MOLECULAR EVOLUTION IN THE LAMINARIALES: RESTRICTION ANALYSIS OF CHLOROPLAST DNA.

by

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

May 1986

Approval

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Degree: Doctor of Philosophy

Title of Thesis: **Molecular Evolution in Laminariales: Restriction Analysis of Chloroplast DNA**

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Molecular evolution in the Laminariales: Restriction analysis of chloroplast DNA.

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ABSTRACT

A method for the isolation of total blade, chloroplast and nuclear DNAs from kelp tissues has been developed. Total blade DNA was isolated from kelp blades at high molecular weight and at yields of 0.1-1.3 ugr per gram wet weight of tissue extracted. Purified chloroplast and nuclear DNAs were isolated from Macrocystis integrifolia at respective yields of 0.02-0.05 ugr and 0.1-1.0 ugr per gram wet weight of tissue extracted.

Restriction analysis revealed the size of the chloroplast DNA (ctDNA) molecule of M. integrifolia to be approximately 170 kb. Cross hybridization experiments with heterologous probes for the angiosperm single copy chloroplast genes psbA, rbcL and atpB indicate that these genes are also single copy in the M. integrifolia chloroplast genome. In addition, hybridization with a 23S rRNA gene probe from Macrocystis has determined that there are two copies of the 23S rRNA gene in the M. integrifolia chloroplast genome.

The evolution of specific regions of the kelp chloroplast genome was studied in three populations of M. integrifolia, two species of Macrocystis, and single species of eleven other kelp genera and Fucus. The twelve selected kelp genera represent four of the families of the order Laminariales: Alariaceae, Chordaceae, Laminariaceae, and Lessoniaceae. Two different regions of the chloroplast genomes of these specimens were investigated. An 8.7 kb Pst I single copy sequence and an 11.4 kb Pst I restriction fragment containing a portion of the 23S

rRNA repeat region were cloned from the chloroplast genome of M. integrifolia and used as hybridization probes. Differences in the sequences homologous to each probe were assessed by comparing the fragment patterns produced by 6-9 restriction endonucleases. Sequence variation was lowest in population/species comparisons (0.56%) and increased at higher levels of taxonomic comparison: intergenus (3.9-9.7%) and interfamily (10-16%).

Chloroplast DNA restriction fragment patterns were used to investigate phylogenetic relationships among the kelp genera examined in this study. In general, ctDNA phylogeny is in agreement with the previously established taxonomy for the group. However, the restriction fragment patterns of northern hemisphere M. integrifolia and M. pyrifera were identical, whereas northern and southern hemisphere M. integrifolia differed. Dictyoneurum californicum and Dictyoneuropsis reticulata were also identical.

ACKNOWLEDGEMENTS

The encouragement and assistance of Dr. Louis D. Druehl, in the continuance of a long line of phycological mentors, has been integral to my further development as a marine botanist. I gratefully acknowledge Dr. David Baillie for introducing me to molecular biology, for his valuable encouragment and consultation, and for the use of laboratory facilities.

My thanks are also due to the many other people who have made this project work: Dr. Andrew Beckenbach, Dr. L. Kemp, Karen Beckenbach, Dr. Mike Smith, Dr. Rose Ann Cattolico, Eric Cabot, Kelly Thomas, Dr. Terry Snutch, Steve Bressler, and Dr. Radovan Popavic, for consultation and technical assistance; Dr. Mike Foster, Dr. Bernabi Santilices, Dr. John Van der Meer, Gabriella Hannach, Dr. Mirielle Amat and Debashish Battacharya for the provision of plant materials from both near and far; Dr. Jeff Palmer for the generous gift of the angiosperm chloroplast gene probes used in this study; Bamfield Marine Station, Beverly Dayton, Dr. David Epel and Dr. Roy Britten for the use of facilities during collecting trips; Simon Fraser University for a President's Research Scholarship; National Science and Energy Research Council for funding (L. D. A2918); and to Lynn Fain for her supportive ways, dependable presence and willing hands.

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GENERAL INTRODUCTION

Kelp exhibit the highest degree of morphological complexity and anatomical specialization achieved among the algae. They range in proportion from the relatively undifferentiated Chorda to the intricate giant kelp Macrocystis. They exhibit a heteromorphic life cycle with dominant sporophyte and independant dioecious gametophyte stages and display a common, highly conserved embryogeny. The kelps are of the chlorophyll a+c-chromophyte line of chloroplast evolution, having evolved separately from the chlorophyll a+b-chlorophyte line for at least 500 million years (Scagel et al. 1982). They include species of both annual and perennial strategy and are commonly the dominant component of the shallow littoral and sublittoral communities of the world's temperate and polar coasts.

Taxonomically, the twelve orders of Phaeophyta are in the single class Phaeophyceae, having two evolutionary lineages represented by the subclasses Phaeophycidae and Cyclosporidae (Papenfuss 1955). Work defining the order Laminariales as a cohesive group within the subclass Phaeophycidae has included the description of the kelp life history and embryogeny (Sauvageau 1915), the cytology of reproductive structures (Fritsch 1945), pheromonal specificity (Luning & Muller 1978, Muller et al. 1985), and the ultrastructure of motile cells (Henry & Cole 1982a&b).

For some time now, researchers have attempted to add to

our understanding of the phylogeny of the Laminariales from tests of interfertility between morphological types. However, the results to date are few and confusing. Although crossability tests generally demonstrate that reproductive isolation exists between morphologically distinct families (Yabu 1964), within the family Lessoniaceae, successful hybridization is routinely observed between morphologically distinct genera (Sanbonsuga & Neushul 1978). Thus far then, the phylogenetic relationships of kelp are poorly understood. Clearly, another means of assaying genetic relatedness is needed.

Isozyme analysis of metabolic proteins, so widely employed in evolutionary studies of other plant groups (Allard et al. 1975), is a likely method of choice. However, the extraction of biologically active kelp proteins is not only prohibitively complicated by the chemistry of kelp tissues (Marsden et al. 1983), but electromorphs are rare compared with variants not affecting the charge of polypeptides (Selander 1976, Kreitman 1983), and they can also be subject to environmental effect. In addition, isozyme analysis can only be effectively employed in comparisons below the species level (Nei 1975).

Alternatively, evolutionary biologists have employed the molecular biological technique of restriction analysis to compare organisms by the nucleotide sequences of their respective DNAs (Rose et al. 1982, Brown et al. 1979, Palmer et al. 1985, Pruitt & Meyerowitz 1986). Restriction fragment pattern analysis quantifies the sequence variation that has

occurred in the DNA of related organisms since they last shared a common ancestor. The application of such an analysis to kelp would provide a directly quantifiable feature for phylogenetic comparisons that is not subject to environmental effects and can be applied at most taxonomic levels.

Many features of chloroplast structure and biochemistry have been used historically in studies of algal systematics. Chloroplast shape, size, distribution within the cell, photosynthetic pigments and reserve products, and photosynthetic membrane arrangement are all distinguishing characteristics of the major classes of the algae (Bisalputra 1974, Meeks 1974).

The chloroplast genome has become a major focus for studies of plant molecular evolution. Restriction fragment pattern analysis has been used to demonstrate species specific nucleotide sequence diversity among chloroplast DNA molecules in several groups of vascular plants, including Nicotiana, Lycopersicon, Oenothera, Brassica and Pisum species (Kung et al. 1982, Palmer & Zamir 1982, Gordon et al. 1982, Palmer et al. 1983a, Palmer et al. 1985). Such data has revealed the occurrence of mutational events corresponding to the separation of different lineages within these groups.

Conservation of the plant chloroplast genome.

Comparisons of the chloroplast genomes of species as widely diverse as angiosperms, ferns, and bryophytes have demonstrated a marked similarity in base composition

(G+C=35%), in size(110-180 kb), and a highly conserved structural organization (Herrmann & Possingham 1980). Prominent among these conserved structural features are the circularity of the chloroplast molecule, the 10-27 kb inverted repeats that encode the rRNA genes, and the small (12-27 kb) and large (83-103 kb) single copy regions that separate the repeat elements. In addition, the positions within the chloroplast molecule of the tRNA, rbcL, psbA, and ATPase complex genes are also highly conserved (Palmer & Thompson 1981a, de Heij et al. 1983). This arrangement has been maintained in all instances examined save 1) A small group of legumes differ in having undergone extensive sequence rearrangements that include the loss of one copy of the inverted repeat. 2) The Euglenophyta lack the inverted repeat and exhibit one large single copy region punctuated by from 1-5.5 tandemly repeated copies of the rRNA genes. 3) Twelve genera in 6 tribes in the Fabaceae have undergone a 50 kb inversion of the large single copy region that places the rbcL and psbA genes only 5 kb apart whereas, in spinach and the majority of dicots and monocots these genes are some 50 kb apart. 4) In Chlamydomonas the orientation of the inverted repeats is reversed with respect to most chlorophytes, and the atpB gene is located in the small single copy region.

Similar characterizations of the chloroplast genomes of the chromophytic (i.e., algal forms with chl a,c) and rhodophytic (i.e., algal forms with chl a and phycobilins) are almost nonexistant. Linne von Berg et al. (1982) have

reported genome sizes for the chromophytes Botrydium granulatum (108 kb), Odentella sinensis (120 kb), Tribonema verde (129 kb), and Vaucheria sp. (116 kb); while Dalmon et al. (1983) have reported that Pylaiella littoralis and Sphacelaria sp. exhibit ctDNA molecules that are heterogeneous in size. However, considerable chloroplast genome organization data are available for the chromophytic alga Olisthodiscus luteus (Ersland et al. 1981, Aldrich & Cattolico 1981, Aldrich et al. 1982, Reith & Cattolico in press). The physical map of the 150 kb O. luteus chloroplast genome is similar to those of chlorophyte plants (i.e., algal and higher plant forms with chl a,b) in that it is comprised of large (22 kb) inverted repeat sequences that define small (37 kb) and large (73 kb) single copy regions. Interesting differences from the chlorophyte pattern have been noted, however, in both the organization and kinds of genes encoded on the O. luteus chloroplast genome. The gene for the small subunit of ribulose bisphosphate carboxylase (rbcS) is encoded on the inverted repeat of the ctDNA of O. luteus along with both the rbcL and psbA genes. The rbcS gene is multi-allelic and nuclear encoded in all higher plants and green algae so far investigated. In addition, save for Chlamydomonas and Pelargonium, the rbcL and psbA genes are encoded on the large single copy region of the several hundred chlorophytic plants so far characterized (Palmer 1985). The differences in chloroplast gene organization between higher plants and Euglena, Chlamydomonas, and Olisthodiscus have inspired some

authors (Lemieux et al. 1984, Reith & Cattolico in press) to suggest that the chloroplast genomes of algae are evolving faster than those of higher plants. However, too little is yet known regarding the evolutionary age of these algal lines; e.g., Walton (1953) states that there is no conclusive evidence that the Phaeophyceae existed before the Jurassic (180 million years ago). In addition, Reith & Cattolico (in press) have observed that all of the algae for which chloroplast genome organization information is available are unicellular forms with comparatively short life spans. They suggest that genome evolution in these organisms may be different than in macroscopic algae with longer life cycles and complex tissue organization. In any event, additional algal representatives, definitely those among the Phaeophyceae and Rhodophyceae, must be investigated before any general understanding of chloroplast evolution can be attained.

Objectives of thesis.

The taxonomy and phylogeny of taxa of the Laminariales (Division Phaeophyta) is essentially based upon phenetic characteristics of extant representatives. This has resulted, in part, from the fact that there are no significant fossil data. The development of the molecular biological technique of restriction analysis has provided researchers with a way to quantitatively determine genetic interrelatedness.

The objectives of the present study were as follows: 1) to develop a method for the extraction and isolation of nuclear

and chloroplast DNA from kelp tissue using Macrocystis integrifolia Bory as the type model; 2) to clone and restriction map different segments of the chloroplast genome of M. integrifolia; 3) to use these cloned ctDNA sequences to probe homologous sequences in the ctDNAs of a number of different kelp species in order to determine the level of sequence diversity that exists between the ctDNA molecules of kelps at the species, genus, and family levels and 4) to suggest a phylogeny for the Laminariales based on these data.

Kelp are particularly well suited to an analysis such as this. In contrast to the relatively diverse higher plant groups that have been the focus of restriction analysis to date (i.e., the family Leguminoceae has about 500 genera, Robbins et al. 1957), the kelps are a small, well defined order of only five families and thirty genera (Druehl unpublished). Consequently, the entire group can be effectively described by a relatively small sample size. As kelp are chromophytic plants, information obtained from their restriction analysis may provide a unique perspective on plant evolution. One study concerning the sequence organization of the chloroplast genome of a chromophytic alga in the Division Chrysophyta has recently been completed (Reith & Cattolico in press). However, this study is the first to investigate ctDNA sequence variation in the chromophytic Division Phaeophyta.

Morphological features that distinguish the studied families and genera of kelp.

The taxonomy of the order Laminariales (5 families, 30 genera, 99 species) is a morphological classification based upon the different ways that the sporophyte form is generated.

Interfamily distinctions. The four studied families are distinguished by characteristic modifications that occur at the transition zone between stipe and blade during development. In the family Laminariaceae, the transition zone is plane and unmodified. The resulting frond (i.e., stipe with associated blade) is therefore simple and unbranched. In the family Lessoniaceae, the transition zone is split longitudinally at the base of the initial juvenile blade. As the blade grows, the split elongates and eventually divides the initial blade. The secondary blades that result retain their own stipes and meristematic transition zones. This initial dichotomous branching and subsequent divisions result in the generation of a compound frond. In the family Alariaceae, the frond is pinnately compound as a result of sporophyll blades arising as outgrowths from the lower portion of the meristematic region at the transition zone. The Chordaceae differ significantly in morphology from other kelp. They consist of long-hollow-cylindrical fronds that are described by meristematic activity that is dispersed over the entire thallus. They do not exhibit a differentiated stipe.

Intrafamily distinctions. The characteristic morphology of the lessoniacean sporophyte is an array of dichotomously branched stipes that each terminate in a single blade. Although the occurrence of pneumatocysts are not exclusive to the Lessoniaceae, they are prominent structures among several genera (Macrocystis, Nereocystis, Pelagophycus).

The Lessoniaceae consist of two basic habitat morphs: exposed coast and subtidal forms. In each morphotype the stipe and blade parts have taken a different course of development. The exposed coast forms developed thickened, rigid, erect stipes conducive to the dissipation of wave shock and capable of supporting the blade parts above the substrate (Lessonia, Lessoniopsis and Postelsia). The subtidal forms also exhibit an erect stipe, but it is a much elongated, elastic, and buoyant structure that supports the blade parts within the photic zone (Macrocystis and Nereocystis). In Dictyoneurum and Dictyoneuropsis the stipe is relatively reduced, and both genera occur in low intertidal to subtidal depths. Blade morphologies that are characteristic of intertidal and subtidal habitats have also evolved. The blades of intertidal plants are relatively short and narrow, typically with longitudinally arrayed bullae, veins or midribs (Dictyoneuropsis, Lessoniopsis, Postelsia), whereas they are generally longer, wider, and without as highly developed secondary surface structures in subtidal genera.

Descriptions of the kelp species examined in this study.

There are eight extant genera and one fossil genus in the family Lessoniaceae. Seven of these genera were included in this study and they are characterized as follows: Lessonia exhibits numerous blades that are born at the extremities of repeatedly branched stipes. Blades are narrowly linear and without midribs. The stipe is erect and characteristically woody and thickened at the base. There are three species in the genus (Searles 1978); L. nigrescens Bory was the studied species. Lessoniopsis exhibits an erect stipe that branches repeatedly. Each branch is continued in a single, narrow vegetative blade and two pinnate sporophyl blades. Vegetative blades exhibit a characteristic flattened midrib. The lower portion of the stipe is conical and considerably woody. The genus is monotypic [L. littoralis (Tild.) Reinke, Nicholson 1976]. The genera Dictyoneurum and Dictyoneuropsis display a prostrate that is markedly flattened. Blades are wide, linear, and reticulated. Dictyoneuropsis exhibits a midrib-like veination pattern, whereas Dictyoneurum does not. Both genera are monotypic [Dictyoneurum californicum (Rupr.) and Dictyoneuropsis reticulata (Saund.) Smith, Nicholson 1976]. Postelsia has a stipe that is erect and hollow. At the apex of the stipe there are many short branches that each terminate in a single narrowly linear blade. Blades are covered with deep-parallel-longitudinal grooves. The genus is monotypic (P. palmaeformis Ruprecht, Nicholson 1976). Nereocystis stipes are long and erect and are inflated

distally into a single large pneumatocyst. Narrow, smooth-margined blades arise from short, flattened branches. The genus is monotypic [N. luetkeana (Mert.) Post. & Rupr., Nicholson 1976]. Macrocystis exhibits many long stipes that branch from an initial primary stipe. Blades are wide, each with a basal pneumatocyst, and they are unilaterally arranged at regular intervals along the stipe. There are three species in the genus; M. integrifolia Bory and M. pyrifera (L.) C. Ag. were the studied species (Nicholson 1976).

There are eight genera in the family Alariaceae. Two of these were included in this study. They were selected because they represent morphologically distinct tribes within the family. They are characterized as follows: Alaria exhibits a single undivided blade that is broadly linear and includes a conspicuous midrib. The stipe is erect, cylindrical, and unbranched, and sporangia are produced on sporophylls attached laterally at the base of the blade. There are fourteen species in the genus (Widdowson 1971); A. marginata Post. & Rupr. was the studied species (Nicholson 1976). In Egregia the stipe is erect, flattened, and branched. The lateral margins of the stipe are fringed with papillae-like blades, and certain of these develop pneumatocysts. The sporophyll blades are intermingled with sterile blades along the length of the stipe. The genus is monotypic [E. menziesii (Turn.) Aresch., Nicholson 1976].

The family Laminariaceae is comprised of eleven genera. Two of these were included in this study. These genera were

also selected because they represent morphologically distinct tribes within the family. They are characterized as follows: Costaria exhibits an undivided, broadly-linear blade that includes five characteristic longitudinal ribs. The stipe is erect, cylindrical, and unbranched. There is a single species in the genus [C. costata (C. Ag.) Saund., Nicholson 1976]. In Laminaria the broadly linear blade is undivided and, in some species, includes characteristic marginal rows of bullae. The stipe is erect, cylindrical, and unbranched. There are thirty-two species in the genus (Druehl unpublished); L. saccharina (L.) Lamouroux was the studied species (Druehl 1968).

The family Chordaceae contains a single genus. The genus Chorda is characterized as follows: The long cylindrical fronds are unbranched and hollow, with diaphragms at irregular intervals. The fronds are covered with hairs. There is no distinction between stipe and blade as is the case in all other members of the order. There are two species in the genus (Setchell 1893); C. filum (L.) Stackh. was the studied species.

Plant collections.

Specimens of Macrocystis integrifolia, Lessoniopsis littoralis, Postelsia palmaeformis, Laminaria saccharina, Costaria costata, Alaria marginata, Egregia menziesii, and Fucus distichus were collected from localities around Vancouver Island, BC, Canada (Table 1). Specimens of M. integrifolia, M. pyrifera, Dictyoneurum californicum, and Dictyoneuropsis reticulata were collected from localities along the central and southern coast of California (Table 1). Specimens of M. integrifolia and Lessonia nigrescens were collected from the central coast of Chile (Table 1); and Chorda filum was collected from the southern coast of eastern Canada (Table 1).

The fifteen algal samples examined in this study comprise the range of morphological variation exhibited within the order Laminariales and include, as well, all of the genera (save Pelagophycus) in the family Lessoniaceae and M. integrifolia population samples from both hemispheres. Fucus distichus, with its clear cytological, ultrastructural, and reproductive differences from the kelps, is the most evolutionary distant species in the analysis. The spinach (Spinacia oleracea) samples used in this study were obtained from a local produce grocer.

Table 1. Taxonomic relationships and collection sites of laminarialean and fucalean species studied.

Family	Tribe	Genus	Species	Collection Site
Lessoniaceae[1]	Macrocysteae	Macrocystis	integrifolia Bory	3, 4, 5
			pyrifera (L.) C. Ag.	6
	Lessonieae	Dictyoneurum	californicum Rupr.	4
		Dictyoneuropsis	reticulata (Saund.) Smith	7
		Lessonia	nigrescens Bory	5
		Nereocystis	luetkeana (Mert.) Post. & Rupr.	8
		Postelsia	palmaeformis Ruprecht	9
	Lessoniopseae	Lessoniopsis	littoralis (Tild.) Reinke	9
Alariaceae[1]	Alarieae	Alaria	marginata Post. & Rupr.	8
	Egregieae	Egregia	menziesii (Turn.) Aresch.	10
Laminariaceae[1]	Laminarieae	Laminaria	saccharina (L.) Lamouroux	11
	Agareae	Costaria	costata (C. Ag.) Saunders	11
Chordaceae[1]		Chorda	filum (L.) Stackh.	12
Fucaceae[2]		Fucus	distichus (L.)	11

Superscript Notations: 1=Subclass Phaeophycidae-Order Laminariales; 2=Subclass Cyclosporidae-Order Fucales.
Collection Sites: 3=Port Desire, Bamfield, BC., CAN.; 4=Stillwater Cove, Monterrey, CA., USA.; 5=Horcon, Chile, SA.; 6=Catalina Is., CA., USA.; 7=Pacific Grove, Monterrey, CA., USA.; 8=Edward King Is., Bamfield, BC., CAN.; 9=Botany Beach, Van. Is., BC., CAN.; 10=Aguilar Pt., Bamfield, BC., CAN.; 11=Brockton Pt., Van., BC., CAN.; 12=Paddy's Head, Halifax, NS., CAN.

Overview of kelp cell structure.

Requirements of DNAs used in restriction analysis are that they be obtainable at acceptable yields, be free of contaminants that inhibit restriction endonuclease activity, and that they be of high molecular weight. The DNA need not be full length but should be significantly larger than the largest fragments produced by the restriction enzymes used in the mapping analysis. Thus, when isolating DNA, it is essential that the the nuclear membrane and chloroplast envelope be stabilized against disruption during the isolation process in order to prevent the access of naturally occurring degradative nucleases.

All tissues of the laminarialean thallus exhibit a similar tissue organization: a meristematic epidermal layer (i.e., meristoderm), a parenchymatous cortex and a central medulla that, in some species, expands into hollow pneumatocysts. The three tissue types vary in cell size and organellar complement (Smith 1939).

The cell walls of the kelps consist of an inner layer of cellulosic fibrils and a thick outer gelatinous layer of pectic substances (Dawes et al. 1961). The pectic substances of the cell wall are the gel forming, high molecular weight structural polysaccharides alginate and fucoidan which together comprise almost 1/3 of the total dry weight of kelp tissue (Rossel & Srivastava 1984).

The physodes are membranous vesicles that are a common component of kelp meristoderm cells. They contain

phloroglucinol compounds and other tannins that constitute from 0.02-0.3% of tissue fresh weight (McInnes et al. 1984). Polyphenolic compounds and their breakdown products are powerful inhibitors of subcellular organelles (Loomis 1974).

The gel forming polysaccharide wall materials and the reactive contents of the physodes are the principal features of the kelp cell to be surmounted in organelle and DNA isolation from kelp tissue.

Chloroplast morphology and isolation.

The majority of the chloroplasts in a kelp thallus are located in the blades and are found within the meristoderm and outer cortical cell layers. Kelp chloroplasts are oval-shaped and situated parietally within the cytoplasm. The thylakoid membranes of kelp chloroplasts are not partitioned into grana as in higher plants but are appressed into sheets of three thylakoid bands. The thylakoids and stroma are sequestered by an encompassing thylakoid sheet called the girdle lamellae. The chloroplast genophore occurs as a continuous ring-shaped structure just within the girdle lamellae (Bisalputra & Bisalputra 1967), not as scattered or single loci within the stroma as in chlorophyte plants (Ris & Plaut 1962). In the kelps, as in all brown algae, an unusual double-membraned outer envelope arises as an outfolding of the outer membrane of the nuclear envelope and extends around each of the chloroplasts of the cell. This enveloping membrane has been called the chloroplast endoplasmic reticulum (Bouck 1965,

Gibbs 1981).

In this study, algal chloroplasts and nuclei were isolated using an adaptation of the method of Nordhorn et al. (1976). The protocol presented here utilizes: 1) Liquid nitrogen to freeze and grind blade tissues to achieve effective trituration; 2) A high extraction buffer volume to tissue weight ratio to dilute the copious amount of water soluble mucilage exhuded by kelp tissue; 3) High centrifugation speeds to sediment organelles from the viscous tissue suspensions and; 4) Cesium chloride-density gradient centrifugation to concentrate and isolate the DNA contents of the organelle preparations.

This protocol has been used to isolate nuclear and chloroplast DNAs from many different kelp genera as well as Fucus. Best results were obtained with blade tissues from which any thickened midrib or fascia structures had been removed. This protocol has been routinely applied to tissue samples as small as 20 grams and as large as 2 kilograms fresh weight. Large samples are most conveniently processed in 100 gram subsamples. More than enough DNA for the analysis of chloroplast DNA sequence divergence described in this study was obtained from 100 gram tissue samples. Another important feature of the protocol is that samples can be held at -70 C either as whole-frozen or frozen-and-ground blade tissue until further processing is convenient. Samples have been held as long as one year at -70 C with good results.

All buffers and centrifugation runs were maintained at 0 C

except where noted. All transfer steps were performed as rapidly as possible and on ice except where noted. Whenever possible, healthy young blades free of epiphytes were selected for organelle extraction. However, older tissue was used, but it required that all sclerotic tissue and visible epiphytes be removed (a razor blade and cork borer worked well in this application). In addition, when necessary, fascia or midrib structures were removed.

Once the material for DNA extraction was selected, the blades (100 gr) were immersed in distilled water, a few at a time, and scrubbed with a cheesecloth pad. The blades were then blotted dry and stripped of surface mucous by rubbing vigorously with a dry cheesecloth pad; sectioned with a clean razor blade into 2 cm squares; and quick frozen by immersing in a wide mouthed (7-10 cm diameter) Dewar flask containing 500 ml of liquid nitrogen. The frozen tissue pieces were then poured, 25 gr at a time, into a chilled (-70 C) mortar (11 cm diameter) and ground to a fine powder by hand with a pestle (4.5 cm diameter). The capacity of this size of mortar in this application is about 25 gr. When larger sample sizes were attempted, it was difficult to obtain the required particle size without spilling a considerable amount of sample in the process. For this application, a larger mortar proved unwieldy. During the early stages of the grinding process, the liquid nitrogen that was poured into the mortar, along with each tissue sample, was replenished as it boiled away. However, as the tissue approached a powder consistency,

it boiled out of the mortar along with the liquid nitrogen. Consequently, the grinding process was typically completed in about a minute in order to avoid excessive warming. As each of the four 25 gr subsamples were ground, they were combined in a plastic bag that was buried in crushed dry ice.

The protocol was continued by transferring the ground blade tissue in 50 gr portions into two chilled (-20 C) mortars (11 cm diameter) and warming to -10 C at room temperature by constant stirring with frozen spoons. If the samples were not stirred constantly and, if the stirring spoons were not kept frozen, the tissue powder warmed unevenly and this resulted in a reduced yield. It was also determined that the 100 gr tissue sample warmed more evenly and more rapidly when divided into two 50 gr subsamples. The tissue was warmed to -10 C before being suspended in extraction buffer because it freezes into a solid lump if it is transferred immediately after grinding (i.e., the temperature of liquid nitrogen is -195.8 C).

The two 50 gr tissue samples were each suspended in 400 ml of Buffer A [Buffer A is: 1.65 M Sorbitol, 50 mM MES, 10 mM EDTA, 2% (w/v) Polyvinylpyrolidone (PVP), 0.1% (w/v) BSA, 5 mM B-Mercaptoethanol, pH 6.11. This ratio of buffer volume to tissue weight (8:1) was determined empirically. A lower ratio (6:1) resulted in a suspension with a gel strength that precluded efficient filtration and centrifugal fractionation. A higher ratio (10:1) did not change yield appreciably and, since the cost of the extraction buffer ($6.00 CDN/liter) was

not inconsiderable, the stated ratio of 8:1 was selected. The extraction buffer was contained in 1 liter beakers that included 1 X 4 cm stir bars. The tissue samples were slowly sifted onto the surface of the buffer volume while it was being mixed with a magnetic stirrer set at high speed. The tissue was added slowly so that it melted into an even suspension. About 30-45 seconds were required for the transfer. Because the magnetic stirrer could not provide the rate of mixing required for the rapid suspension of 100 gr of tissue in the larger volume, two separate 400 ml extraction buffer volumes were used instead of a single 800 ml volume. In addition, it was determined that the filtration step of the process was more efficiently accomplished with smaller suspension volumes (see below).

The resulting homogenates were each expressed through individual cheesecloth filter pads. Although the gel strength of the tissue suspensions varied by species and season, they were always quite viscous and required some force to be expressed through the filter. A filter pad consisted of eight layers of cheesecloth, each 45 cm on a side. The center of each filter pad was pressed into the mouth of a 15 cm diameter funnel whose pipe end was directed into a collection beaker buried in an ice bucket. The filter pad and funnel were supported over the collection beaker with a ring stand. Typically, 50 gr of tissue sample was combined with 400 ml of extraction buffer to yield a suspension volume of about 500 ml. This volume would almost fill the filter lined funnel. A

greater tissue load was beyond the carrying capacity of the filter. While the filter was wetting, its draping edges were gathered together and closed at the top with a few twists. Then, by applying a steady wringing pressure with the hands, the tissue suspension was squeezed through the filter mesh.

The volume of the pooled filtrates from the extraction of 100 gr of tissue generally ranged from 600-700 ml. This volume was dispensed into four 250 ml centrifuge bottles and centrifuged at 4,000 X g for 10 minutes in a Sorval GSA rotor. The pellets, consisting of cell debris, nuclei, chloroplasts, and probably some mitochondria, were pooled and gently resuspended in two 200 ml volumes of Buffer B (Buffer B is: Buffer A without PVP) with a clean camel hair brush (1 cm width) and centrifuged as above. The resulting pellets were each gently resuspended in 40 ml of Buffer B with a clean camel hair brush, and the nuclear/cell debris fraction was pelleted by centrifugation at 120 X g for 10 minutes in a Sorval SS34 rotor. The chloroplast fraction was collected from the supernatant by centrifugation at 3,000 X g for 5 minutes in a Sorval SS34 rotor. For nuclear and total blade DNA preparations, the differential centrifugation step was skipped.

DNA isolation.

The two pellets were each resuspended in 20 ml of Buffer C (Buffer C is: 1.65 M Sorbitol. 50 mM Tris-HCl, 25 mM EDTA, pH7.5) with gentle vortexing and pelleted by centrifugation at

3,000 X g for 5 minutes in a Sorval SS34 rotor. The pellets were each resuspended in 5 ml of Buffer C with gentle vortexing and combined in a single clean 50 ml capped tube (Falcon 2098). Proteinase K (Sigma P0390; 2 ml of a 5 mgr/ml solution prepared in buffer C) was added to the approximately 20 ml suspension volume for a final concentration of 0.5 mgr/ml and incubated for 2 minutes on ice. The organelle suspensions were lysed by adding one third of the suspension volume (about 7 ml) of Buffer D [Buffer D is: 15% (w/v) sodium sarcosinate, 50 mM Tris-HCl, 25 mM EDTA, pH 8.0] and gently mixing by inverting occasionally on ice for 1 hour.

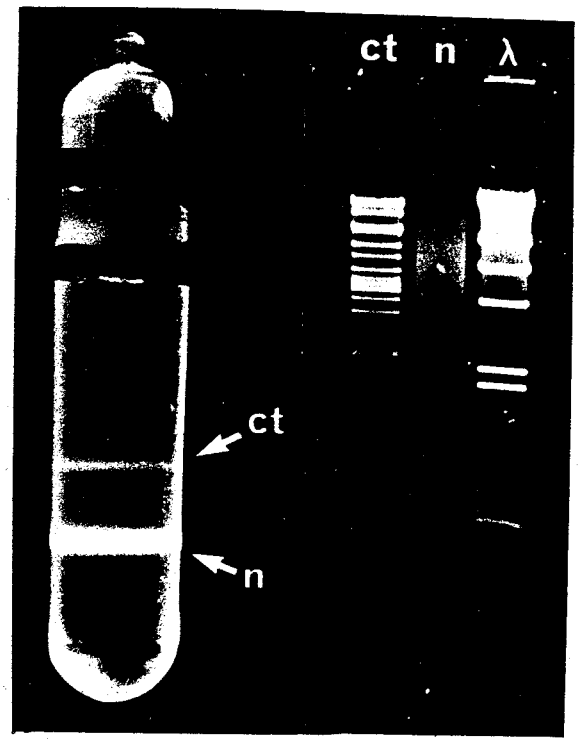
Solid cesium chloride (CsCl, 1 gr/ml) was dissolved in each lysate volume (density=1.7 gr/cc), and the protein, lipid, and polysaccharide that precipitated with this increase in salt concentration were pelleted from the lysates by centrifugation at 27,000 X g for 30 minutes at room temperature in a Sorval SS34 rotor. The supernatants were pooled, and Hoechst dye (Sigma B2883) in a 10 mgr/ml sterile distilled water stock solution was added to a final concentration of 40 ugr/ml. The supernatant plus dye was then partitioned into three 12.5 ml polyallomar ultra-centrifuge tubes and centrifuged at 45,000 rpm for 30 hours at 20 C in a Beckman Ti-80 rotor.

The DNA appeared within the gradients as two ultraviolet-fluorescent bands. The chloroplast DNA fraction was contained in the upper band while the nuclear DNA fraction was contained in the lower band (Fig. 1). Each of these DNA

Figure 1. Separation of total blade DNA into nuclear (n) and chloroplast (ct) components.

A. The figure is of a Hoechst dye-CsCl gradient at equilibrium. The designations of the respective DNA fractions are indicated with arrows.

B. Fluorescence photograph of the restriction fragment patterns of the respective DNA fractions in panel A digested with the restriction enzyme Pst I. Lambda DNA digested with Hind III is included as a restriction fragment size standard.


ct
n
λ
ct
n
A
B

fractions was collected with a separate hypodermic syringe fitted with a #18 needle. The chloroplast and nuclear DNA fractions collected from the three gradients (about 0.5-1.0 ml/gradient) were pooled separately and centrifuged a second time in an ethidium bromide (Sigma E8751; 150 ugr/ml)-CsCl (1.0 gr/ml) density gradient. The chloroplast and nuclear DNA fractions were each recentrifuged in a single Ti-80 tube. Ethidium bromide was added to each gradient as a 10 mgr/ml solution prepared in sterile distilled water.

Ultracentrifugation through a second CsCl density gradient was neccessary in order to remove a contaminant(s) from the DNA fractions (i.e., most likely a polysaccharide, Aldritch et al. 1982) that exhibited a similar density as DNA in cesium chloride density gradients and that inhibited the activity of restriction endonucleases. As the inhibition of restriction endonuclease activity was no longer observed after a second passage of the DNA fractions through a CsCl density gradient, the contaminant was judged to have either been completely removed or sufficiently diluted from the DNA samples. Total blade DNA preparations were not passed through Hoechst dye-CsCl gradients, but were passed through two consecutive ethidium bromide-CsCl density gradients. Following the second ultracentrifugation passage, the DNA samples were gently isopropanol extracted 4-7 times to separate the DNA from the dye. Following this, the DNA was precipitated by adding 2 volumes of water and 6 volumes of cold 95% ethanol while incubating at -20 C for 1-2 hours (Davis et al. 1980). The

precipitated DNA was collected by centrifugation at 25,000 X g in a Sorval SS34 rotor for 20 minutes at 0 C. Small amounts of ctDNA were collected by ultracentrigugation in a Beckman SW-40 rotor at 30,000 rpm for 30 minutes at 5 C. The DNA pellets were washed with 70% ethanol at room temperature, air dried, and resuspended overnight without agitation at 4 C in Buffer D (Buffer D is: 10 mM TRIS, 1 mM EDTA, pH 8.0). Once the DNA was completely resuspended, it was brought to a concentration of 500 ngr/ml with Buffer D and stored at 4 C over a few drops of chloroform. DNA samples stored in this fashion were stable in fragment length for over 1 year. If DNA samples were to be stored for longer periods, they were precipitated as above and stored at -20 C in 70% ethanol. DNA samples stored in this fashion have been stable in fragment length for over 2 years.

Techniques for molecular analysis.

Restriction enzyme digestions. Total blade DNA, chloroplast DNA and plasmid DNA were digested with various restriction enzymes (Pharmacia). Single enzyme digests (except Kpn I & Sac I) were performed in either high, medium, or low salt restriction buffers (Maniatis et al. 1982). Restriction digests with Kpn I & Sac I were performed according to the suppliers instructions. Complete digestion was generally accomplished by incubating 6 units of restriction enzyme/ugr of DNA for 3 hours at 37 C. The reaction conditions required for the complete digestion of kelp genomic DNAs were

determined by co-restricting equal amounts of kelp DNA and plasmid pUC9 DNA. A set amount of restriction enzyme (i.e., 6 units of the restriction enzyme Eco RI) was used, and the reaction was allowed to run for 5 hours with aliquots of the reaction volume being removed and stopped with EDTA every half hour. The time series of reaction products was then electrophoretically separated on the same gel, transferred to a nitrocellulose filter, and hybridized with a kelp ctDNA Eco RI fragment cloned into pUC9. The hybridization patterns were assessed by autoradiography. The time sample wherein both the plasmid and kelp DNAs were resolved into their respective linear fragment lengths was determined to be the appropriate duration for complete digestion of the genomic DNA. Double enzyme digests were carried out consecutively; first digesting with the restriction enzyme with the lowest salt requirements, and then changing the conditions to that of the higher salt restriction enzyme of the pair. Restriction enzyme concentrations and incubation times were as for single restriction digests. Digests were stopped by adding EDTA to a final concentration of 100 mM.

Agarose gel electrophoresis. Between 0.5-1.0 ugr of digested DNA was loaded per lane of 0.5-2.0% neutral agarose (BDH 44302) horizontal slab gels (18 X 20 cm). All gels contained ethidium bromide at a concentration of 1 ugr/ml. Electrophoresis was for 16-24 hours at 25 volts in 89 mM Tris-Borate (pH 8.0), 2.5 mM EDTA. Molecular size

standards of 200 ngr uncut Lambda DNA, 1 ugr Eco RI-Hind III digested Lambda DNA, and 1 ugr Bam HI digested Lambda DNA were included on each gel. Electrophoretically separated DNA fragments were visualized and photographed (Kodak Pan-X 100) using a 260 nm wavelength transilluminator.

Transfer of DNA to nitrocellulose filters.
Electrophoretically separated DNA was acid hydrolyzed, denatured, and transferred bidirectionally from agarose gels onto nitrocellulose filter paper (Schleicher & Schuell, BA85, 45 um) according to the method of Smith & Summers (1980).

Preparation of radio-labelled probes. Probe DNA (100 ngr) was labelled with 10 uCi [α-^{32}P]dATP (Amersham), 1 Unit of DNA polymerase I, and 125 pgr DNAse I to a specific activity of 1-2 X 10^7 cpm/ugr by the modified (Baillie pers. com.) nick-translation reaction according to Rigby et al. (1977). Reactions were incubated at 12 C for 1-2 hours and stopped with 1/10 volume of a 2% SDS, 0.2 M EDTA solution. Unincorporated nucleotides were separated from labelled DNA by passing the reaction volume through a 1.5 ml Sephadex G-50 spin column (Maniatis et al. 1982).

Filter hybridizations. Nitrocellulose filters were placed in heat-sealable plastic bags and pre-hybridized at 65 C in 8 ml/100 cm^2 of 5 X SSPE, 5 X Denhart and 0.1% SDS (1 X SSPE is 0.18 M NaCl, 10 mM Sodium Phosphate, 1 mM EDTA, pH 7.4; 1 X

Denhart is 0.02% BSA, 0.02% Ficoll, 0.02% PVP). Hybridization was carried out in a fresh aliquot of the above solution (4 ml/100 cm^2 filter area) along with the heat-denatured, nick-translated probe (200-300 X 10^3 cpm/ml). Hybridizations were at 60-65 C (depending upon the homology of the probe and target DNAs) for 16-24 hours. Following hybridization, filters were washed in several changes of 0.2-2 X SSPE/0.1% SDS for 1 hour at 60-65 C (depending upon the homolgy of the probe and target DNAs). Hybridization criteria were as follows: 1) Low stringency was hybridization in 1 M Na at 60 C, wash in 0.4 M Na at 60 C; 2) Medium stringency was hybridization in 1 M Na at 65 C, wash in 0.4 M Na at 65 C; and 3) High stringency was hybridization in 1 M Na at 65 C, wash in 0.04 M Na at 65 C.

Autoradiography. After drying the filters under a heat lamp, the filter bound kelp ctDNA fragments homologous to the Macrocystis integrifolia ctDNA fragment probes or heterologous angiosperm ctDNA probes were detected by autoradiography (1-20 days) at -70 C or room temperature with Kodak X-Omat XR1 and XAR-5 films.

Cloning of Macrocystis chloroplast DNA.

Construction of a Macrocystis total blade DNA library into Lambda gt10.

a) Preparation of vector and insert DNA. Lambda gt10 DNA was prepared as follows. Phage were plaque purified on LB

agar plates with E. coli strain C600 plating bacteria (Maniatis et al. 1982). Several individual turbid plaques were used to prepare separate plate stocks which were each titred to determine their content of contaminating clear-plaque phage. The stock containing the lowest percentage of clear-plaque phage (i.e., less than 1 in 10,000 plaques) was selected to inoculate five LB-agar plates, and phage were grown to confluence by incubating for 5-6 hours at 37 C (Davis et al. 1980). Phage harvesting and DNA isolation were as discussed below. Macrocystis total blade DNA was prepared as previously described. Two ugr of Lambda gt10 DNA and 1 ugr of Macrocystis total blade DNA were digested to completion with Eco RI in separate 20 ul reaction volumes. Restriction digest conditions were as previously described. Each digest was made 20 mM for EDTA and heated to 65 C for 10 minutes to stop the reaction. About 500 ngr of each sample was electrophoretically separated to check for completion of the restriction digest (i.e., 5 X 10 cm - 0.5% agarose gel, 40 volts, 2 hour separation time). One ugr of Eco RI digested Lambda gt10 DNA and 250 ngr of Eco RI digested Macrocystis total blade DNA were combined, and the volume was brought to 18 ul with TE. The DNA solution was then made 0.3 M NaOAc with 2 ul of a 3 M stock, and the DNA was precipitated by adding 40 ul ETOH while incubating on dry ice for 30 minutes. The precipitated DNA was sedimented for 15 minutes in a microfuge at 4 C, washed briefly with 70% ETOH and left to air dry. The DNA pellet was then dissolved in 4.5 ul of Phage

Ligation Buffer (Phage Ligation Buffer is: 10 mM Tris-Cl, 10mM $MgCl_2$, 50 mM NaCl, pH 7.5).

b) Ligation of Lambda gt10 and Macrocystis total blade DNA. Prior to annealing the cohesive ends of the insert and vector DNAs, the DNA mixture was heated to 70 C for 3 minutes, lowered to 50 C for 15 minutes and then cooled on ice. Next, 0.5 ul of 10 mM ATP, 0.5 ul of 0.1 M DTT and 0.1 ul of 1.6 mgr/ml T4 ligase were added and the ligation reaction, now 5.6 ul in volume, was incubated at 14 C overnight. To test the completion of ligation, 1 ul of the ligation reaction mixture (about 200 ngr DNA) was separated on a 0.4% mini-agarose gel. The ligated material runs at or greater than the size of intact Lambda DNA.

c) In vitro packaging of the ligated DNA. Essentially, in vitro packaging of the library was accomplished by the method of Maniatis et al. (1982). Prepared packaging extract was generously supplied by Terry Snutch [E. coli Lambda lysogens NS 428 (N205; Lambda Aam11 b2 red3 cTts857 Sam7) and NS 433 (N205; Lambda Eam4 b2 red3 cIts857 Sam7)]. A 50 ul aliquot of packaging extract was thawed on ice until it attained a slushy consistency (i.e., for about 3 minutes). Immediately thereon,1.5 ul of 100 mM ATP, 20 ul of CH Buffer (CH Buffer is: 40 mM Tris-Cl, 1 mM spermidine, 1 mM putrescine, 0.1% B-mercaptoethanol, 7% DMSO, pH 8.0) and the ligated DNA sample were added to the packaging extract and the mixture was stirred with a sealed-5 ul-glass micropipette. This mixture was then incubated at 37 C for 60 minutes. Afterward, a

second 50 ul aliquot of packaging extract was thawed and combined with 10 ul of 0.1 mgr/ml DNAse and 2.5 ul of 1 M $MgCl_2$. Twenty-five ul of the second extract mix was added to the first and the two were incubated at 37 C for 30 minutes. The packaging reaction was stopped by diluting with 900 ul of Lambda diluent (Lambda diluent is: 1 mM Tris-Cl, 1 mM $MgCl_2$, pH 8.0) and adding 4 drops of chloroform. The diluted mixture was then centrifuged for 60 seconds in a microfuge, and the supernatant was collected in a 1.5 ml microfuge tube. Again, 4 drops of chloroform were added.

d) Amplification of Lambda gt10 phage. There is no size selection for recombinant phage when using the Lambda gt10 vector. As a result, recombinant phage may constitute only a small percentage of the phage complement in the *in vitro* packaging reaction. Recombinant phage were distinguished from the non-recombinant background on the basis of plaque morphology; recombinant phage form clear plaques whereas religated parent phage form turbid plaques. Recombinant phage were amplified above the non-recombinant background on the bacterial strain MA150. An hfl deletion mutant, MA150 suppresses plaque formation of cI^+ parent phage by lysogenizing them with extreme efficiency. Recombinant phage are not lysogenized and form plaques normally. However, MA150 is a restricting strain. Because of this, the *in vitro* packaged phage were methylated by passaging through the bacterial strain C600 (rK^- mK^+) before plating on MA150.

The C600 and MA150 bacterial strains were prepared as described by Davis et al. (1980). About 1-2 X 10^5 phage were incubated for 15 minutes in 60 ul of a fresh 10 ml overnight culture of C600 cells that had been resuspended in 5 ml of Lambda diluent. This suspension was mixed with 2.5 ml of LB top agar and then poured onto fresh LB agar plates (LB top agar & LB agar were prepared as in Maniatis et al. 1982). The top agar was allowed to set for 10 minutes at room temperature, and then the plates were incubated 5-6 hours at 37 C. Phage were harvested by placing the plates overnight at 4 C with 10 ml of Lambda diluent and 4 drops of chloroform (Davis et al. 1980). This plating and harvest process was repeated with MA150 cells where more than 98% of the turbid phage were eliminated from the library. The final titre of the library was 10^8 plaque forming units (pfu)/ml. Lambda gt10 is an Eco RI cloning vector that contains a single Eco RI site in the phage receptor gene. Lambda gt10 can accept DNA insert fragments that range in size from 0-7 kb. Because this vector is designed to accept small DNA inserts, the library described here contains only a subset of the Macrocystis integrifolia nuclear and chloroplast genomes. However, in Eco RI digests of M. integrifolia ctDNA, about 100 kb is represented in restriction fragments of approximately 7 kb or less in length. The largest ctDNA insert so far isolated from the library was 4 kb in length.

e) Isolation of phage DNA. Phage DNA was isolated by the

plate lysate method (Maniatis et al. 1982). Approximately 10^5 phage were mixed with 60 ul of C600 plating bacteria (i.e., 10 ml overnight culture of C600 cells resuspended in 5 ml of Lambda diluent) and incubated at room temperature for 15 minutes. Ten such suspensions were each mixed with 2.5 ml of LB top agarose and poured onto fresh LB agar plates. After letting the soft top agarose set for 15 minutes at room temperature, the ten plates were incubated at 37 C in a closed plastic container lined with wet paper towels until confluent lysis occurred (i.e., 8-10 hours). Phage were collected by adding 5 ml of Lambda diluent to each plate and incubating overnight at 4 C (Davis et al. 1980). The Lambda diluent and eluted phage were collected, and the phage were precipitated in 1 M NaCl and 10% polyethylene glycol. Finally, the phage were purified from the precipitates by cesium chloride density centrifugation in 0.75 gr/ml CsCl at 45,000 rpm for 16 hours at 20 C in a Beckman Ti80 rotor. Phage DNA was isolated from phage in CsCl solution by the formamide method of Davis et al. (1980) with an added 70% ethanol wash to remove residual formamide. Phage DNA was isolated at yields of 10-25 ugr DNA/plate.

f) Subcloning. Macrocystis ctDNA-Lambda gt10 clones containing sequences homologous to heterologous total Spinacia chloroplast genome and mung bean 23S rRNA gene probes were isolated from the library by plaque purification as described above. The selected Lambda clones were subcloned into the E. coli plasmid vector, pUC8 (Viera & Messing 1982) for

restriction mapping and use as hybridization probes. Cloning from Lambda phage into pUC8 was accomplished by digesting 1 ugr of phage DNA and 0.2 ugr of pUC8 DNA in the same 10 ul reaction volume with 5 units of Eco RI for 2 hours at 37 C. Enzyme activity was stopped by heating the reaction volume to 65 C for 10 minutes. To the 10 ul reaction volume was added 6 ul of 2X Plasmid Ligation Mix (2X Plasmid Ligation Mix is: 2.5 mM ATP, 250 ugr/ml BSA, 25 mM DTT, 25 mM $MgCl_2$, 125mM Tris, pH 7.4) and 1 Unit of T4 ligase and the mixture was incubated at 15 C for two hours. After 2 hours, 104 ul of 1X Plasmid Ligation Mix and 0.5 Units of T4 ligase were added and the reaction was left overnight at 15 C.

Competent E. coli strain JM83 cells were transformed with recombinant pUC8 plasmids after the method of Dagert & Ehrlich (1979). Positive transformants were selected on LB agar plates containing 50 ugr/ml Ampicillin (Amp), 40 ugr/ml 5-Brom-4-chlor-3-indolyl-B-D-galactopyranoside (X-gal) and 160 ugr/ml Isopropyl-B-D-thiogalactoside (IPTG). Clones were restreaked twice on LB-Amp plates (50 ugr/ml) and then maintained at 4 C on LB-Amp plates.

Cloning the Macrocystis ctDNA restriction fragments used as hybridization probes in sequence divergence studies. Two recombinant DNA molecules, each containing different regions of the M. integrifolia chloroplast genome, were used as hybridization probes in this study. The cloning of these ctDNA sequences and others was accomplished by the

construction of an M. integrifolia ctDNA-pUC19 recombinant clone bank (Palmer & Thompson 1981b). Purified ctDNA from M. integrifolia and plasmid vector pUC19 DNA were digested with Pst I. Macrocystis insert DNA (1.0 ugr) was ligated to pUC19 vector DNA (0.2 ugr) and used to transform E.coli strain JM83 cells as described above under subcloning methods. Positive transformants were colony selected on Amp, XGAL, IPTG, LB plates (Viera & Messing 1982). Mini-DNA plasmid preparations were according to Davis et al. (1980). Large scale plasmid preparations were in M-9 media as described by Maniatis et al. (1982).

The clones used as hybridization probes in the sequence divergence analysis of this study were selected from M. integrifolia ctDNA-pUC19 clone banks by screening with homologous M. integrifolia and heterologous angiosperm chloroplast DNA probes. Clone pMiP73 was selected with a 1.5 kb Eco RI M. integrifolia ctDNA fragment cloned in a Lambda gt10 phage library and which was itself selected with a mung bean 23S rRNA chloroplast gene probe (see Results). Clone pMiP52 was selected with a Spinacia total chloroplast genome probe.

Sequence divergence data assessment and analysis.

Site mapping and fragment pattern determinations. The purification of ctDNA from kelp tissue is a low yield process. On the other hand, total blade DNA is readily obtained and, for this reason, it was prepared from the twelve

species examined in this study and cloned ctDNA probes were used to visualize specific ctDNA fragments in each sample. The digestion of kelp ctDNA with a restriction enzyme having six bases in its recognition site typically resulted in some 25 fragments of different lengths. Specific ctDNA fragments containing sequences homologous to the radio-labelled probes were identified by filter hybridization and autoradiography.

The central focus of this study was to assess the amount of sequence divergence that has occurred between the ctDNAs of selected genera within the family Lessoniaceae. This was accomplished by developing restriction site maps for homologous regions in each of the sample ctDNAs and comparing them after the method of Nei & Li (1979). For a similar application of this method see Helm-Bychowski et al. (1986).

In all, seven genera in the family Lessoniaceae were investigated (Table 1). Restriction site maps were prepared for each of the seven ctDNAs by using two cloned Macrocystis ctDNA restriction fragments as hybridization probes and 6-7 restriction enzymes (Table 2). Each probe was positioned within a ctDNA molecule by identifying restriction sites that were conserved in both the probe sequence and its region of homology in the sample ctDNA. These conserved restriction sites served as markers from which restriction site maps of the region of homology were developed.

For example, of the restriction enzymes used to map the region of the Macrocystis chloroplast genome that was homologous to the probe pMiP52, Hpa I, Kpn I, Pvu II, Sal I,

Table 2. Restriction endonucleases used in the analyses of this study.

Enzyme	Recognition Site	Interpopulation & Interspecies	Intergenus	Interfamily
Eco RI	GAATTC	X	X	X
Bam HI	GGATCC	X	X	X
Hind III	AAGCTT			X
Pst I	CTGCAG	X	X	X
Sal I	GTCGAC	X	X	X
Xho I	CTCGAG	X		X
Hpa I	GTTAAC		X	X
Pvu II	CAGCTG	X	X	X
Xba I	TCTAGA	X	X	X
Kpn I	GGTACC		X	
Sac I	GAGCTC		X	

and Xba I have recognition sites within the probe sequence. For this reason, two or more fragments were identified in hybridizations with restriction digests with these enzymes. For Eco RI and Sac I, a single ctDNA fragment hybridized with the probe pMiP52. This fragment contains the region of homology to pMiP52 bounded by flanking sequences on either side. The flanking sequences contain the restriction sites identified by these enzymes. A comparison of the Macrocystis ctDNA sample double digested with an enzyme cutting internally to the probe (e.g., Kpn I) and an enzyme cutting externally to the probe (e.g., Sac I), with the same sample digested with Kpn I alone, shows that one of the fragments identified in the double digest is shorter than the corresponding single digest fragment. The size of the shorter fragment gives the distance between the internal and external restriction sites.

Restriction sites were positioned within the regions of homology between the sample ctDNAs and the probe sequences by determining the lengths of the restriction fragments identified in filter hybridizations. The hybridization of a given probe to restriction fragments of similar length in different ctDNA samples indicated the similar placement of sites in those samples. Alternatively, if restriction fragments of different length were identified, the ctDNA samples being compared were determined to have lost or gained a restriction site in the time since the species they represent last shared a common ancestor. In this fashion, the lengths of the restriction fragments identified in Macrocystis

ctDNA by hybridization with the two cloned Macrocystis ctDNA probes were compared to those identified in the six other lessoniacean ctDNAs and the corresponding restriction site maps were developed.

A more preliminary survey of the relative degree of ctDNA sequence divergence extant within and between four of the families in the order was determined by the simpler method of restriction fragment length comparison (Upholt 1977, Nei & Li 1979). The relative degree of sequence divergence that has occurred between the ctDNAs of representative samples is correlated with the proportion of DNA fragment lengths shared by them. For a similar application of this method see Avise et al. (1979) and Clegg et al. (1984). For interfamily (i.e., Lessoniaceae, Laminariaceae, Alariaceae, and Chordaceae) and intrafamily (i.e., Lessoniaceae, Laminariaceae, and Alariaceae) ctDNA comparisons, restriction fragment patterns for each probe were determined for 8-9 restriction enzymes (Table 2).

Sequence divergence determinations. I have assumed that all restriction fragment length differences observed in this study are the result of restriction site changes due to nucleotide substitutions (see Discussion for the basis of this assumption). The number of base substitutions per base pair (i.e., p) between pair-wise comparisons of kelp samples was estimated in two ways. Estimates of p within and between the ctDNAs of lessoniacean genera were made from comparisons of

cleavage maps by using equation 16 of Nei & Li (1979):

$p=(2/r)(1/S^{1/2} - 1)$, where

r=number of nucleotides in restriction enzyme cleavage site, and

$S=2n_{XY}/(n_X + n_Y)$, which is an estimate of the proportion of ancestral sites that remain unchanged in both lines.

In addition, p values for both intrafamily (Lessoniaceae, Laminariaceae, and Alariaceae) and interfamily (Alariaceae, Chordaceae, Laminariaceae, and Lessoniaceae) ctDNAs were estimated from the proportion of restriction fragment length differences observed among the sample digests after Upholt (1977) and Nei & Li (1979):

$p=1 - [-F + (F^2 + 8F)^{1/2}/2]^{1/n}$, where

$F=2n_{XY}/(n_X = n_Y)$, or the fraction of fragment lengths shared between the two digests, and

n=number of nucleotides in restriction enzyme cleavage site.

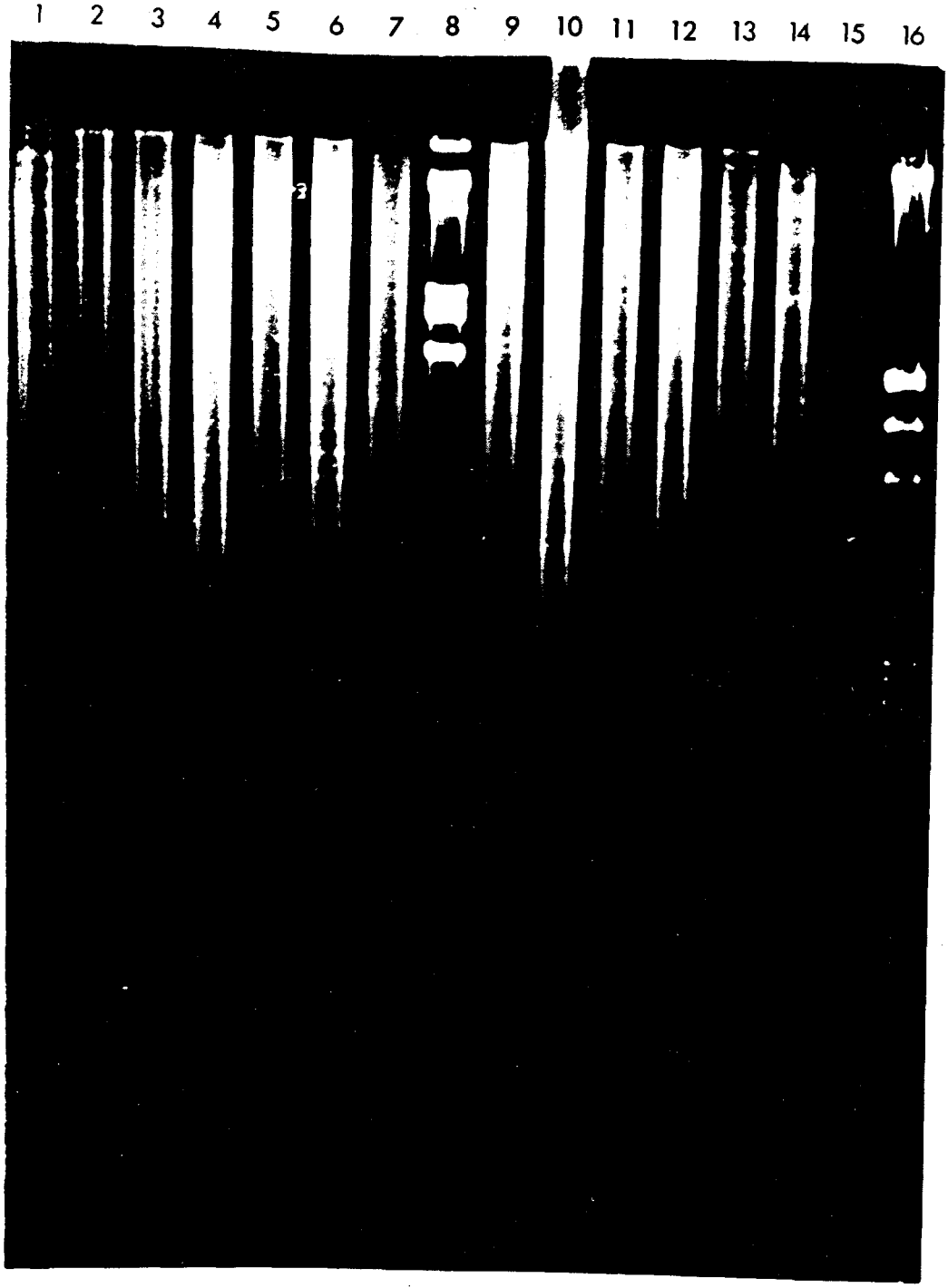
Estimates are based on the fraction of shared restriction enzyme cleavage sites and restriction fragment lengths observed for all restriction enzymes used in a comparison.

Efficiencies of extraction protocols.

Chloroplast extraction. Chloroplasts were retrieved from Macrocystis integrifolia at a yield of 2.7 ugr chlorophyll a+c/gr fresh weight of blade tissue. The chlorophyll a+c content of the blade tissue of M. integrifolia ranges from 1.75-3.5 mgr/gr fresh weight (Druehl 1984). Based on this value, this method of chloroplast extraction provides chloroplasts at an efficiency of approximately 0.1% of the total chloroplast content of the starting tissue homogenate.

DNA isolation. Total blade DNA (Fig. 2) was isolated from kelp tissues at high molecular weight (>100 kb) and at a yield of 1.0 ugr/gr wet weight of blade tissue extracted. Purified chloroplast DNA (Fig. 2) was isolated from M. integrifolia at a yield of 0.05 ugr/gr wet weight of blade tissue extracted and approximately 0.02 ugr/ugr chl a+c extracted. These values are roughly 10% of the yields obtained in extractions of total blade DNA. By applying the chloroplast extraction efficiency calculated above for this method, the nuclear and ctDNA content of a wet gram of Macrocystis blade tissue can be approximated at 200 ugr and 20 ugr respectively. Macrocystis plants collected in early spring and fall months yielded 1.0 ugr total blade DNA/gr wet weight blade tissue. Those collected in late spring and summer yielded 0.5 ugr total blade DNA/gr wet weight blade tissue.

Figure 2. Fluorescence photograph of the restriction fragment patterns of total blade DNA from various kelp plants generated by digestion with Pvu II (Lanes 1-7 and Pst I (Lanes 9-15). Fucus distichus (Lanes 1 & 9), Chorda filum (Lanes 2 & 10), Egregia menziesii (Lanes 3 & 11), Alaria marginata (Lanes 4 & 12), Costaria costata (Lanes 5 & 13), Laminaria saccharina (Lanes 6 & 14) and Macrocystis integrifolia (Lanes 7 & 15). Molecular weight standards are uncut and Bam HI restricted (Lane 8) and Hind III/Eco RI restricted (Lane 16) Lambda DNA. Electrophoretic separation was on a 0.7% agarose gel.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Chloroplast DNA characterization.

Restriction fragment patterns indicated the total size of the ctDNA molecule of M. integrifolia to be about 170 kb (Fig. 3, Table 3). The respective portions of the Macrocystis and Spinacia chloroplast genomes that were sufficiently homologous as to hybridize were estimated from reciprocal, low-stringency-hybridization experiments. Filter blots of Macrocystis and Spinacia ctDNA restriction fragments were each hybridized with a total chloroplast genome probe prepared from the other plant, and the lengths of the restriction fragments that hybridized were summed. These experiments demonstrated an approximate homology between the 170 kb Macrocystis and the 150 kb Spinacia chloroplast genomes of 20% (i.e., 34 and 30 kb respectively).

In Spinacia, as in almost all chlorophytic plants, the chloroplast 16S-5S-23S rRNA cistrons are encoded on inverted repeat structures that are highly conserved in both organization and sequence (Whitfeld & Bottomley 1983). Those Spinacia ctDNA restriction fragments that hybridized with the Macrocystis total chloroplast genome probe and that were part of the Spinacia inverted repeat structure (Crouse et al. 1978) were identified in filter hybridizations with A 23S rRNA gene specific probe from mung bean (Palmer & Thompson 1981a). The same experiment was performed on Macrocystis ctDNA to determine how much of the Macrocystis-Spinacia homology was comprised of Macrocystis ctDNA sequences that occur on the inverted repeat structures of angiosperm ct DNAs. Based on

Figure 3. The size of the chloroplast genome of *Macrocystis integrifolia*. Restriction fragment patterns of *M. integrifolia* chloroplast DNA fractionated on a 0.7% agarose gel after digestion with Sal I (Lane 1), Sal I & Kpn I (Lane 2), Sac I (Lane 3), Sac I & Kpn I (Lane 4), Kpn I (Lane 5), Kpn I & Pst I (Lane 6) and Pst I (Lane 7). Uncut and Hind III digested Lambda DNA (Lane 8) was included as a restriction fragment size standard. Sums of restriction fragment sizes, accounting for fragment stoichiometries, and the sizes of individual restriction fragments are listed in Table 3.

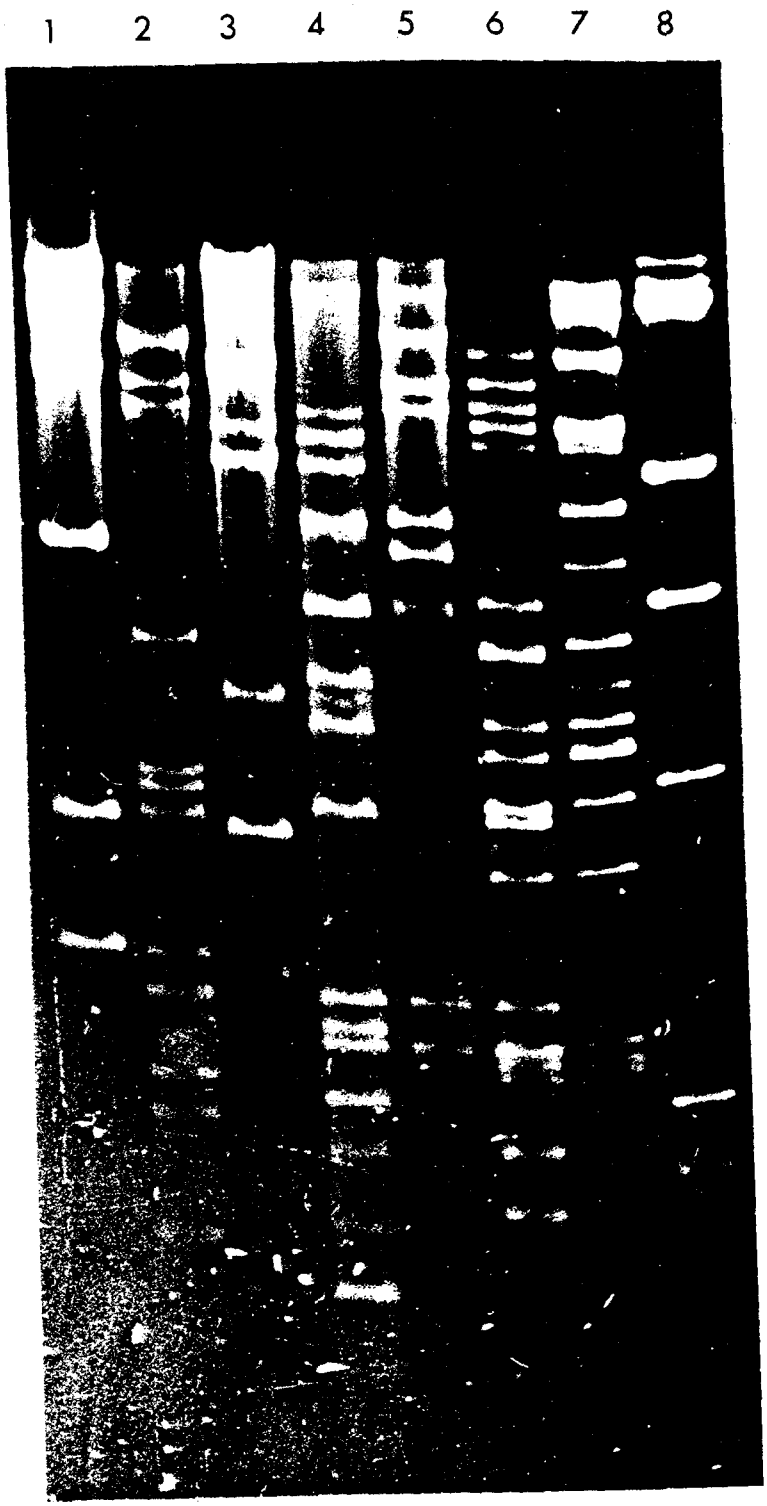

1
2
3
4
5
6
7
8

Table 3. Length (in kilobase pairs) and stoichiometry of the restriction fragments of Macrocystis integrifolia chloroplast DNA digested with a number of restriction enzymes. Restriction fragments of M. integrifolia chloroplast DNA (Fig. 3, Lanes 1-7) were fractionated on 0.7% agarose gels and their lengths were estimated relative to size standards of uncut Lambda DNA and Lambda DNA cut with Hind III (Fig. 3, Lane 8). Stoichiometries, (i.e., double or single molar equivalents) were estimated by a visual assessment of the relative fluorescence intensity of EtBr stained restriction fragments in destained agarose gels. Restriction fragments that were determined to be of double molar intensity are underlined; those of single molar intensity are not underlined. Based on the mean of the fragment sizes obtained in each of the seven digests in Figure 3, the size of the chloroplast genome of M. integrifolia was assessed at 173 kb.

Fragment Number	Restriction Enzyme						
	Sal I	Sal I/Kpn I	Sac I	Sac I/Kpn I	Kpn I	Kpn I/Pst I	Pst I
1	44.0	34.0	40.0	34.0	34.0	18.0	25.0
2	23.1	18.3	14.7	22.6	22.6	14.2	21.7
3	22.7	14.0	12.5	13.4	18.3	13.4	18.0
4	21.0	13.0	10.5	10.5	14.2	11.4	11.4
5	15.9	12.4	9.3	9.3	13.4	9.6	9.6
6	7.6	6.4	6.1	8.3	8.3	7.2	8.7
7	4.0	5.9	5.0	6.4	7.4	6.7	7.2
8	3.1	4.4	3.8	5.3	6.4	6.1	6.1
9	1.5	4.2	1.6	5.0	5.0	5.5	5.5
10		4.0	1.3	4.7	2.8	5.0	5.0
11		3.1		4.0	2.6	4.7	4.7
12		2.8		2.8		4.2	4.2
13		2.5		2.7		4.1	3.7
14		2.2		2.6		3.9	2.6
15		1.8		2.2		3.7	2.5
16		1.5		2.0		2.8	2.2
17				1.7		2.6	1.8
18				1.5		2.5	1.3
19						2.2	1.2
20						1.9	
21						1.8	
22						1.3	
	164	169	173	181	178	171	177

these comparisons, it was estimated that 30% of the homology between Macrocystis and Spinacia ctDNA (i.e., 10 of 34 kb and 9 of 30 kb respectively) could be attributed to restriction fragments carrying the 23S rRNA gene.

A 1.5 kb Eco RI ctDNA restriction fragment from M. integrifolia was selected from a recombinant M. integrifolia total blade DNA-Lambda gt10 phage library with the 23S rRNA gene specific probe from mung bean (Palmer & Thompson 1981a). In that an additional 0.8 kb fragment was observed in filter hybridizations of the mung bean 23S rRNA gene probe to Eco RI digests of Macrocystis ctDNA (and that the 23S rRNA gene is 2.1 kb in length, Whitfeld & Bottomley 1983), it was determined that the 1.5 kb Eco RI ctDNA clone comprised approximately two-thirds of the M. integrifolia 23S rRNA gene. The cloned fragment, when hybridized to filter blots of Eco RI digests of Macrocystis ctDNA, did not hybridize to the 0.8 kb fragment. It is not known whether the cloned fragment contains the 5' or 3' end of the gene.

The cloned 1.5 kb Eco RI ctDNA fragment did not contain internal Pst I restriction sites but hybridized to two large (21.7 & 11.4 kb) Pst I restriction fragments in digests of M. integrifolia ctDNA (Fig. 4). The same fragments hybridized to the mung bean 23S rRNA gene specific probe. This was interpreted to indicate that the 23S rRNA gene is repeated in the Macrocystis chloroplast genome. The relative orientation of these Pst I restriction fragments was not determined. The atpB, rbcL (Fig. 4) and psbA (Fig. 5) chloroplast genes were

Figure 4. The hybridization of the heterologous angiosperm gene probes for 23S rRNA, atpB and rbcL to Macrocystis integrifolia ctDNA. The radiolabelled gene probes: (Panel A) 23S rRNA-a 3.5 kb Sac I fragment from mung bean which is specific for the 23S rRNA gene (Palmer & Thompson 1981a); (Panel B) atpB-a 1.95 kb Eco RI fragment from spinach which contains the entire atpB gene plus 5' flanking sequence (Zurawski et al. 1982); and (Panel C) rbcL-an 830 bp Eco RI fragment from mung bean which is internal to the rbcL gene (Palmer & Thompson 1981b) were hybridized against filter blots of M. integrifolia Sac I (Lane 1), Kpn I (Lane 2), Pvu II (Lane 3), Pvu II/Pst I (Lane 4), Pst I (Lane 5), Pst I/Sal I (Lane 6) and Sal I (Lane 7) ctDNA restriction fragments. Macrocystis ctDNAs were separated on a 0.7% agarose gel. All three autoradiographs are of repeated hybridizations to a single nitrocellulose filter blot. Filters were stripped of hybridized probe by boiling for thirty minutes in 0.1 X SSPE-0.1% SDS. Filters were hybridized at 60 C in 5 X SSPE-0.3% SDS-5 X Denhardt for 24 hours. Filters were washed to 2 X SSPE-0.1% SDS at 60 C. These are the low stringency criteria discussed in the Methods.

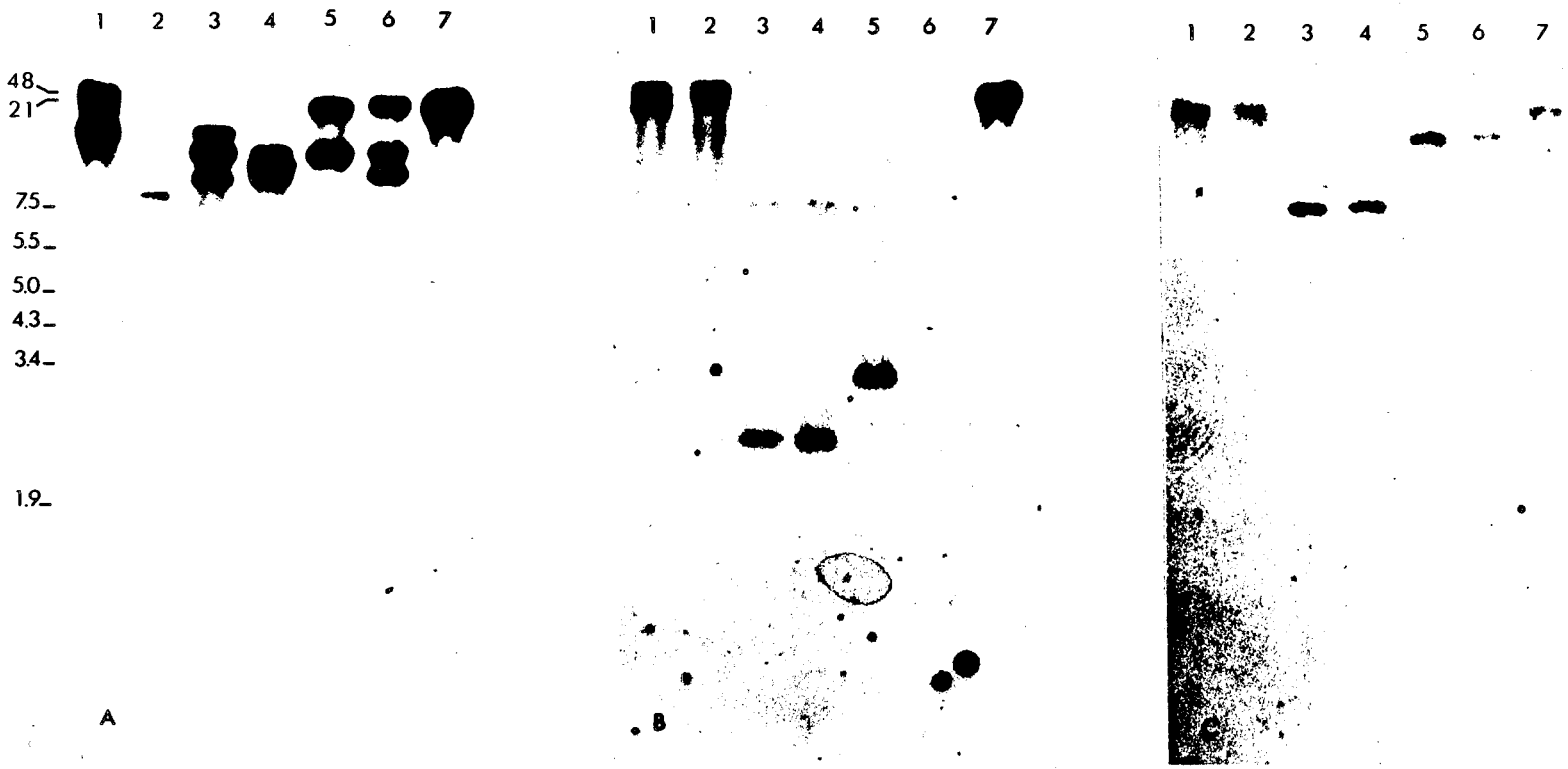
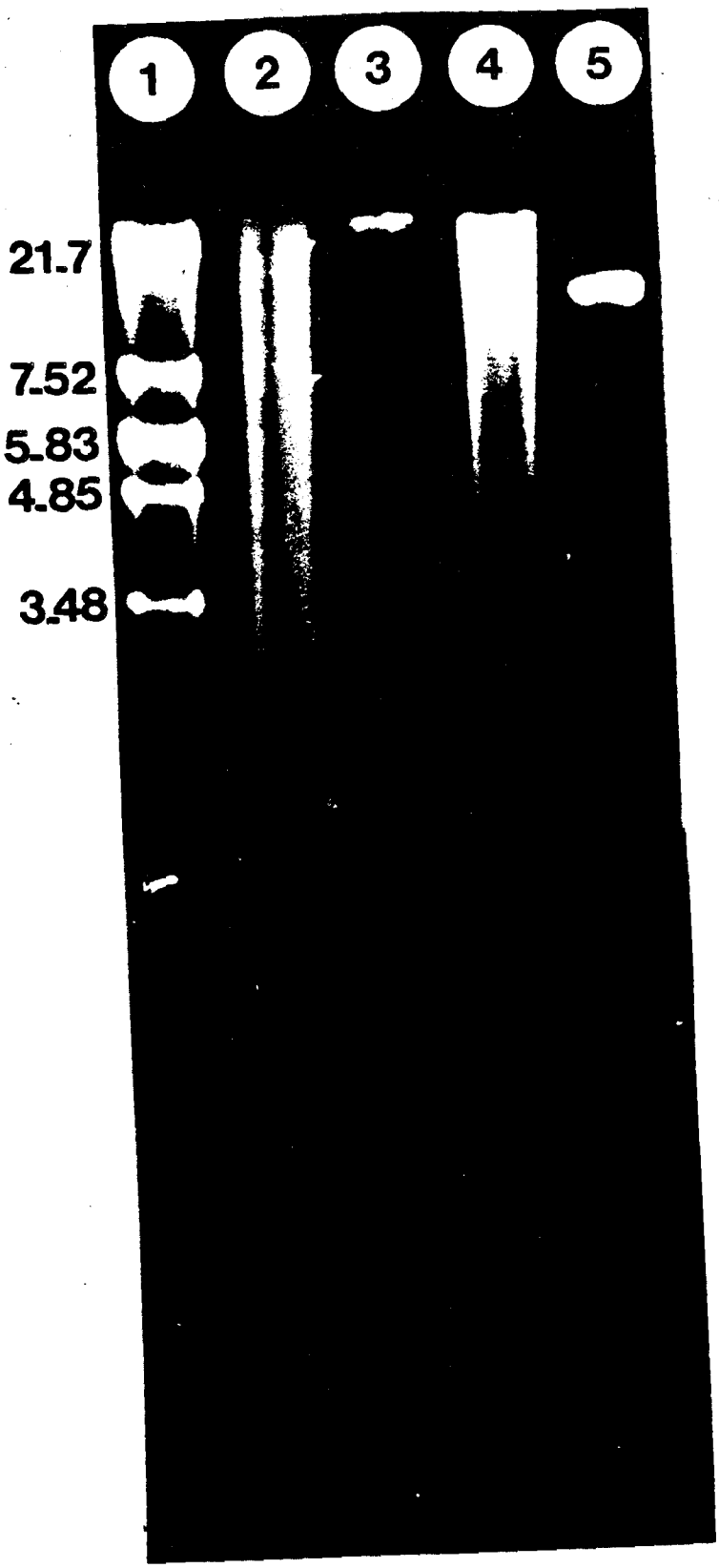

1 2 3 4 5 6 7
48
21
7.5
5.5
5.0
4.3
3.4
1.9
A
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7
C

Figure 5. The hybridization of the heterologous angiosperm chloroplast gene probe for psbA to Macrocystis integrifolia ctDNA. The radiolabelled psbA gene probe-a 1.83 kb Bgl II fragment from mung bean which contains the entire psbA gene plus flanking sequences (Palmer & Thompson 1981a) was hybridized to filter blots of M. integrifolia total blade DNA. Lane 1: Eco RI restriction digest of Lambda DNA, fragment sizes are indicated in kilobase pairs. Lane 2: Fluorescence photograph of the restriction fragment pattern of M. integrifolia total blade DNA digested with Sal I. Lane 3: Autoradiograph of filter blot of DNA in Lane 2 hybridized with the radiolabelled psbA gene probe. Lane 4: Fluorescence photograph of the restriction fragment pattern of M. integrifolia total blade DNA digested with Eco RI. Lane 5: Autoradiograph of filter blot of DNA in Lane 4 hybridized with the radiolabelled psbA gene probe. The Macrocystis total blade DNAs were separated on a 0.5% agarose gel. The filter was hybridized at 60 C in 5 X SSPE-0.3% SDS-5 X Denhardt for 24 hours. The filter was washed to 2 X SSPE-0.1% SDS at 60 C. These are the low stringency criteria discussed in the Methods.


1
2
3
4
5
21.7
7.52
5.83
4.85
3.48

also located on Macrocystis ctDNA restriction fragments, but this was accomplished with the use of heterologous angiosperm gene probes (Table 4).

Hybridization probes used in restriction analyses. The cloned M. integrifolia ctDNA restriction fragment pMiP73 (11.4 kb Pst I fragment, Fig. 6) included single copy sequence as well as part of one of the conserved 23S rRNA gene encoding repeat regions of the Macrocystis ctDNA molecule. When the 23S rRNA gene specific probe from mung bean ctDNA was hybridized to filter blots of Eco RI digested pMiP73 DNA, it hybridized to the same 1.5 and 0.8 kb fragments as in chloroplast genome blots. The cloned 1.5 kb Eco RI fragment (that encodes part of the Macrocystis 23S rRNA gene) hybridized only to itself on filter blots of Eco RI digested pMiP73 DNA. In addition, when the cloned 11.4 kb Pst I fragment of pMiP73 was hybridized to filter blots of Pst I digested Macrocystis ctDNA, the same 21.7 and 11.4 kb fragments that hybridized to the 1.5 kb Macrocystis 23S rRNA probe were identified. This demonstrates that an entire 23S rRNA gene is encoded on the 11.4 kb Pst I fragment of pMiP73 and that a second copy resides on the 21.7 kb Pst I fragment of the Macrocystis chloroplast genome. Therefore, the 11.4 and 21.7 kb Pst I fragments each contain part of the repeat regions of the Macrocystis ctDNA molecule.

The restriction patterns demonstrated in hybridization reactions with the pMiP73 probe were interpreted as follows:

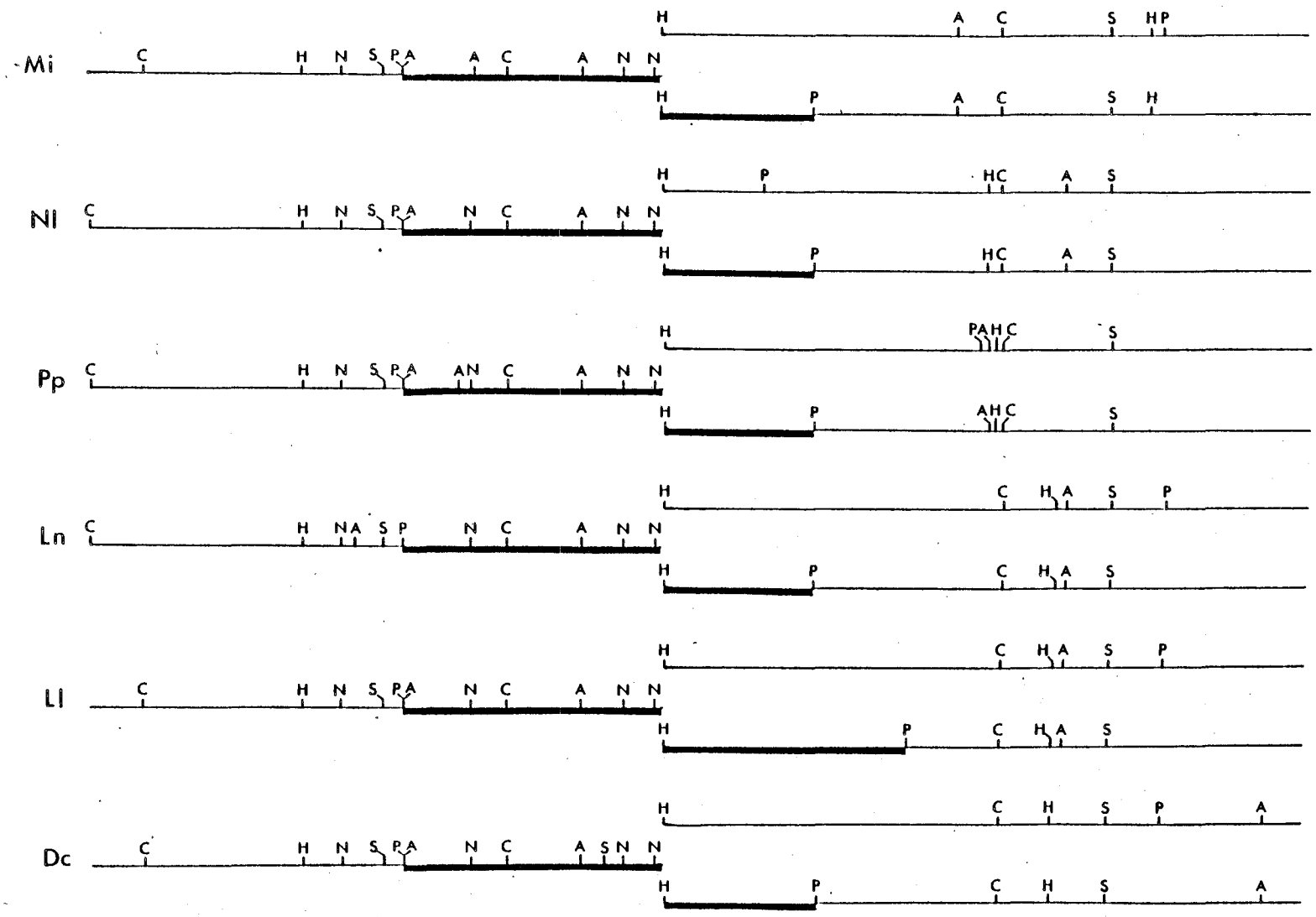
Table 4. Summary of the *Macrocystis integrifolia* ctDNA restriction fragments that hybridized to the heterologous angiosperm chloroplast gene probes.

	Filter-bound ctDNA restriction fragments				
Gene Probe	Sac I	Kpn I	Pst I	Sal I	EcoRI
23SrDNA	44,15	8.0	21.7,11.4	22	1.5,0.8
atpB	44	38	3.7	50	14.5
rbcL	44	38	18	23	
psbA				21	12.7

Values are in kilobase pairs. Autoradiographs of Eco RI restriction fragments were not included in text.

Figure 6. Restriction site maps of the pMiP73 homologous regions of the chloroplast genomes of lessoniacean kelp. The species are Macrocystis integrifolia (Mi), Nereocystis luetkeana (Nl), Postelsia palmaeformis (Pp), Lessonia nigrescens (Ln), Lessoniopsis littorallis (Ll) and Dictyoneurum californicum (Dc). The thick black bars represent the Pst I restriction fragment homologous to the M. integrifolia ctDNA Pst I restriction fragment in pMiP73. The maps of the respective ctDNAs were alligned with each other at these conserved Pst I sites. The thick black bar at the lower left is a scale representing 5kb of DNA sequence. The code for restriction enzyme recognition sites is C, Sac I; H, Hpa I; N, Kpn I; S, Sal I; P, Pst I and A, Xba I.

The left hand sides of the maps are single lines that represent parts of the 23S rRNA encoding repeat sequences of the respective molecules. The right hand sides of the maps are double lines that represent the single copy sequences adjacent to each copy of the repeated sequences. The conserved Hpa I site in each molecule was arbitrarily chosen as the break point between repeat and single copy sequence. See Methods for a discussion of how these maps were constructed.


Mi
C H N S P A A C A N N
H A C S HP
H P A C S H
Nl
C H N S P A N C A N N
H P HC A S
H P HC A S
Pp
C H N S P A AN C A N N
H PAHC S
H P AHC S
Ln
C H NA S P N C A N N
H C H A S P
H P C H A S
Ll
C H N S P A N C A N N
H C H A S P
H P C H A S
Dc
C H N S P A N C A S N N
H C H S P A
H P C H S A

1) Two restriction sites internal to the repeat regions were represented by two identically sized restriction fragments that appeared as a single band in restriction profiles (Fig. 7B-lane 5). 2) Restriction fragments defined by two conserved restriction sites internal to the repeat region and two assymetric restriction sites occurring in the adjacent single copy regions were dissimilar in size (Fig. 7B-lane 7). 3) In instances where both the repeat region restriction sites and the single copy region restriction sites were conserved, the resulting restriction profile contained a single band representing two restriction fragments of identical size (Fig. 7B-lane 1).

The second hybridization probe, pMiP52, contained an 8.7 kb Pst I restriction fragment of the chloroplast genome of M. integrifolia (Fig. 8). When hybridized to the chloroplast genome of Spinacia (Fig. 7C), pMiP52 mapped to Pst I fragment 2 in the large single copy region (Herrmann et al. 1980). This fragment of the Spinacia chloroplast genome encodes the genes psbB, petA, petB, petD and rbcL (Palmer 1985). The restriction profiles generated in hybridizations of this probe to Macrocystis ctDNA (Fig. 7A) were all interpretable as representing single copy sequence.

Interpopulation and interspecies chloroplast DNA sequence comparisons. Restriction analysis of chloroplast DNA has been used to estimate percent sequence divergence values between three populations of Macrocystis integrifolia and a

Figure 7. Restriction fragment patterns for Macrocystis integrifolia and Spinacia oleracea ctDNA homologous to pMiP52 and pMiP73.

A & B. Restriction digests of Macrocystis integrifolia ctDNA with (1) Sal I, (2) Sal I/Kpn I, (3) Sac I, (4) Sac I/Kpn I, (5) Kpn I, (6) Kpn I/Pst I and (7) Pst I.

C & D. Restriction digests of Spinacia oleracea ctDNA with (1) Sma I, (2) Kpn I, (3) Pst I, and (4) Sal I.

Macrocystis and Spinacia fragments were transferred to nitrocellulose and hybridized with radiolabelled probe DNA.

A & C. Autoradiographs of Macrocystis and Spinacia ctDNAs hybridized with the M. integrifolia ctDNA clone pMiP52.

B & D. Autoradiographs of Macrocystis and Spinacia ctDNAs hybridized with the M. integrifolia ctDNA clone pMiP73.

Filters were hybridized at 65C in 5 X SSPE-0.3% SDS-5 X Denhardt for 16 hours. Filters were washed to 2 X SSPE-0.1% SDS at 65 C. These are the medium stringency criteria discussed in the Methods.

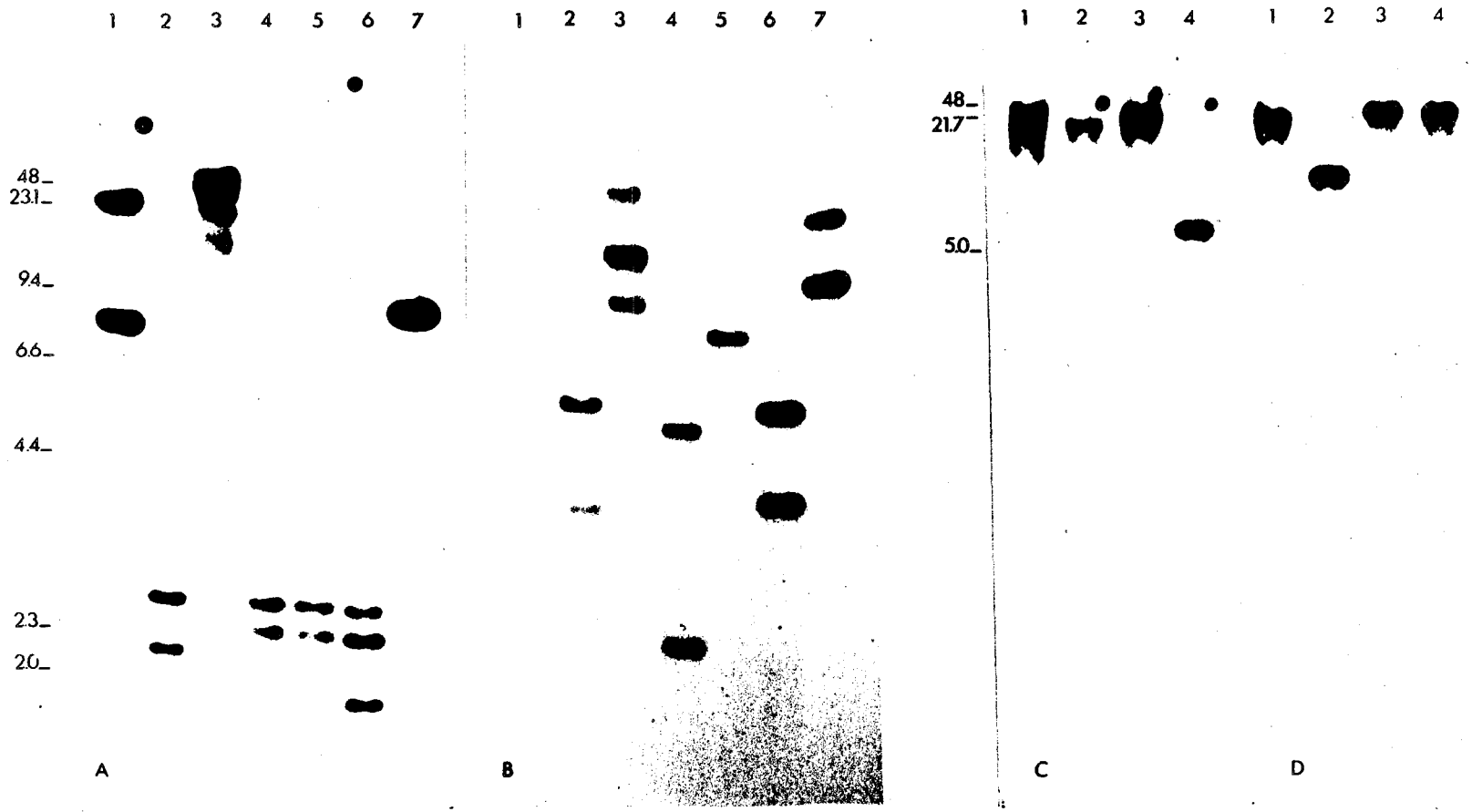
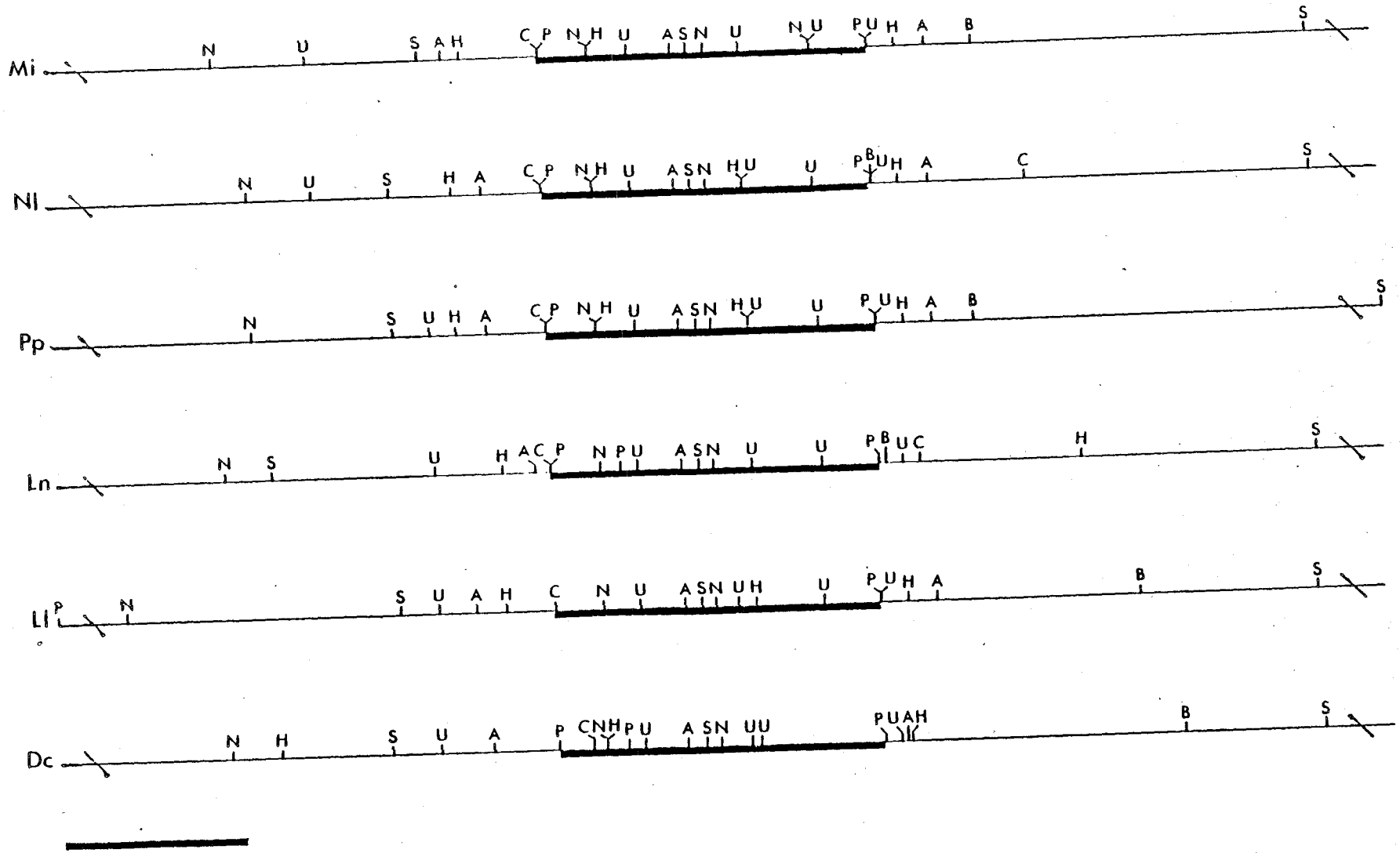

1 2 3 4 5 6 7
48
23.1
9.4
6.6
4.4
2.3
2.0
A
1 2 3 4 5 6 7
B
1 2 3 4
1 2 3 4
48
21.7
5.0
C
D

Figure 8. Restriction site map the pMiP52 homologous regions of the chloroplast genomes of lessoniacean kelp. The species designations are as in Figure 6. The thick black bars represent the Pst I restriction fragment homologous to the M. integrifolia ctDNA Pst I restriction fragment in pMiP52. The maps of the respective ctDNAs were alligned with each other at these conserved Pst I sites. The thick black bar at the lower left is a scale representing 5 kb of ctDNA sequence. The code for restriction enzyme recognition sites is P, Pst I; N, Kpn I; S, Sal I; H, Hpa I; U, Pvu II; A, Xba I; C, Sac I and B, Bam H1. The diagonal marks at the ends of each map represent 10 kb of sequence that was removed in order to bring the leftward Pst I site of Ll and the rightward Sal I site of Pp into the format dimensions of the other maps. See Methods for a discussion of how these maps were constructed.


Mi
N U S A H C P N H U A S N U N U P U H A B S
Nl
N U S H A C P N H U A S N H U U P B U H A C S
Pp
N S U H A C P N H U A S N H U U P U H A B S
Ln
N S U H A C P N P U A S N U U P B U C H S
Ll
P N S U A H C N U A S N U H U P U H A B S
Dc
N H S U A P C N H P U A S N U U P U A H B S

second closely related species M. pyrifera (Table 1). These values have been compiled from restriction digests with seven different six-base enzymes (Figs. 9 & 10; Table 2). The number of nucleotides assessed in this way was estimated from the number of restriction fragments observed in all enzyme digests and with both hybridization probes. A total of 91 different restriction sites were identified (39 for pMiP52 and 52 for pMiP73) totaling 546 base pairs. This number comprises 0.32% of the base pairs in the 170 kb chloroplast genome of Macrocystis. Two substitutions were detected (Fig. 9B-lane 3) between the two northern hemisphere M. integrifolia populations and the southern hemisphere M. integrifolia population. These differences were observed in the region of the M. integrifolia chloroplast genome that was homologous to the single copy probe pMiP52. However, no differences were observed between the two northern hemisphere species M. integrifolia and M. pyrifera. Interpopulation percent sequence divergence values (100p=percent of nucleotides in a comparison having undergone substitution), as determined by the fragment method (Upholt 1977, Nei & Li 1979), ranged from 0 between British Columbia and California populations of both M. integrifolia and M. pyrifera, to 0.46 between British Columbia-California and Chile populations of M. integrifolia. Interpopulation percent sequence divergence (100p), as determined by the site method (Nei & Li 1979), ranged from 0 between British Columbia-California populations of M. pyrifera and M. integrifolia, to 0.56 between British

Figure 9. Restriction fragment patterns for Macrocystis ctDNA sequences homologous to pMiP52. Autoradiographs of radiolabelled pMiP52 hybridized to Eco RI (A), Pst I (B), Xba I (C) Xho I (D) and Pvu II (E) restriction digests of Macrocystis integrifolia-BC, CAN (1), M. integrifolia-CA, USA (2), M. integrifolia-Chile, S.A. (3) and M. pyrifera-CA, USA (4) total blade DNA.
Restriction digests were separated on 0.7% agarose gels, transferred to nitrocellulose filters and hybridized with the M. integrifolia ctDNA sequence probe pMiP52.
Filters were hybridized at 65 C in 5 X SSPE-0.3% SDS-5 X Denhardt for 16 hours. Filters were washed to 0.2 X SSPE-0.1% SDS at 65 C. These are the high stringency hybridization criteria discussed in the Methods.
The highest molecular weight band in Panel D-Lane 3 is uncut ctDNA.

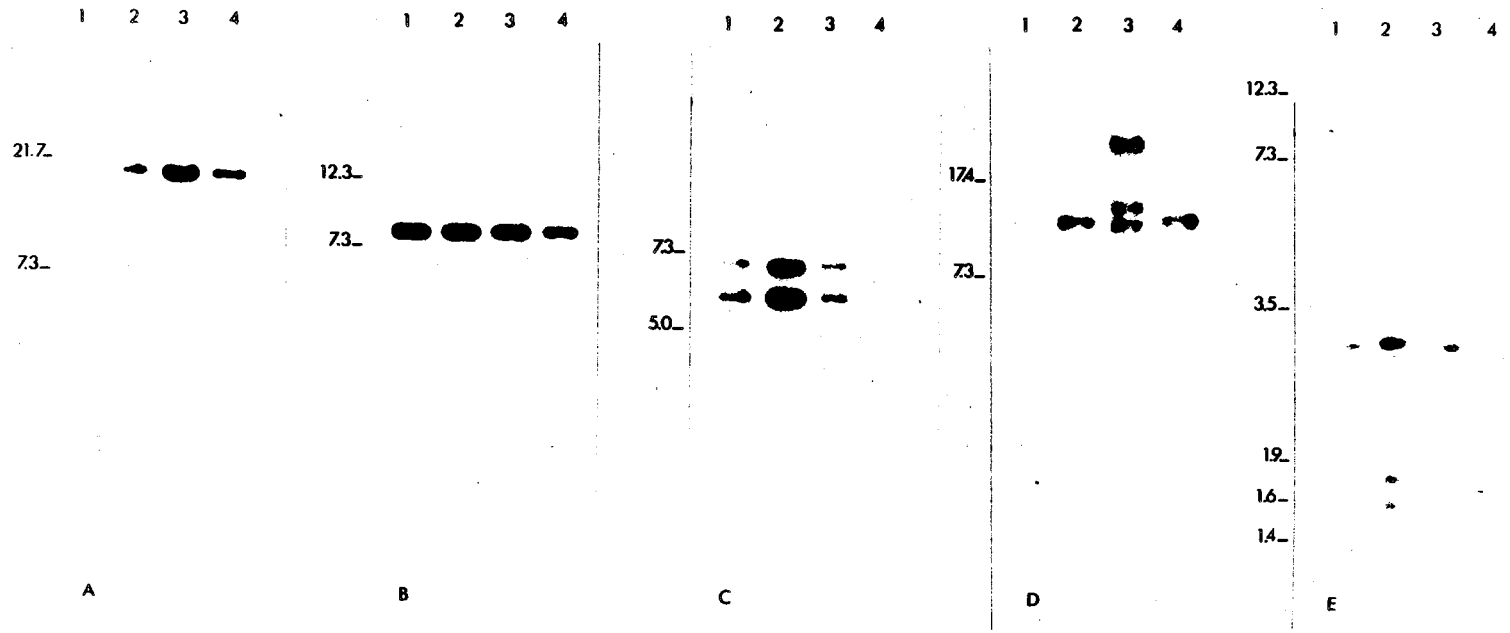
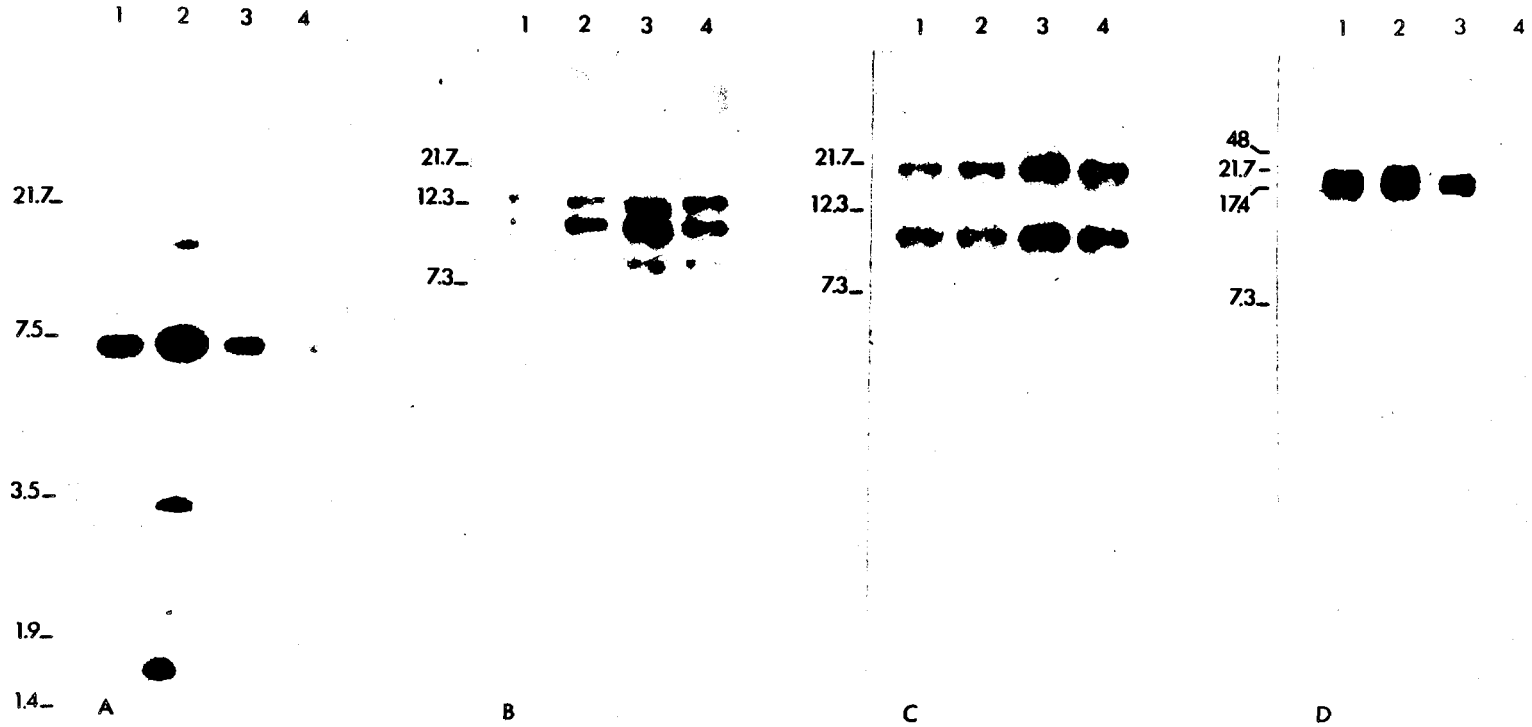

1 2 3 4
21.7
7.3
A
1 2 3 4
12.3
7.3
B
1 2 3 4
7.3
5.0
C
1 2 3 4
17.4
7.3
D
1 2 3 4
12.3
7.3
3.5
1.9
1.6
1.4
E

Figure 10. Restriction fragment patterns for Macrocystis ctDNA sequences homologous to pMiP73. Autoradiographs of radiolabelled pMiP73 hybridized to Eco RI (A), Pvu II (B), Pst I (C), and Xho I (D) restriction digests of Macrocystis integrifolia-BC, CAN (1), M. integrifolia-CA, USA (2), M. integrifolia-Chile, S.A. (3) and M. pyrifera-CA, USA (4) total blade DNA. Restriction digests were separated on 0.7% agarose gels, transferred to nitrocellulose filters and hybridized with the M. integrifolia ctDNA sequence probe pMiP73.
Filters were hybridized at 65 C in 5 X SSPE-0.3% SDS-5 X Denhardt for 16 hours. Filters were washed to 0.2 X SSPE-0.1% SDS at 65 C. These are the high stringency hybridization criteria discussed in the Methods.


1 2 3 4
21.7
7.5
3.5
1.9
1.4
A
1 2 3 4
21.7
12.3
7.3
B
1 2 3 4
21.7
12.3
7.3
C
1 2 3 4
48
21.7
17.4
7.3
D

Columbia-California and Chile populations of M. integrifolia.

Intrafamily chloroplast DNA sequence comparisons.

Restriction analysis has been used to estimate the degree of sequence divergence that has occurred between the chloroplast genomes of seven different kelp genera within the family Lessoniaceae (Table 1).

Figures 6 & 8 illustrate the restriction maps of the regions of the ctDNAs of the seven studied genera. Sequence divergences (100p) for each of the pairwise comparisons in the test, as determined by both fragment and site methods of comparison, are presented in Table 5 (Upholt 1977, Nei & Li 1979). With the exception of Dictyoneurum californica and Dictyoneuropsis reticulata, all intergeneric comparisons demonstrated significant differences in sequence divergence values. Intergeneric sequence divergence (100p) ranged from a low of 0 between D. californica and D. reticulata to a high of 9.7 (site method) / 5.4 (fragment method) between D. californica and Postelsia palmaeformis. The average intra-family sequence divergence (100p) was 7.7 (site method)/ 3.8 (fragment method). These values are averages of 15 separate comparisons (i.e., D. californica- D. reticulata were treated as a single sample). The average sequence divergence values (100p) calculated from sequences homologous to the hybridization probes pMi52 and pMiP73 were 10.4 and 6.4 (site method) / 6.0 and 3.1 (fragment method) respectively.

Restriction sites for Bam HI and Sac I flanked the Pst I

Table 5. Matrix of sequence divergence between the ctDNAs of genera in the family Lessoniaceae.

	Mi	Dc	Ll	Pp	Nl	Lx
Mi		8.7	8.2	6.6	6.6	9.2
Dc	5.2		7.7	9.7	9.7	7.3
Ll	4.7	2.8		7.7	6.4	6.8
Pp	4.4	5.4	4.5		3.9	9.5
Nl	4.5	4.3	3.3	1.6		7.3
Lx	4.2	3.0	3.2	3.6	2.6	

Genus-species abbreviations are: Mi=*Macrocystis integrifolia*; Dc=*Dictyoneurum californicum*; Ll=*Lessoniopsis littoralis*; Pp=*Postelsia palmaeformis*; Nl=*Nereocystis luetkeana* and Ln=*Lessonia nigrescens*. Estimates use the combined data sets from hybridizations with the Pst I fragments in probes pMiP52 and pMiP73 and are listed as 100p (i.e., the percent of all nucleotides in a comparison having undergone substitution). Estimates made by restriction site comparisons (Nei & Li 1979) are in the boxhead and those made by restriction fragment pattern comparisons (Upholt 1977, Nei & Li 1979) are in the stub.

sites delimiting the pMiP52 probe fragment. In an attempt to obtain a set of partial overlap fragments with which to reference the flanking sites, the restriction fragments generated with Bam HI were digested a second time with Sac I. An overlap was observed that positioned the Sac I and Bam HI flanking sites with respect to each other. These sites were positioned with repect to the probe sequence by finding a partial overlap fragment defined by a Sal I site within the probe sequence and one of the flanking Sac I sites (Fig. 8). The remaining flanking Bam HI site was not included in the maps because in all cases save Lessonia it defined a restriction fragment that was too large to unambiguously size. For the probe pMiP73, partial overlap fragments for the flanking Sal I sites were generated with Hpa I. The external sites of large (>20 kb) Kpn I restriction fragments obtained with both probes (Fig. 7) were not positioned due to their size and degradation problems with the Kpn I enzyme.

The maps in Figures 6 and 8 demonstrate that certain restriction sites are shared by different genera. These shared sites describe the different evolutionary lineages that have defined what we now recognize as the family Lessoniaceae. In all, a total of 92 different restriction sites were identified. Phylogenetically informative sites (i.e., restriction sites shared by two or more genera) totalled 52 whereas 40 sites were identified on only a single DNA. This number of six-base restriction sites comprises a total of 552 bp or about 0.3% of the 170 kb Macrocystis

genome. Among the shared sites were those defining the 8.7 & 7.0 kb Pst I (Fig. 11A), and the 3.0, 1.9 & 1.7 kb Pvu II (Fig. 11C) ctDNA fragments homologous to pMiP52; and the 21.7 & 11.4 kb Pst I (Fig. 11D) and the 13.7, 11.7 & 10.2 kb Sac I ctDNA fragments homologous to pMiP73.

Table 6 includes the results of the intra-Alariaceae and intra-Laminariaceae comparisons. All comparisons demonstrated significant differences in sequence divergence values. Estimated sequence divergence (100p) was 5.6 (fragment method) between two genera within the Laminariaceae and 5.8 (fragment method) between two genera within the Alariaceae.

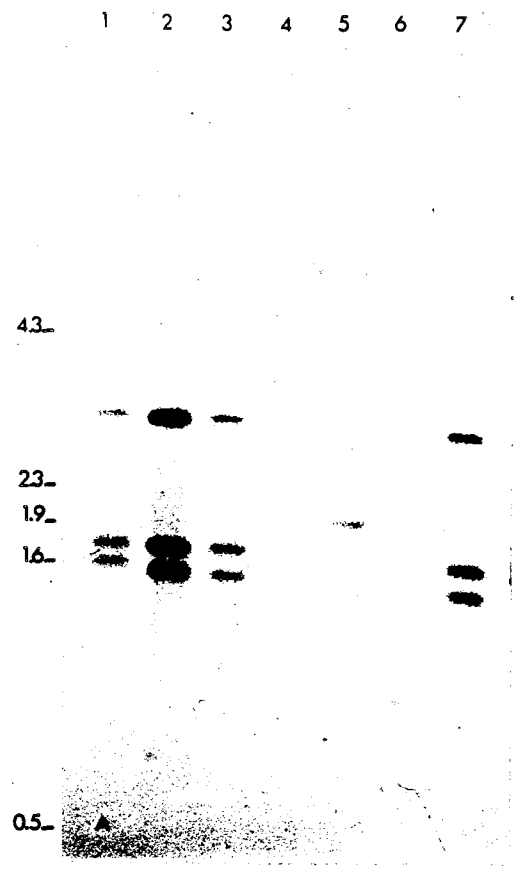
Interfamily chloroplast DNA sequence comparisons.

Restriction analysis has been used to estimate the amount of sequence divergence that has occurred between representative samples of the four families of the Laminariales. Comparisons were also made between the laminarialean samples and a sample from the order Fucales.

Among the shared restriction sites were those defining the 7.0 kb Pst I (Fig. 12A), the 9.0, 2.7 & 1.9 kb Pvu II (Fig. 12B) and the 21.7 kb Sal I (Fig. 12C) ctDNA fragments homologous to pMiP52; and the 21.7 & 11.4 kb Pst I (Fig. 13D) and the 14.8, 7.3 and 4.8 kb Xba I (Fig. 13C) ctDNA fragments homologous to pMiP73. Alternatively, Xba I & Hpa I ctDNA restriction sites in the regions homologous to pMiP52 were broadly divergent (Figs. 13A & B).

Table 6 presents the results of the interfamily and

Figure 11. Restriction fragment patterns for lessoniacean kelp ctDNA sequences homologous to pMiP52 and pMiP73. Autoradiographs of radiolabelled pMiP52 hybridized to Pvu II (A) and Pst I (B) restriction digests of Lessonia (1), Nereocystis (2), Postelsia (3), Lessoniopsis (4), Dictyoneuropsis (5), Dictyoneurum (6), and Macrocystis (7) total blade DNAs. Autoradiographs of radiolabelled pMiP73 hybridized to Sac I (C) and Pst I (D) restriction digests of Macrocystis (1), Dictyoneurum (2), Dictyoneuropsis (3), Lessoniopsis (4), Postelsia (5), Nereocystis (6) and Lessonia (7) toal blade DNAs. Restriction digests were separated on 0.7% agarose gels, transferred to nitrocellulose filters and hybridized with the M. integrifolia ctDNA sequence probes pMiP52 and pMiP73.
Filters were hybridized at 65 C in 5 X SSPE-0.3% SDS-5 X Denhardt for 16 hours. These are the high stringency hybridization criteria discussed in Methods.
Longer autoradiograph exposures revealed additional 1.7 kb Pst I restriction fragments homologous to pMiP52 in Lanes 1, 5 and 6 of Panel B.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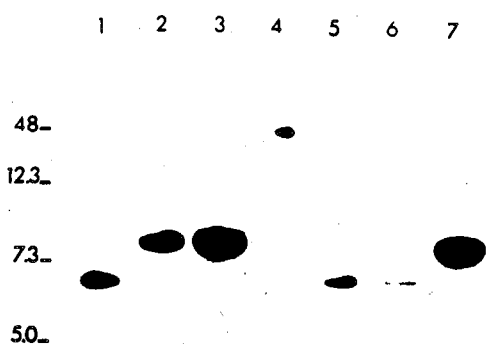
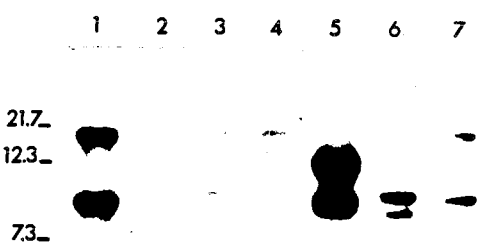
4.3
2.3
1.9
1.6
0.5


1 2 3 4 5 6 7
48
12.3
7.3
5.0

B

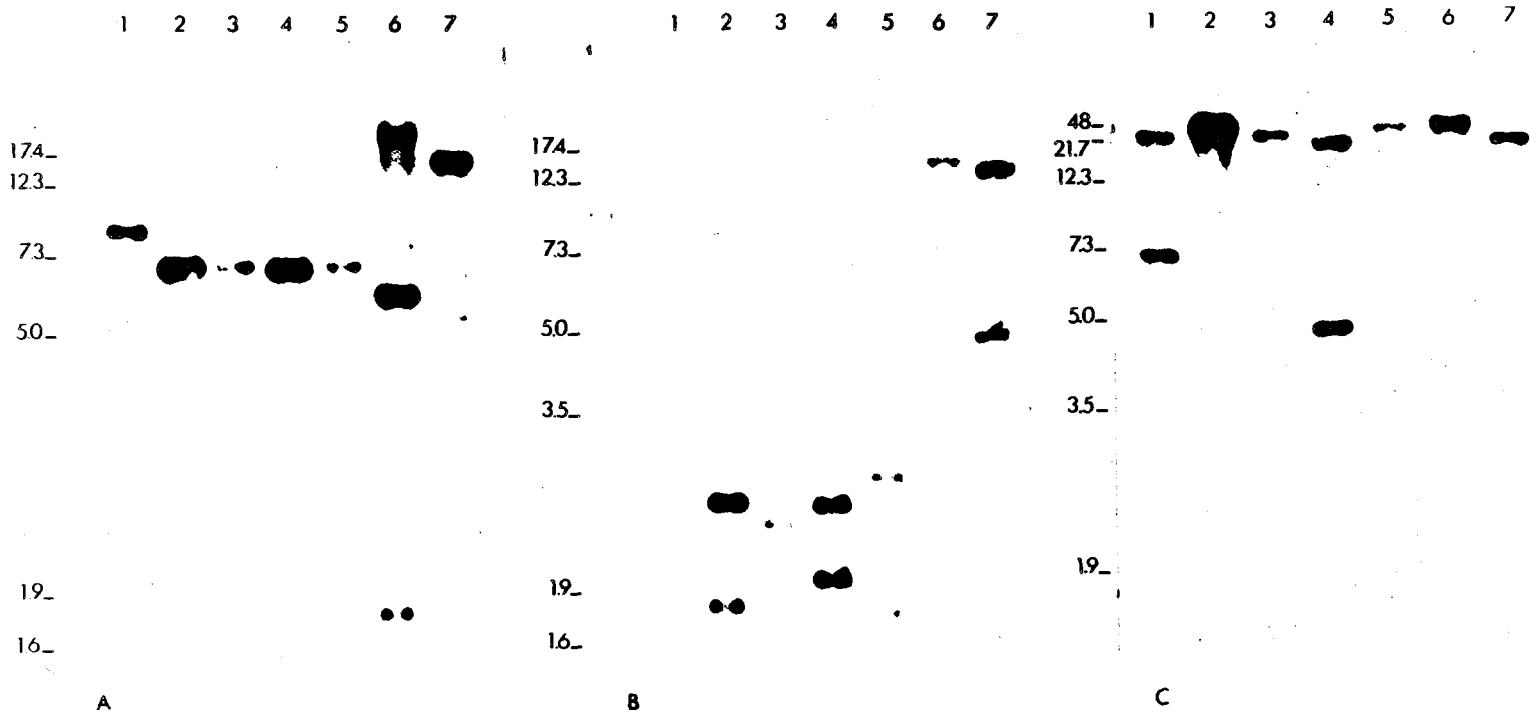

1 2 3 4 5 6 7
48
17.4
12.3
7.3

C


1 2 3 4 5 6 7
21.7
12.3
7.3

D

Figure 12. Restriction fragment patterns for alariacean, chordacean, laminariacean, lessoniacean and fucalean ctDNA sequences homologous to pMiP52. Autoradiographs of radiolabelled pMiP52 hybridized to Pst I (Panel A), Pvu II (Panel B) and Sal I (Panel C) restriction digests of Macrocystis (Lane 1), Laminaria (Lane 2), Costaria (Lane 3), Alaria (Lane 4), Egregia (Lane 5), Chorda (Lane 6) and Fucus (Lane 7) total blade DNAs. Restriction digests were separated on 0.7% agarose gels, transferred to nitrocellulose filters and hybridized with the M. integrifolia ctDNA sequence probe pMiP52.
Filters were hybridized at 65 C in 5 X SSPE-0.3% SDS-5 X Denhardt for 16 hours. Filters were washed to 0.2 X SSPE-0.1% SDS at 65 C. These are the high stringency hybridization criteria discussed in the Methods.
The highest molecular weight band in Panel A-Lane 6 is uncut ctDNA.


1 2 3 4 5 6 7
17.4
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A
1 2 3 4 5 6 7
17.4
12.3
7.3
5.0
3.5
1.9
1.6
B
1 2 3 4 5 6 7
48
21.7
12.3
7.3
5.0
3.5
1.9
C

Figure 13. Restriction fragment patterns for alariacean, chordacean, laminariacean, lessoniacean and fucalean ctDNA sequences homologous to pMiP52 and pMiP73. Autoradiographs of radiolabelled pMiP52 hybridized to Xba I (Panel A) and Hpa I (Panel B) restriction digests, and pMiP73 hybridized to Xba I (Panel C) and Pst I (Panel D) restriction digests of total blade DNAs. Sample order in Panels A, B and D: Macrocystis (Lane 1), Laminaria (Lane 2), Costaria (Lane 3), Alaria (Lane 4), Egregia (Lane 5), Chorda (Lane 6) and Fucus (Lane 7). Sample order in Panel C: Fucus (Lane 1), Chorda (Lane 2), Egregia (Lane 3), Alaria (Lane 4), Costaria (Lane 5), Laminaria (Lane 6) and Macrocystis (Lane 7).
Restriction digests were separated on 0.7% agarose gels, transferred to nitrocellulose filters and hybridized with the M. integrifolia ctDNA sequence probes pMiP52 and pMiP73.
Filters were hybridized at 65 C in 5 X SSPE-0.3% SDS-5 X Denhardt for 16 hours. Filters were washed to 0.2 X SSPE-0.1% SDS at 65 C. These are the high stringency hybridization criteria discussed in the Methods.

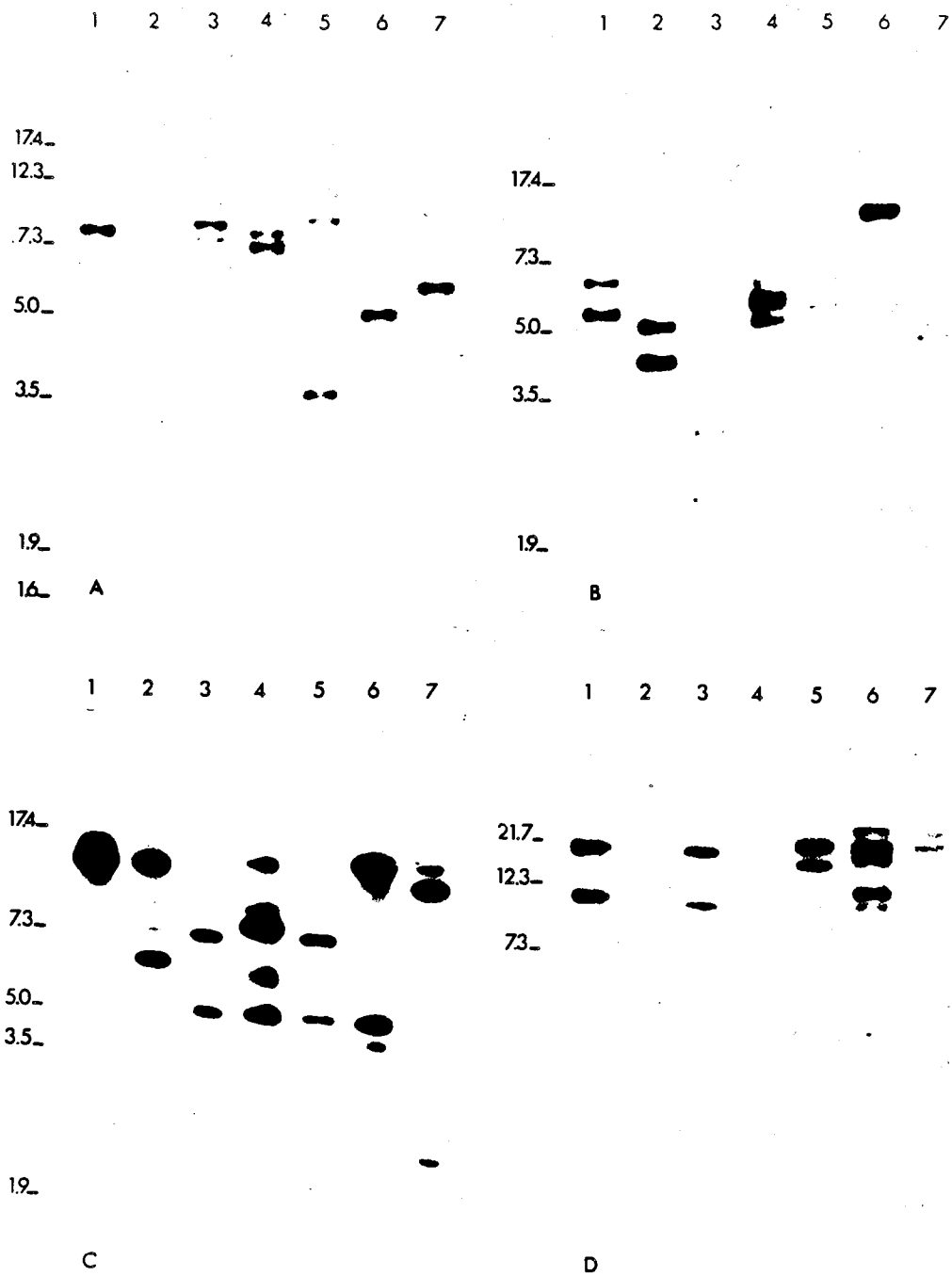

1 2 3 4 5 6 7
17.4
12.3
7.3
5.0
3.5
1.9
1.6
A
1 2 3 4 5 6 7
17.4
7.3
5.0
3.5
1.9
B
1 2 3 4 5 6 7
17.4
7.3
5.0
3.5
1.9
C
1 2 3 4 5 6 7
21.7
12.3
7.3
D

Table 6. Matrix of sequence divergence between the ctDNAs of genera in the laminarialean families Alariaceae, Chordaceae, Laminariaceae and Lessoniaceae; and in the fucalean family Fucaceae.

	Mi	Ls	Cc	Am	Em	Cf	Fd
Mi		5.4	6.9	6.6	6.6	7.4	10.3
Ls			5.8	5.8	5.9	8.6	12.2
Cc				5.6	6.9	11.0	10.6
Am					5.8	10.8	10.4
Em						8.2	11.3
Cf							10.0

Genus-species abbreviations are: Mi=*Macrocystis integrifolia*; Ls=*Laminaria saccharina*; Cc=*Costaria costata*; Am=*Alaria marginata*; Em=*Egregia menziesii*; Cf=*Chorda filum* and Fd=*Fucus distichus*. Estimates use the combined data sets from hybridizations with the Pst I fragments in probes MiP52 and pMiP73 and are listed as values of 100p (i.e., the percent of all nucleotides in a comparison having undergone substitution). Estimates are made by comparisons of restriction fragment patterns (Upholt 1977, Nei & Li 1979).

interorder comparisons. Interfamily sequence divergence (100p) ranged from a low of 5.4 for comparisons between the families Lessoniaceae and Laminariaceae, to a high of 11.0 between the Laminariaceae and Chordaceae. Estimated sequence divergence (100p) ranged from 10.0-12.2 in interorder comparisons. The average interfamily sequence divergence (100p) was 6.7 (average of 13 comparisons). The average sequence divergence values (100p) calculated from sequences homologous to the hybridization probes pMiP52 and pMiP73 were 9.5 and 6.0 respectively. The average interorder sequence divergence (100p) was 11.0 (average of 6 comparisons). The average sequence divergence values (100p) calculated from sequences homologous to the hybridization probes pMiP52 and pMiP73 were 14.8 and 8.8 respectively.

A total of 133 restriction sites or 798 bp were identified in intra-laminarialean comparisons. This sample represents about 0.5% of the laminarialean chloroplast genome [132 kb, Laminaria hyperborea (Kowallik pers. com.); 170 kb, Macrocystis integrifolia (this study)].

Discussion overview.

Although the taxonomy of the order Laminariales is considered to be one of the most clearly defined among the algae (Druehl 1981), the actual phylogenetic relations within the group are poorly understood (Scagel 1966). The phylogeny presented in this study was determined by the restriction analysis of specific regions of the ctDNAs of 12 kelp genera selected from four of the families that comprise the order. This is the first comprehensive analysis of chloroplast DNA evolution within the Phaeophyceae.

Chloroplast thylakoids have been isolated from kelp tissues by classic methods for fluorescence studies or to examine pigment composition or characterize pigment protein complexes (Goedheer 1970, Sugahara et al. 1971, Barret & Anderson 1977, Alberte et al. 1981). However, attempts to isolate intact chloroplasts from kelp tissue had not been successful prior to the onset of this study, although since then, two other groups (Barret & Anderson 1980, Popovic et al. 1983) have been successful.

In general, this study finds the evolutionary relations of the members of the order Laminariales as determined from restriction analysis of ctDNA to be in agreement with the previously established taxonomy for the group.

Sequence variation (100p) as determined by restriction analysis was lowest in population/species comparisons (0.46% by fragment method, 0.56% by site method). These comparisons (and those listed below) are based on individual samples from a population. However, additional experiments with M. integrifolia failed to find any sequence variation at all, either among individuals within a population, or between populations from Bamfield, BC (10 individuals); Nootka Island, BC (2 individuals), or Malcolm Island, BC (3 individuals). These populations were separated from each other by a distance of 400 km. Moreover, they were separated from the southern California populations (Table 1), to which they were also identical, by over 2500 km.

Sequence variation (100p) increased at higher levels of taxonomic comparison: intergenus (1.6-5.4% by fragment method, 3.9-9.7% by site method), interfamily (5.4-11.0% by fragment method) and interorder (10.0-12.2% by fragment method). Although there are no other reports of algal ctDNAs for comparison, these values are consistant with earlier findings (Table 7) for various groups of angiosperm plants. Fluhr & Edelman (1981) and Palmer et al. (1983b) reported respective sequence variation values of about 3% and between 6-10% in intergenus comparisons within the families Solanaceae and Leguminoceae. Palmer et al. (1983a, 1985) examined interspecies sequence variation within the genera Brassica (Cruciferae) and Pisum (Leguminoceae) and reported values of 3% and 0.8% respectively. Interpopulation sequence variation

Table 7. Angiosperm ctDNA sequence divergences from restriction analyses at various taxonomic levels.

Species Compared	Taxonomic Level	100p	Reference
Lycopersicon esculentum	Population	0	1
Pennisetum americanum	Population	0	2
Aegilops	Species	0-1.0	3
Pennisetum	Species	0.34	2
Brassica	Species	0.3	4
Pisum	Species	0.8	5
Nicotiana	Species	0.2	6
Phaseolus, *Glycine*, *Vigna*	Intrafamily: Leguminoceae	6-10	7
Nicotiana, *Atropa*, *Petunia*	Intrafamily: Solanaceae	3	8
Aegilops, *Triticum*	Intrafamily: Gramineae	0.7	3
Pennisetum, *Cenchrus*	Intrafamily: Gramineae	0.0-1.2	2
Lycopersicon, *Solanum*	Intrafamily: Solanaceae	0.7	1
Nicotiana, *Lycopersicon*	Intrafamily: Solanaceae	13	6
Nicotiana, *Spinacea*	Interfamily: Solanaceae-Chamelopodaceae	30	6

References: 1=Palmer & Zamir 1982; 2=Clegg *et al.* 1984; 3=Terachi *et al.* 1984; 4=Palmer *et al.* 1983a; 5=Palmer *et al.* 1985; 6=Rhodes *et al.* 1981; 7=Palmer *et al.* 1983b;

has been found to be consistently low (0-0.4%) in all instances examined (Solanaceae: Palmer & Zamir 1982; Graminaceae: Clegg et al. 1984; Leguminoceae: Palmer et al. 1985).

Sequence divergence values for kelp chloroplast DNA (Tables 5 & 6) are considerably lower than those measured at similar taxonomic levels for animal mitochondrial DNA (Table 8). The range of chloroplast DNA variation observed in this study for interfamily comparisons (5.4-11.0%) is most similar to that found for mitochondrial DNA variation between congeneric animal species (15%). This observation has been made previously (Palmer & Zamir 1982; Palmer et al. 1985; Zurawski et al. 1984) in investigations of the chloroplast DNA of angiosperm plants. Thus, it would appear that the kelp chloroplast genome is also evolving slowly relative to animal mitochondrial genomes.

The proportion of nucleotide substitutions per nucleotide position (i.e., p) can be estimated from the proportion of fragment length differences observed among sample digests (Upholt 1977, Nei & Li 1979). However, these estimates should be interpreted with some caution. The estimation models upon which they are based assume that all restriction site changes are due to nucleotide substitution and that the distribution of variant sites occurs randomly. Detailed analyses of chlorophyte ctDNAs have revealed that small additions and deletions are common, and that the distribution of variant sites is nonrandom, with the inverted repeat region evolving

Table 8. Animal mitochondrial DNA sequence divergences from restriction analyses at various taxonomic levels.

Species Compared			100p	Reference
Human (*Homo sapiens*)	–	Baboon (*Papio papio*)	29.0	Brown *et al.* 1979
	–	Rhesus (*Macaca mulatta*)	27.0	
	–	Quenon (*Cercopithecus aethiops*)	23.0	
Salmo gairdneri	–	*Salmo clarki*	2–3.5	Wilson *et al.* 1985
Peromyscus polionotus	–	*Peromyscus maniculatus*	15.0	Avise *et al.* 1979

more slowly than single copy regions (Gordon et al. 1982, Bowman et al. 1983, Palmer et al. 1983a, Clegg et al. 1984). In addition, data from Nicotiana and Brassica species indicate a clustering of variable sites at the juncture between the large single copy region and the inverted repeats (Kung et al. 1982, Palmer et al. 1983a). The upshot is that estimates of sequence divergence from restriction fragment pattern analysis are ambiguous, in that no distinction is made between the different kinds of mutational events that may have contributed in the observed restriction fragment patterns. However, such estimates do provide an approximation of the magnitude of the sequence divergence that has occurred between the DNAs of organisms sharing common ancestors and hence, have been used in this study. In order to achieve a truly unambiguous characterization of the kinds of mutational events that may have occurred, it is necessary to compare complete DNA sequences.

Chloroplast DNA sequence divergence.

Intergenus sequence divergence was of consistant magnitude between families: Lessoniaceae 0-5.4%, Laminariaceae 5.8, and Alariaceae 5.8% (by fragment method). One might expect to find less sequence variation maintained within families containing fewer species. Palmer & Zamir (1982) have reported that a significantly greater amount of sequence variation was found in the genus Atriplex (250 species) than in the genus Lycopersicon (10 species). Within the order Laminariales, the

Laminariaceae and Alariaceae are some four-fold more diverse than the Lessoniaceae (Table 9). However, it is not clear from this limited assessment, (Laminariaceae-Laminaria & Costaria; Alariaceae-Alaria & Egregia), that there is more or less ctDNA sequence divergence within either of the families. Although the genera compared in this study are placed in different tribes (Table 1) and as such might be expected to reflect the amount of sequence variation maintained in their respective families, a more comprehensive assessment will be necessary for certain determination.

The laminarialean families Lessoniaceae, Alariaceae, and Laminariaceae appear to have diverged from each other at approximately the same time (5.4-6.9% sequence divergence by fragment method). However, all three are collectively as divergent from the Chordaceae (7.4-11.0% by fragment method) as from Fucus (10.3-12.2% by fragment method) in the order Fucales. Chorda is similarly divergent (10.0% by fragment method) from Fucus. This result indicates that restriction analysis of ctDNA, as applied in this study, is not an accurate indicator of sequence divergence in comparisons at the taxonomic level of subclass. This result may be explained by the more mutable sequences within the respective molecules undergoing substitution at a consistant and regular rate, whereas those under greater selective pressure are resistant to significant divergence even over extended lengths of time. It is unfortunate that a representative of an order within the subclass Phaeophycidae (e.g., Sporochnus in the Sporochnales)

Table 9. The kelp families investigated in this study and the number of genera and species that they contain (Druehl unpublished).

Family	Genera	Monotypic Genera	Species
Alariaceae	8	3	34
Chordaceae	1	0	2
Laminariaceae	11	5	48
Lessoniaceae	8	5	12

was not included in this assessment. Such a comparison would be a more accurate assessment of the feasibility of extending restriction analysis of ctDNA to interordinal taxonomic levels.

These results (Table 6) indicate an early separation of the Chordaceae from the line leading to the Lessoniaceae, Laminariaceae, and Alariaceae. Muller et al. (1985) have demonstrated developmental similarities between the orders Dictyosiphonales and Laminariales and have emphasized their close evolutionary relatedness. On the basis of these analyses, the authors have suggested that Chorda tomentosum be removed from the Laminariales and referred to the Dictyosiphonales. In this light, it is also unfortunate that a representative of the Dictyosiphonales was not included in the present analysis for comparison with Chorda filum.

Kelp chloroplast DNA substitution rate.

The high levels of sequence divergence observed within angiosperm families (Table 7) have been interpreted as indicating an ancient origin for these groups. The substitution rate determined by Zurawski et al. (1984) between the ctDNAs of Zea and Hordeum is consistent with this interpretation (i.e., 1.1 substitutions/bp/billion years). In this context, the high levels of within and between family sequence divergence that kelp exhibit can be explained in one of two ways: 1) kelp are also of ancient origin and have

accrued the observed levels of base substitution at a similarly slow rate, or 2) kelp are of a more recent origin and the observed sequence divergence is the result of a fast rate of substitution; either within the molecule as a whole or within the regions investigated in this study.

In lieu of a fossil record of kelp evolution, the only way to test these hypotheses is to correlate the observed sequence divergences between kelp species with some documented geologic event such as continental drift (Valentine & Moores 1974) or environmentally induced extinction phenomena (Stanley 1984). A possible candidate is the well documented global warming episode that occurred some 2-4 million years ago (mya) in the early Pliocene (Buchardt 1978, Stanley 1984).

Beginning in the Cretaceous (135 mya) and continuing throughout the Cenozoic (70-2 mya), the entire world underwent a devastating climatic cooling episode. The fossil record of this period documents the effect of a sustained global temperature reduction of at least 10-15 C in the catastrophic extinction of most of the warm water plant and animal species in the oceans. Temperature is the most important physical factor regulating the geographic distribution of plants in the oceans. A particular species can survive only within a certain range of temperatures (Fain & Murray 1982), and a discontinuity in temperature often marks the boundary of that species' geographic range (Druehl 1970). The sustained climatic cooling of the Cretaceous-Tertiary eliminated species that could not accomodate cooler temperatures and that could

not migrate to warmer refugia (Stanley 1984). Conversely, warm sensitive species such as kelp could have vastly expanded their normal distribution and, if sufficient cooling had occurred, cross the tropical latitudes into the southern hemisphere. A sustained global reduction of 10 C in mean annual temperature would have been sufficient to connect the northern and southern hemisphere temperate biotas via a cold water "corridor" through the tropics (Setchell 1915). Oxygen isotope and faunal extinction data (Flint 1971, Buchardt 1978, Stanley 1984) indicate that such conditions probably existed in the middle Oligocene (35-30 mya) and late Miocene (10-5 mya). The aforementioned Pliocene warming episode would have been of sufficient magnitude to dissolve the late Miocene "corridor" and cause the poleward migration of warm sensitive kelp. The present day distribution of the giant kelp Macrocystis suggests such a scenario. Unique among kelp, Macrocystis is one of only a few genera to occur in both hemispheres. It is feasible that the reproductive isolation of the North American and Chilean Macrocystis populations investigated in this study can be estimated from the biogeographical record of the last occurrence of a cold water corridor connecting the temperate regions of the two hemispheres. With that event fixed in time, the amount of sequence divergence observed between northern and southern hemisphere Macrocystis populations today can be used to estimate the rate at which the kelp chloroplast genome is evolving.

Although it is likely that a Pliocene warming event was responsible for separating northern and southern hemisphere Macrocystis populations, was it the most recent instance? The Pliocene era culminated with the onset of the Pleistocene Ice Ages (Buchardt 1978), and it might seem likely that the northern and southern hemisphere biotas would have again become confluent with the world-wide reduction in seawater temperature that occurred during this time. However, the Ice Ages were not static periods of consistent climate patterns. They were comprised of intervals of cold, periglacial, and full glacial conditions that alternated with warm interglacial periods from the end of the Pliocene (1.8 mya) to the end of the last ice event (8300 BC). The amplitude of temperature alterations during the Quaternary was at least three fold greater than during the Miocene (Hodell et al. 1986). Oxygen isotope analysis of fossil foraminiferal shells from the western equatorial Pacific, the Caribbean, and equatorial Atlantic have revealed that seawater temperature in the tropics fell to a minimum of about 22 C during this time period. These data indicate that low temperature intervals alternated with periods where seawater temperature warmed to above 27 C in cycles of about 40,000 years duration. These cycles were repeated some nine times in the last 700,000 years, with the most recent temperature minima occurring about 20,000 years ago (Emiliani & Shackleton 1974). The duration of the cooling portion of these cycles was on the order of ten thousand years, although temperature minima persisted for less

time.

It is concievable that the warming events that occurred before and interspersed between the glaciation events of the Pleistocene created an unstable temperature climate that precluded the reformation of the cold water bridge between northern and southern hemisphere biotas that had persisted into the early Pliocene (about 4 mya). The recently documented (Dayton 1984, North et al. 1985) devastation of Macrocystis in southern California in the wake of the warming effects of the 1982-84 El Nino event is convincing evidence of the sensitivity of this species to warm water conditions. In addition, although Macrocystis has been observed to migrate at rates of 30-50 miles a year under favorable temperature conditions (i.e., 15-20 C; Wheeler North, pers. comm.), the slow reestablishment capability of Macrocystis documented by North (1967) following an earlier (1957-59) warm water devastation in the same region is evidence of the relatively limited range extention capacity of this species in response to unstable temperature conditions. In this light then, a possible date for the reproductive isolation of the northern and southern hemisphere Macrocystis populations would be some 4 mya in the early Pliocene.

A substitution rate of 1.17 substitutions/bp/billion years can be calculated from the inter-hemisphere Macrocystis ctDNA sequence divergence values assessed in this study (p=0.0056, Nei & Li 1979) and the estimated reproductive isolation of the northern and southern hemisphere Macrocystis populations at

the beginning of the Pliocene some 4.8 mya (Hodell et al. 1986). This figure is in agreement with the 1.1 substitutions/bp/billion years estimate of Zurawski et al. (1984) for the substitution rate of angiosperm ctDNA. Alternatively, if the reproductive isolation of northern and southern hemisphere Macrocystis occurred during the last tropical minima (i.e., about 20,000 years ago; Emiliani & Shackleton 1974) a substitution rate of 23 substitutions/bp/million years can be derived. This value is two orders of magnitude faster than the rate calculated for angiosperm ctDNA (Zurawski et al. 1984). It should be understood that the sequence divergence value used in this calculation is based on the assessment of a limited portion of the Macrocystis chloroplast genome. However, as a first approximation, this analysis provides an interesting basis that may be addressed by further investigations. In conclusion, it is my estimation that it is most likely that the chloroplast genome of the kelps is not evolving at an accelerated rate, but that it is evolving at a similar rate to that of the angiosperm plants.

Phylogenetic relationships in the Laminariales.

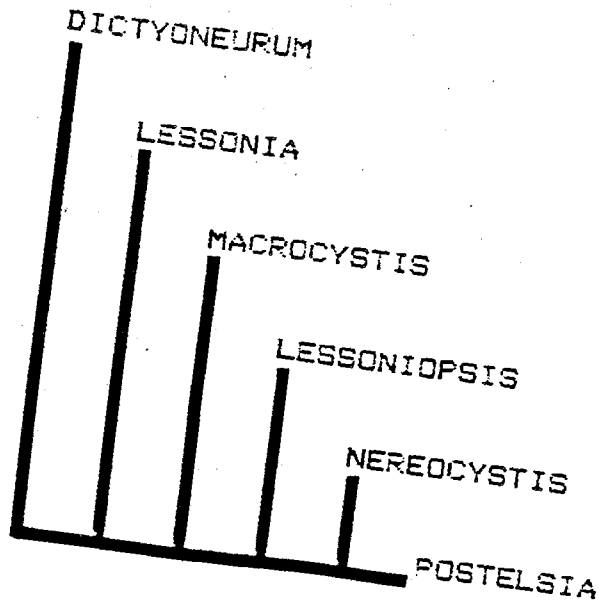
Chloroplast DNA evolutionary trees were constructed for intrafamily and interfamily comparisons by parsimony analysis (Phylip program of Joe Felsenstein, Univ. of Washington). The number of phylogenetically informative restriction site

phenotypes (i.e., shared by two or more DNAs) for intrafamily comparisons (7 enzymes) were 54, whereas 40 sites were unique to a single DNA. In interfamily comparisons (9 enzymes), the number of phylogenetically informative restriction fragments was 40 while 116 were unique to a single DNA.

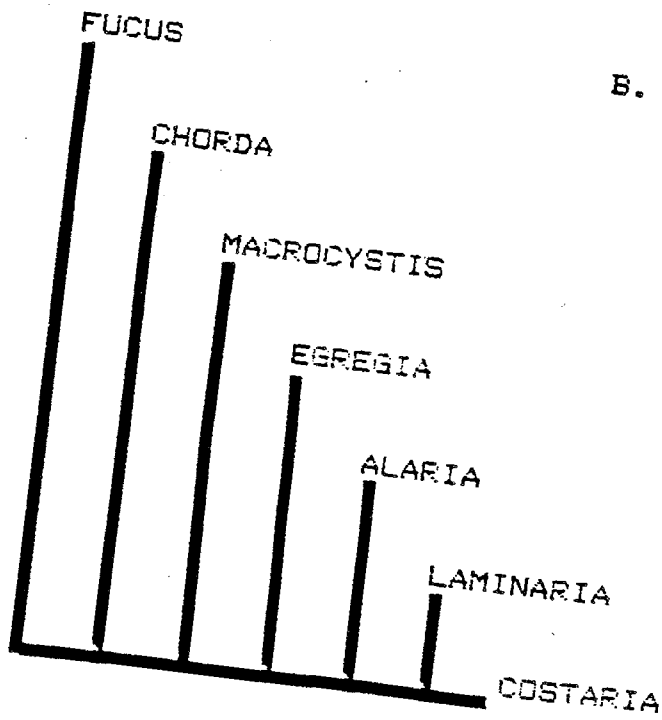
Parsimony analysis does not result in a rooted tree. Phylogenetic information is obtained from the branching relationships of the resulting network. A priori, any group in the network could be chosen as the root and a consistant branching pattern calculated in reference to it. However, by incorporating such justifiable reference features as known or assumed ancestors or outlying species, the shape of the tree can be adjusted internally to reveal the best approximation of the true evolutionary relationships. The trees in Figure 14 (Panel A-intrafamily; Panel B-interfamily) best reflect the evolutionary history of these taxa as inferred from the chloroplast DNA divergences reported in this study.

The ctDNAs of Dictyoneurum and Dictyoneuropsis were identical for the present comparisons, and the intrafamily tree (Fig. 14-Panel A) includes only a single branch representing their phylogenetic lineage (labelled Dictyoneurum). The Dictyoneurum branch is indicated as the most ancient lineage in the group by its placement at the base of the tree on the far left. The most recent divergence within the group occurred in the line leading to the Nereocystis and Postelsia branches, and it is indicated by its terminal position on the far right. The interfamily

Figure 14. Phylogenetic trees for six lessoniacean genera (Panel A) and four laminarialean families (Panel B). The tree in (Panel A) depicts the most parsimonious branching order from ctDNA restriction site maps (Figures 6 & 8). The tree in (Panel B) depicts the most parsimonious branching order from ctDNA restriction fragment length data. Trees are compiled from the combined hybridization results of both probe sequences pMiP52 and pMiP73. Relative time is indicated in the figures by more ancient to more recent divergences being drawn from left to right.


DICTYONEURUM
LESSONIA
MACROCYSTIS
LESSONIOPSIS
NEREOCYSTIS
POSTELSIA

A.


FUCUS
CHORDA
MACROCYSTIS
EGREGIA
ALARIA
LAMINARIA
COSTARIA

B.

phylogenetic lineages within the Laminariales (Fig. 14-Panel A, Figure 14-Panel B) are referenced to each other by the ancient divergence of the fucalean line from the base of the tree. Laminarialean lineages branch to the right with the Chordaceae diverging early from the line leading to the Lessoniaceae, Alariaceae, and Laminariaceae. Although ctDNA sequence divergences indicate that the families Lessoniaceae, Alariaceae, and Laminariaceae are equally divergent, parsimony analysis of shared restriction fragment lengths indicates that the Alariaceae and Laminariaceae have diverged most recently. The Lessoniaceae diverged earlier from the common ancestor to the Alariaceae and Laminariaceae. The comparatively similar unbranched morphologies of the Alariaceae and Laminariaceae (with the exception of Egregia) support this association. In that both the Lessoniaceae and Alariaceae/Laminariaceae lineages exhibit specialized soral blades, sorus formation on vegetative blades, and midrib structures in vegetal blades, it is likely that these characters were present in the common ancestor to the three families. The most significant discriminating feature between the families would then remain thallus branching format (i.e., unbranched, pinnate, or true branching).

Biogeographical distribution and evolution in the Lessoniaceae.

Primarily of an anti-tropical distribution, the

Lessoniaceae are found circum-subantarctic and along the West coast of South America in the southern Hemisphere, and along the West coast of North America. The place of origin of a taxon is considered to be that location in which it is most diverse. It is thought that the richest centers produced competitively advanced individuals superior to the parental forms and that the subsequent interaction tended to force an outward dispersal of the more primitive forms. Today the greatest diversity of kelp species is encountered in the southern Kuriles-northern Hokkaido region of Japan and in the vicinity of Vancouver Island in southern British Columbia, Canada (Druehl 1981).

The differences between the absolute magnitudes of the sequence divergences obtained by fragment and site analysis (Table 5) were consistantly observed. However, the relationships observed between samples by site comparisons were maintained by fragment comparisons. Sequence divergence determined by site analysis was on average about 1.8 times that determined by fragment analysis. This is likely due to the fact that the fragment method does not consider back mutation (Nei & Li 1979). As a result, the use of the fragment method is most accurate when the number of substitutions per nucleotide site is relatively small. This is seen in the similarity of the respective sequence divergence estimates of the site and fragment methods in comparisons of populations and species of Macrocystis [i.e., 100p=0.46 (fragment method) and 0.56 (site method)].

Therefore, the magnitudes of the sequence divergence estimates obtained in comparisons of more distantly related plants (e.g., genera both within and between families) are underestimations. Accordingly, the 1.8 times difference between the two methods was used to equate the magnitudes of the interfamily fragment method sequence divergence values with the more accurate site method values determined in intrafamily comparisons. This was done solely to estimate divergence times between the four kelp families. The following interpretation of the evolutionary history of the kelps was determined from the ctDNA sequence divergence data obtained in this study, and the ctDNA substitution rate derived for Macrocystis from biogeographical and ctDNA restriction analysis data (see above). About 150 mya the lineage leading to the present day kelp family Chordaceae diverged from the line that eventually led to the families: Lessoniaceae, Laminariaceae, and Alariaceae. Some 80-100 mya the diversification of these three families occurred.

Within the family Lessoniaceae, the early divergence (70-80 mya) of the Dictyoneurum/Dictyoneuropsis lineage is of particular interest, as it would have been concurrent with the diversification of the three principle kelp families discussed above. This, in conjunction with its relatively unspeciallized morphology, may indicate it as being representative of the ancestral lessoniacean "type". Subsequent divergences of Lessonia, Lessoniopsis, Macrocystis, and the line that led to the present day Nereocystis and

Postelsia occurred about 60-65 mya. The combination of the movement of cold water to lower latitudes and the formation of a cold water connection along the West coast of the Americas allowed for the southward dispersal of Macrocystis and Lessonia. In the Northern hemisphere, Nereocystis and Postelsia apparently diverged from a common ancestor about 30 mya.

Again it should be stressed that these estimates are based on a substitution rate calibration that has been determined from a limited assessment of the Macrocystis chloroplast genome and are therefore tentative at best. The only other ctDNA substitution rate estimate available was determined from angiosperm ctDNA sequence comparisons. As mentioned previously, the estimation of kelp family and genus divergence times based on the angiosperm ctDNA substitution rate would not yield significantly different results.

DNA extraction yields.

Total cellular DNA was isolated from kelp blades at yields of about 1mgr per gram wet weight of tissue extracted. Purified nuclear and chloroplast DNAs were isolated from M. integrifolia at respective yields of about 50 ngr and 1mgr per gram wet weight of tissue extracted. DNA yields from kelp tissues by my protocol are 1/25-1/10 those obtained from angiosperm plants by various other protocols.

Chloroplast genome characterization.

The size of the chloroplast genome of Macrocystis integrifolia is about 170 kb. The chloroplast genome of M. integrifolia is comprised of both repeat regions and single copy regions. The repeat regions encode the rRNA genes and, consequently, the chloroplast genomes of kelp species encode two copies of the 23S rRNA gene (Fig. 4, Table 4). The chloroplast genes atpB, rbcL, and psbA appear to occur only once on the M. integrifolia chloroplast genome and hence are presumed to be encoded in the single copy regions of the molecule (Figs. 4 & 5, Table 4). The repeat regions of the chloroplast genomes of kelp undergo nucleotide substitutions less frequently than do the single copy regions.

Chloroplast DNA sequence divergence at comparable taxonomic levels in kelp and angiosperm plants.

With only thirty genera divided into five separate families, the classification of the order Laminariales might appear to be somewhat artificial. However, kelp chloroplast DNA sequence divergences at the species, genus, and family levels is comparable to those determined for similar taxonomic levels among the much more diverse angiosperm plant families (Table 7). Such data may justify the high level of taxonomic distinction accorded to the Laminariales.

Interpopulation/Interspecies sequence divergences.

Northern and southern hemisphere M. integrifolia populations have diverged at about 0.5% of the base pairs in their respective chloroplast genomes. No sequence divergence was observed among or between northern hemisphere M. integrifolia populations. No sequence divergence was observed among or between northern hemisphere M. integrifolia and M. pyrifera species. Thus the northern hemisphere species M. integrifolia and M. pyrifera have shared a common ancestor more recently in their evolutionary past than have the northern and southern hemisphere populations of M. integrifolia.

Intrafamily sequence divergence.

With respect to the analysis employed in this study, the chloroplast genomes of the lessoniacean genera Dictyoneurum

and Dictyoneuropsis are identical. In that at least 0.5% sequence divergence was observed between the ctDNAs of species/populations within the genus Macrocystis, it is likely that genus level distinction between these plants is not justified. On the basis of both the parsimony analysis of shared restriction sites and sequence divergence determinations, Nereocystis and Postelsia are the most closely related genera within the Lessoniaceae, and Dictyoneurum the most distant relation (Fig. 14 and Table 5). Together, Macrocystis and Lessoniopsis comprise a loosely defined subgroup that is more closely alligned with the Nereocystis-Postelsia subgroup than with Lessonia or Dictyoneurum. However, in these comparisons parsimony analysis and sequence divergence determinations give conflicting results. Sequence divergence determinations indicate that Macrocystis is more closely related to Nereocystis and Postelsia than is Lessoniopsis (Table 5). Parsimony analysis of shared restriction sites gives the opposite result (Fig. 14).

The range of ctDNA sequence divergence that exists within the families Alariaceae and Laminariaceae may be similar to that in the family Lessoniaceae (Tables 5 & 6). Two genera from different tribes in the Laminariaceae and Alariaceae were investigated in this study (Table 1). However, the degree of morphological difference that is required to erect taxonomically distinct tribes within a family is not representative of the range of ctDNA divergence extant within

that family. That is, highly diverged morphologies do not necessarily imply highly diverged ctDNAs. The ctDNAs of additional representatives of the Laminariaceae and Alariaceae must be investigated before their relative divergences can be discussed.

Interfamily sequence divergence.

The family Chordaceae diverged early from the line leading to the closely inter-related families Alariaceae, Laminariaceae, and Lessoniaceae (Figure 14 and Table 6). The relative divergences of the Alariaceae, Laminariaceae, and Lessoniaceae is not clear. The sequence divergence of the ctDNAs of selected representatives indicates that these three families are equally divergent (Table 6). On the other hand, parsimony analysis of shared restriction fragment lengths indicates that the Alariaceae may be more closely related to the Laminariaceae than are the Lessoniaceae.

Substitution rates in laminarialean chloroplast DNA.

A substituion rate of 1.17 nucleotide substitutions /bp/ billion years can be calculated from the inter-hemisphere Macrocystis ctDNa sequence divergence values assessed in this study and the estimated reproductive isolation of the northern and southern hemisphere Macrocystis populations 4-8 mya (Hodell et al. 1986). This figure is in close agreement with the 1.1 nucleotide substitutions /bp/ billion years estimate

of Zurawski *et al*. (1984) for the substitution rate of angiosperm ctDNA.

In this light, it has been estimated that the Chordaceae diverged from the main line of Laminarialean evolution about 150 mya. The divergence of the Lessoniaceae, Alariaceae, and Laminariaceae occurred some 80-100 mya, and the lessoniacean genera investigated in this study diverged over the last 30-70 my. For purposes of reference, Muller (1981) has determined from fossil pollen deposits that the monocotyledonous and dicotyledonous subclasses of the Angiospermae diverged some 100-150 mya. In the same study it was estimated that the dicotyledonous families Cruciferae, Solanaceae, Chamelopodaceae, and Leguminaceae have diverged over the last 50-100 my.

PROPOSALS FOR FUTURE RESEARCH

I. Construct a complete restriction map of the chloroplast genome of Macrocystis for overall sequence organization determination and gene location. Are there interesting differences with respect to angiospernm and other algal systems?

II. Examine the question of mutation rate in the nuclear and chloroplast genomes of kelps. Utilize clearly datable biogeographical events as time references (e.g., northern & southern hemisphere segregation of Macrocystis by global temperature fluctuations).

III. Determine the relative divergences between selected representatives of the Alariaceae, Laminariaceae, and Lessoniaceae by sequence analysis of selected regions of their respective ctDNAs.

IV. Use restriction analysis to quantify the range of ctDNA sequence divergence that is extant within the Alariaceae, Laminariaceae, Chordaceae, and Phyllariaceae. Are they equivalent?

V. Examine ctDNA relations between the laminarialean families Chordaceae, Phyllariaceae and a representative of the closely related order Sporochnales (Phaeophycidae). This information would provide insight as to both the early diversification of the Laminariales and the divergence of the Laminariales and Sporochnales.

VI. Determine nuclear sequence divergence and perform crossability tests between Dictyoneurum and Dictyoneuropsis and also between Macrocystis pyrifera and M. integrifolia to determine if they are congeneric species or conspecific ecotypes/forms.

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