employed as a senior research assistant under Dr. W. D. Wicks in the Department of Biochemistry at The University of Tennessee, Knoxville. In 1981 he reentered The Department of Botany and received the Ph.D. degree in 1986 with a concentration in molecular biology.