MOLECULAR EVOLUTION AND PHYLOGENY OF THE LAMINARIALES: ANALYSIS OF THE NUCLEAR CODED RIBOSOMAL CISTRON

by

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Molecular Evolution and Phylogeny of the Laminariales: Analysis of the Nuclear Coded Ribosomal Cistron

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Abstract

Molecular investigations were undertaken for a variety of representative Laminariales (kelp) to obtain insights into their evolution and phylogeny. These aspects of kelp biology are poorly understood owing to phenotypic plasticity and the paucity of a fossil record. For this reason I have undertaken restriction-enzyme mapping of the nuclear coded ribosomal cistron and dideoxy sequencing of the small-subunit (SSU) and internal transcribed spacers (ITS), including the 5.8S gene, of the cistron.

Restriction-enzyme mapping was phylogenetically uninformative for these taxa. The rRNA gene coding regions were too conserved for meaningful comparisons. In contrast, the intergenic spacer (IGS) was too variable and homologous restriction sites could not be assigned with certainty.

Sequencing revealed that the SSU genes for representatives of seven kelp genera were too highly conserved to resolve phylogenetic relationships. By applying SSU divergence estimates to a molecular clock, I proposed that the kelp employed in the current study diverged from a common ancestor as recently as 16-20 mya, rather than 200-300 mya suggested in interpretations of the fossil record. Reevaluation of kelp relationships to other heterokonts, using the first complete kelp SSU sequence, essentially supported earlier cytological and molecular derived phylogenies.

The ITS regions of the ribosomal cistron, including the 5.8S gene, for representatives of the genera Alaria and Postelsia were compared to those of other eukaryotes. Features such as length, G+C balance, distribution of conserved and variable regions, and putative post-transcriptional processing sites are discussed. The 5.8S gene sequence of Alaria was compared to those of other eukaryotes adding the chromophyte lineage to a universal 5.8S phylogeny.

Regions of the SSU (3' terminus), ITS1, 5.8S gene and ITS2 were sequenced for a variety of alariacean and lessoniacean taxa. These data supported the polyphyletic state proposed for the Lessoniaceae based on chloroplast-DNA data and additionally contrasted other aspects of traditional kelp taxonomy.

Dedication

This thesis is dedicated to the carefully planned and legislated conservation of biological diversity.

Life's Paradox. What comes around Goes?

Evolutionists estimate twas three billion years ago
the Earth was quite different from the one we know.

An environment of extreme heat, ultraviolet rays and toxic gas
was certainly a planet where human beings could never last.

Yet in this early world that we can merely speculate about
reigned the golden age of the supposedly lesser Prokaryotes.

Their numbers swelled as they modified to fill every niche
not realizing that they were polluting the planet beyond their reach.

And left in their shadow by mother nature's chemical way
was an abundance of oxygen and eukaryote tolerant solar ray.

They had destroyed their own planet and left a strange place
in this changed world evolved the superior human race.

Father time finds us now in the golden age of man
but still life continues without a long term plan.

So we continue to pollute the planet we need
considering ourselves the most civilized breed.

However this warning I am sending out to all
to change our destructive ways before we also fall.

We would then leave a planet for who knows what
perhaps once again the rise of the primitive Prokaryote?

Acknowledgments

I extend appreciation to Charlene Mayes, Ian Tan, Jackie Schein, Dave Travers, Dr. Debashish Bhattacharya and Larry Mroz for technical support, discussion and most importantly friendship in the kelp cove laboratory. Drs. Jim Brown, Sandra Lindstrom and Mark Ragan have reviewed portions of this thesis and provided insightful comments. Dr. B. Santelices, Dr. S. Fain and Mr. C. Baron provided some of the algal samples used in this study. Vic Bourne has assisted in preparing the visual presentations of my research. In particular I thank Karen Beckenbach for all her advice and assistance in my growth as a molecular biologist.

My committee members, Drs. Andrew Beckenbach and David Baillie, were always available to help with problems and give valuable suggestions and were instrumental in bringing this thesis to completion. I graciously acknowledge the encouragement and advice of Dr. Mike Smith, my adoptive supervisor during absences of my senior adviser, concerning this research and my future endeavours.

Many thanks Dr. Louis Druehl, you have provided an opportunity for me to learn and to develop my academic abilities. We have talked, laughed and had many intellectual and questionable discussions. I will always remember the times we spent together and I hope that we will continue to unravel the wonders of the marine algae together.

Thank you Dolores, my patient and loving wife, for being constantly supportive throughout this research. I leave this university a better person, if for no other reason than I met you.

To a man who believed in me before it was due. It was your inspiration, understanding and dedication to teaching that brings me to the completion of my Ph.D. program. Thank you Dr. Darryl Grund for giving me a chance, I shall never forget your friendship and commitment.

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CHAPTER 1

GENERAL INTRODUCTION

This thesis presents molecular investigations of the nuclear coded ribosomal cistron for a variety of genera from the Laminariales (kelp). The aim of this work was to further understanding of kelp evolution and phylogeny. The kelp display the greatest morphological and anatomical specialization among all the divisions of algae. Currently six families are recognized, three of which contain complex genera. These families, the Alariaceae, Laminariaceae and Lessoniaceae are distinguished on the basis of morphological features produced during development from the intercalary meristem at the stipe-blade transition zone (Setchell & Gardner 1925). In this thesis I use the term kelp in a restricted sense, referring only to these three families.

Kelp have heteromorphic life cycles, characterized by a dominant sporophyte generation alternating with dioecious, filamentous gametophytes. One of the most intriguing features of the kelp is their extensive display of phenotypic plasticity (Mathieson et al. 1981). This latter trait has presented contemporary phycologists with a barrier to understanding the evolution, age and phylogeny of the kelp. This thesis is concerned with these latter aspects of kelp biology.

Molecular Phylogeny

The aim of many biosystematists is to base taxonomy on phylogenetic relatedness. Phenotypic similarity is used to estimate genetic relatedness so that a phylogenetically compatible taxonomy can be inferred. The extensive phenotypic variability common amongst species of kelp has prompted molecular investigations that by-pass the phenotype for phylogenetic analyses (Bhattacharya & Druehl 1988, Fain et al. 1988). Further,

phenotypic characters are usually three dimensional, difficult to interpret and weigh, and the decision on ancestral versus derived character states is difficult to ascertain in the absence of a thorough fossil record, as is the case for the kelp. DNA is linear, with discrete changes occurring along its length enabling direct pair-wise comparisons of characters. Many changes at the DNA level are apparently neutral, and therefore, are not acted upon by selection and are fixed at random (Nei 1987). These factors lend DNA two important properties for biosystematic utilization; DNA generally evolves in a regular clock-like fashion (when homologous regions of DNA are compared from different sources) and similarity is a function of relatedness not selection driven convergent evolution.

When endeavouring to investigate genetic relationships at the molecular level two major questions must be addressed. First, there is the selection of the genome, DNA region (possibly a gene or fraction thereof) or gene product to be investigated and secondly, the choice of the appropriate method of analysis. Two key issues must be considered in making these selections: the technical difficulty of the approach and the level of resolution that can be achieved.

Technical difficulty begins with the initial isolation of the portion of genome or gene product selected for study. Abundance and ease of isolation are the major concerns. In green plants it is generally accepted that the chloroplast is an abundant and easily isolated genome for molecular analysis (Palmer 1987). Nuclear DNA is easy to isolate in pure form but the nuclear genome is very large and difficult to analyze. As a result investigations of the nuclear genome are centred around easily isolated fractions of this genome. One such region is the ribosomal cistron which occurs in thousands of copies per haploid genome in most plants (Appels & Honeycutt 1986).

Restriction enzymes can be employed to estimate DNA divergence. Restriction enzymes are proteins that cleave DNA at or near specific recognition sequences in the DNA. The more similar two DNAs, i.e., the least time since they shared a common ancestor, the

more similar their restriction-enzyme cleavage patterns. We can estimate the number of nucleotide substitutions between the DNAs of two organisms by determining the proportion of restriction sites shared between their DNAs. The simplest method is the restriction-fragment method. The DNA is digested with a variety of restriction endonucleases which recognize different sites distributed throughout the DNA of interest. Digests of a variety of taxa (populations, species, etc.) for an enzyme are simultaneously size fractionated by electrophoresis on a horizontal agarose gel. Differences in fragment patterns are usually the result of the loss or gain of a restriction-enzyme recognition site owing to single base pair mutations within the recognition sites on the DNA. Other types of mutations which occur such as inversions, insertions and deletions also affect the resulting pattern but are more difficult to interpret. The degree of genetic similarity between two DNAs is correlated to the number of restriction fragments shared between them. This method is subject to a variety of errors (see Nei 1987, p. 106-107) and is only reliable for short genetic distances between populations and closely related species.

Restriction-site mapping is a more exact, but time consuming, method of transferring restriction data into divergence estimates. In this case the relative locations of restriction sites are determined on a physical map of the DNA region. These sites will vary as the nucleotide sequence varies between the DNAs of the two taxa being compared. Therefore, the more closely related two taxa, the more similar their restriction-enzyme maps. The number of nucleotide substitutions between two homologous DNAs can be estimated by comparing their restriction maps (Nei 1987, p. 96-105). Mapping is accomplished by digesting DNA with individual, then paired, combinations of restriction enzymes. By comparing the fragments of these single and double digests it is possible to place restriction sites relative to each other on a physical map. Restriction maps improve the accuracy of sequence divergence estimates by reducing incorrectly assumed homologies. At some level

of DNA divergence, mapping also fails because the likelihood of two or more independent mutations occurring in the same restriction site increases.

It is possible to determine the actual DNA sequence of a portion of a gene or genome. DNA sequences can be directly compared enabling more accurate divergence estimates. By selecting genes or DNA regions of different conservation it is possible to determine relationships at varied levels of taxonomy. DNA sequencing, as well as providing divergence and phylogenetic insights, also illucidates the types of mutations that are occurring. DNA sequencing is expensive and time consuming but the technology is rapidly improving and the data are superior to the other methods. One such development is the ability to produce synthetic oligonucleotides to prime DNA sequencing at intervals of 250 to 300 basepairs. By designing primers complementary to highly conserved regions of the target DNA, with divergent regions between adjacent primers, it is possible to complete extensive taxonomic surveys with the same set of primers without the need for subcloning. Elwood et al. (1985) used this approach to sequence the small-subunit ribosomal RNA genes (approx. 1800 basepairs) in a survey that encompassed the entire eukaryotic lineage.

A second major advance, the Polymerase Chain Reaction (PCR), utilizes the technology of synthetic oligonucleotides in addition to a heat stable DNA polymerase (*Taq* I). In PCR a specific DNA region is amplified from small amounts of a complex DNA mixture. The amplified product can be directly sequenced avoiding the cloning and screening procedures traditionally involved in isolating a particular DNA region for subsequent sequencing. Synthetic primers are designed to complement the coding and noncoding strands of DNA, at opposite ends of the region to be amplified. The template is denatured at a temperature (92-95^{o}C) where the *Taq* I polymerase remains stable. The mixture is cooled allowing the primers to anneal to the template DNA and then heated to the optimal temperature for the polymerase which incorporates nucleotides extending the primer

complementary to the template strand. Successive cycles are completed resulting in an exponential increase in the target DNA.

Ribosomal Cistron

There are two main types of genes, those ultimately coding protein products and those coding structural RNAs. Protein genes are transcribed as messenger RNAs (mRNA) that are translated into the protein products. Structural RNA genes transcribe as pre-transfer RNAs (tRNA), small nuclear RNAs (snRNA) and ribosomal RNAs (rRNA). These RNA products, after post-transcriptional modification, directly function in metabolism and are essential to the mRNA translational machinery. Key to this role, the ribosomes, are abundant in the plant cytosol. Ribosomes are a combination of proteins and rRNAs and are assembled in the nucleolus.

Ribosomes consist of small and large subunits, with the major rRNA associated with the former called the small-subunit rRNA (SSU). The major rRNA associated with the latter is called the large-subunit rRNA (LSU). The nuclear SSU and LSU are also called the 18S rRNA and the 25-28S rRNA respectively, owing to their Svedberg sedimentation coefficients. The large-subunit of the ribosome in eukaryotic cytoplasm has two additional associated rRNAs, the 5S and 5.8S rRNAs.

Nuclear coded rRNA genes in plants are arranged, head to tail, in tandem repeat units (Fig. 1). The head to tail arrangement of the ribosomal cistron lends it the quality of a circular molecule for purposes of restriction-enzyme mapping, thus simplifying the mapping procedure. The copy number of these tandem repeats is variable even among closely related taxa. In fact copy number can change within the somatic cells of an individual and up to 90% of the copies are believed to be superfluous (Rogers & Bendich 1987). Plants generally have a higher copy number than animals, containing 500-40000

versus 100-1000 copies per cell respectively (Appels & Honeycutt 1986). The SSU, 5.8S and LSU genes are clustered and cotranscribed producing a transcript that is later processed to yield the mature rRNAs. The 5.8S gene is located between the first and second internal transcribed spacers (ITS1 and ITS2 respectively) in the region between the SSU and LSU genes (Fig. 1). The transcribed gene clusters are separated by an intergenic spacer region (IGS) that consists of transcribed and nontranscribed spacer sequence (Fig. 1). The IGS was traditionally divided into the nontranscribed spacer (NTS) and the external transcribed spacer (ETS) but this terminology is confusing in view of recent investigations that suggest the NTS may in fact be transcribed (see Rogers & Bendich 1987). The term intergenic spacer is used here to prevent confusion. The IGS consists of many rapidly evolving subrepetitive elements that vary in sequence between related species and in copy number between neighbouring repeat cistrons on a chromosome (Appels & Honeycutt 1986).

Besides phylogeny, ribosomal spacer regions are investigated to define post-transcriptional processing sites. It was proposed that processing-sequence motifs should be detectable in the primary RNA structure near the processing sites themselves (see Torres et al. 1990). In the search for consensus sequence patterns, the ITS regions have been sequenced for a select variety of eukaryotes, mostly animals and fungi (Torres et al. 1990). It is not certain if a universal processing system occurs among eukaryotes and recent data suggest that this is probably not the case (Gerbi 1985, Nazar et al. 1987, Torres et al. 1990). During attempts to understand rRNA processing, the phenomenon of G+C balance was noted for the ITS1 and ITS2 of a given organism, for a wide variety of eukaryotes (Torres et al. 1990).

Evolution of the tandem ribosomal cistrons occurs in a concerted fashion that homogenizes the cistron sequence. Regions of the ribosomal cistron are under varying degrees of functional constraint. As such, different regions provide varying limits of phylogenetic resolution spanning the biotic spectrum from populations to kingdoms. The

IGS evolves rapidly being under the least constraint and is useful for intraspecific levels of taxonomy. Conversely, the SSU, 5.8S and LSU genes are the most conserved regions of the cistron. The SSU, 5.8S gene and the 5' region of the LSU have all been employed independently in eukaryote-wide phylogenies (Sogin et al. 1986, 1989, Yokota et al. 1989, Baroin et al. 1988). The relative merits for these different systems are argued in the literature. The strong conservation and functional equivalence of these molecules in all forms of life renders them valuable for distant phylogenetic comparisons (Sogin et al. 1986, Woese 1987). The rRNA genes also have less conserved regions that can be utilized to investigate closely related taxa (McCarrol et al. 1983, Woese 1987). Overall the SSU is more conserved than the LSU, the latter being particularly variable at its 5' end (Appels & Honeycutt 1986). This makes the SSU particularly valuable for more distant phylogenetic comparisons. The 5.8S gene is slightly less conserved than the SSU but has also been used to infer distant phylogenetic relationships. The 5.8S gene is considerably smaller than the SSU or LSU and its usefulness in taxonomic investigations for close and distantly related organisms has been questioned (McCarrol et al. 1983, Sogin et al. 1986). The ITS1 and ITS2 regions are variable, with the ITS2 being the least conserved of the internal spacers. These spacers are valuable for phylogenetic comparisons at the intrafamily and intrageneric levels (Appels & Honeycutt 1986).

Laminariales, Phylogeny and Evolution

The laminarialean families Phyllariaceae, Pseudochordaceae and Chordaceae are distinguished from each other and the families Alariaceae, Laminariaceae and Lessoniaceae by varied anatomical, life history, phermonal and ultrastructural features (Kawai & Kurogi 1985, Henry & South 1987). The latter three families are conserved for these diagnostic features and are divided on the basis of morphological features of the stipe-blade transition

zone. In the Lessoniaceae the transition zone divides, giving rise to branched thalli. The Alariaceae and Laminariaceae have simple unbranched transition zones and the former is discrete from the latter by having pinnately arranged sporophylls arising from the transition zone along the stipe or blade (Setchell & Gardner 1925). Although this system of taxonomy is widely accepted in the literature, Setchell & Gardner (1925) acknowledged inconsistencies in their system that have yet to be reconciled. Phylogeny within the Laminariales has been largely neglected until a recent study by Fain et al. (1988). This initial and important investigation has provided some thought provoking insights into kelp evolution. The paper casts doubt on the traditional taxonomic view of the Laminariales. RFLD (restriction-fragment-length difference) analysis of the chloroplast-DNA (cpDNA) was the basis for the conclusions in this study. The data indicated that Nereocystis, morphologically in the Lessoniaceae, has phylogenetic affinities with Laminaria, morphologically in the Laminariaceae. The study aligned Macrocystis and Lessoniopsis, also of the Lessoniaceae, with the genus Alaria, morphologically defined in the Alariaceae. Fain et al. (1988) concluded that the Lessoniaceae was polyphyletic, and that taxonomy, based solely on morphology, provides an artificial taxonomic system for the kelp.

The interpretation of the above data may present a realistic phylogeny for the Laminariales, but there are two possible explanations for the disparity between the chloroplast and morphological interpretations.

First, chloroplast phylogenies do not always equate to organismal phylogenies particularly in closely related taxa where uniparental chloroplast inheritance occurs (see Palmer 1985, 1987). Confusion occurs when a hybrid organism breeds back into the paternal population, thus introducing the maternal chloroplast into the paternal population. If the maternal chloroplast becomes fixed in the paternal population, the initial two taxa would appear more closely related in a cpDNA than a nuclear phylogeny. Chloroplast genomes, therefore, trace matriarchal lineages (Palmer 1987), thus enabling the

determination of parentage for hybrid plants and the detection of chloroplast introgression when discrepancies occur between nuclear and chloroplast data (the opposite, patriarchal introgression also occurs) (Palmer 1985, 1987). Conversely, if a hybrid event has occurred without detection and phylogenies are based on the cpDNA in the absence of nuclear data, then accurate maternal phylogenies are obtained that argue against nuclear and probably morphological relationships. Intergeneric hybrids have been reported in the Laminariales by Neushul (1971) and Sanbonsuga and Neushul (1978). Hence, the kelp cpDNA phylogenies may contradict classical views owing to maternal inheritance and random fixation of a maternally inherited chloroplast.

Second, Fain et al. (1988) estimated divergence between chloroplast genomes by the proportion of shared restriction fragments. Nei (1987) discusses the merits of this approach and notes that it gives accurate divergence estimates when $d < 0.05$. The divergence estimates of Fain et al. (1988) between Alaria and Macrocystis (0.04) narrowly fall within Nei's margin of error. However, if nonhomologous fragments do result in the error discussed above, then the divergence estimates of Fain et al. (1988) might be underestimated. In summary, chance error resulting from the fragment method of analysis in the chloroplast study may explain the inconsistency of the chloroplast and morphological based phylogenies for diverse taxa such as Macrocystis and Alaria.

Interest in kelp phylogeny has gained momentum following this study, casting doubt on their relationships (Fain et al. 1988); however, aspects of laminarialean evolution remain poorly understood. In recent years attention has focused on evolution with phycologists wondering when and where the kelp radiated (Estes & Steinberg 1988, Stam et al. 1988, Lüning & tom Dieck 1990, Fain & Druehl unpubl, Druehl & Saunders 1991). By more accurately assessing phylogeny through molecular relationships of extant taxa, I hope to define a natural system of taxonomy for the Laminariales. In the absence of a substantial

fossil record (Parker & Dawson 1965, Loeblich 1974, Clayton 1984) these data can also provide insights into kelp evolution.

The morphological diversity of the kelp leads one to suggest they are an ancient assemblage. This is reflected in interpretations of the fossil record that suggest the kelp are 200-300 million years old (see Loeblich 1974, Clayton 1984). However, there has been increasing evidence for a recent radiation. Mathieson et al. (1981) discussed the considerable phenotypic plasticity among "species"-delineating characters for kelp. Intergeneric hybrids have been observed in the laboratory as well as in the wild (Neushul 1971, Sanbonsuga & Neushul 1978). Estes & Steinberg (1988) used a variety of observations, biogeographical distribution, habitat, numbers of monotypic genera and the fossil record of kelp associates, to suggest a late Miocene (10-15 million years ago, mya) to as recent as the late Cenozoic (3-5 mya) divergence of the kelp. Julescrania, a fossil kelp hypothesized as ancestral to the morphologically divergent genera Nereocystis and Pelagophycus, was isolated from Mohnian, Miocene sediments (7-10 mya, Stam et al. 1988, interpreted from Parker & Dawson 1965). Employing chloroplast derived divergence estimates (Fain et al. 1988, Fain & Druehl unpubl) it was noted that intergeneric divergence for kelp equate to interspecific distances in the angiosperms while intergeneric distances in the latter extend past interfamilial divergence for the kelp (Druehl & Saunders 1991). These varied observations suggest rapid morphological evolution over a short evolutionary time.

The Taxa Employed in this Study

Taxa investigated in this study were restricted to the order Laminariales and emphasized members of the Alariaceae and Lessoniaceae (Table 1). Sargassum muticum (Yendo) Fensholt of the Fucales was used as an outgroup in one study.

Lessoniaceae Setchell & Gardner: the base of the juvenile plant splits at the transition zone. As the plant grows from an intercalary meristem, located at the transition zone, the split elongates and eventually divides the blade. Subsequent, similar divisions produce a compound frond.

Lessonia Bory: this plant has numerous narrowly linear blades each of which terminates a branch of the repeatedly divided stipe (Fig. 2). This plant lacks any type of midrib on the vegetative blades. The reproductive sori are produced on the vegetative blades and sporophylls are absent. This plant clearly meets the criteria for the Lessoniaceae. This taxon is unique among the Lessoniaceae in its absence from the Pacific coast of North America and isolation to the southern hemisphere.

Nereocystis Postels & Ruprecht: this plant has a long, flexible stipe that is hollow and terminates in an expanded pneumatocyst (Fig. 3). Crowning the pneumatocyst are large strap-like vegetative blades on which the reproductive sori are produced. The transition zone consists of a compacted series of branches that bear the blades. Although strikingly different in habit from Lessonia, Nereocystis also fits the lessoniacean criteria.

Postelsia Ruprecht: this plant has a short, strong but hollow stipe that supports a tuft of narrow strap-like, deeply grooved blades (Fig. 4). The transition zone is as described above for Nereocystis. Although Postelsia superficially appears different from the other kelp the overall pattern is similar to that observed for Nereocystis.

Dictyoneurum Ruprecht/Dictyoneuropsis Smith: these genera have a flattened stipe that is prostrate along the substratum (Fig. 5). The characteristic splitting occurs at the base of strap-like, reticulate vegetative blades that bear the reproductive sori. Dictyoneurum lacks a midrib on its vegetative blades that is present in Dictyoneuropsis.

Macrocystis Agardh: this plant has long slender stipes bearing unilaterally arranged vegetative blades that are subtended by pneumatocysts (Fig. 6). These blades are split from an apically positioned intercalary meristem rather than the primary intercalary meristem,

which gives rise to the individual fronds, positioned at the transition zone. The transition zone is, however, characterized by splitting and Macrocystis was therefore placed in the Lessoniaceae. This genus has discrete sporophylls, an alariacean character.

Lessoniopsis Reinke: this plant is similar in branching habit to its namesake Lessonia, with which it was originally classified (Fig. 7). It differs in that its vegetative blades have midribs and are subtended by paired sporophylls. Setchell & Gardner (1925) noted that because of this latter feature this plant could be placed with equal priority in the Lessoniaceae or the Alariaceae.

Alariaceae Setchell & Gardner: this family is generally characterized by plants with simple fronds that terminate in single blades. This family was intended to include all those laminarialean algae with sporophylls arising from the stipe and blade except Lessoniopsis (Setchell & Gardner 1925).

Alaria Greville: this plant usually produces a single, undivided, vegetative blade with a prominent midrib (Fig. 8). The stipe is also simple and unbranched. The reproductive sori are produced on pinnately arranged sporophylls borne on the stipe immediately below the transition zone.

Pterygophora Ruprecht: when this plant has its vegetative blade in tact it gives the appearance of an Alaria with a woody stipe (Fig. 9). The midrib is less distinct in Pterygophora, while the sporophylls are more prominent. The vegetative blade is usually degenerate occurring as a necrotic strap of tissue terminating the stipe.

Eisenia Areschoug: this plant has a woody stipe with a prominent dichotomy (Fig. 10). This 'splitting' of the stipe does not initiate in the transition zone, as in the Lessoniaceae, and is rather an erosive process of an initial vegetative frond. This erosive process leaves a split meristem that continues to produce sporophylls on both sides of the split stipe, above the transition zone on the vegetative blade remnants.

Egregia Areschoug: this plant has an as-yet-uncharacterized branching pattern of a flattened stipe (Fig. 11). The lateral margins of the stipe and blade are fringed with pinnately arranged blades that occasionally differentiate to form pneumatocysts. The lateral blades on the stipe irregularly differentiate to function as sporophylls.

Laminariaceae Bory: this family is characterized by members with simple fronds with an undifferentiated transition zone. Reproductive sori are produced on the vegetative blade and sporophylls are absent. True splitting (sensu Lessoniaceae) resulting in branching does not occur in this group (Setchell & Gardner 1925). However, splitting by the same ontogenetic means results in split blades in the Digitatae section of Laminaria.

Costaria Greville: I have studied this genus as a representative member of this family. This plant meets all the conditions for this family and is distinct from other laminariacean algae in producing five distinct midribs on its vegetative blade (Fig. 12).

Fig. 1. The ribosomal cistron. a) Arrows indicating tandemly repeated ribosomal cistrons. b) Close up of an individual cistron displaying the relative location of the rRNA genes and spacers. ? indicates uncertainty of NTS-ETS boundary in kelp See text for abbreviations.

Fig. 1.

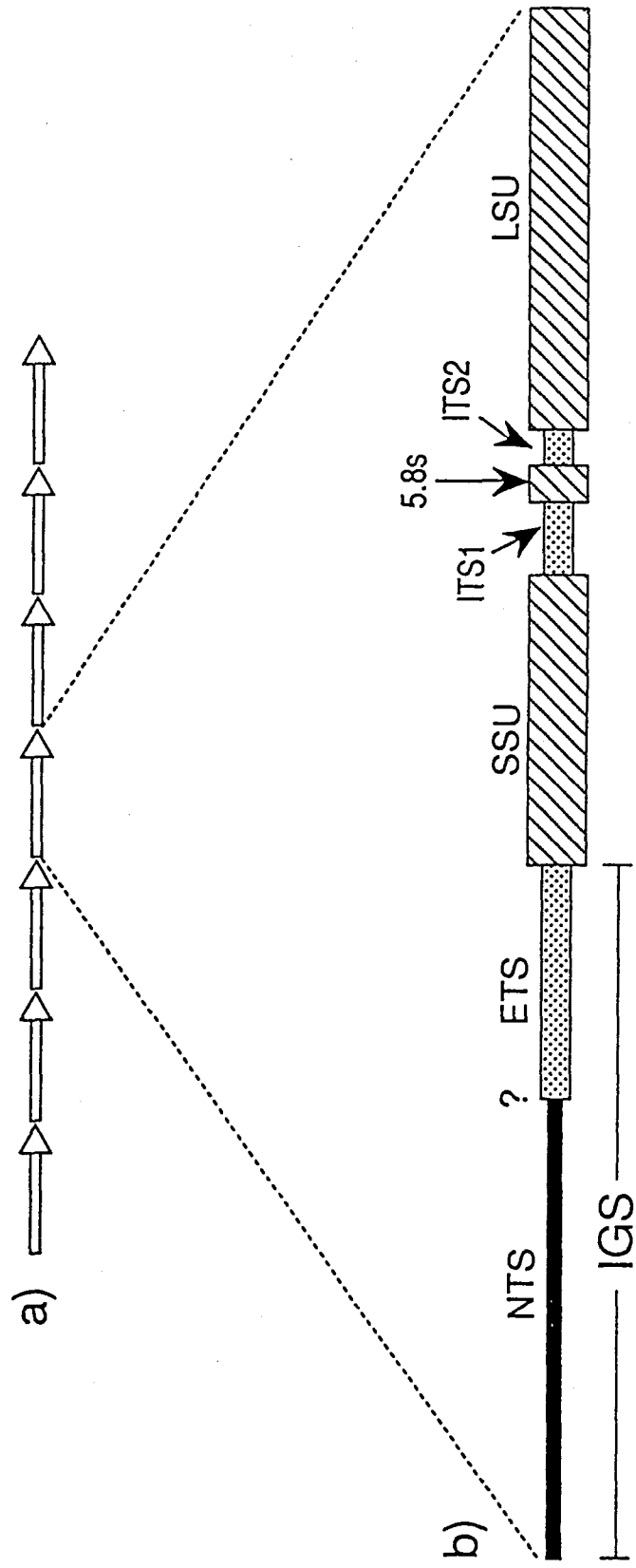


Fig. 2. Lessonia nigrescens Bory from Chile. This drawing was from dried rather than fresh samples.

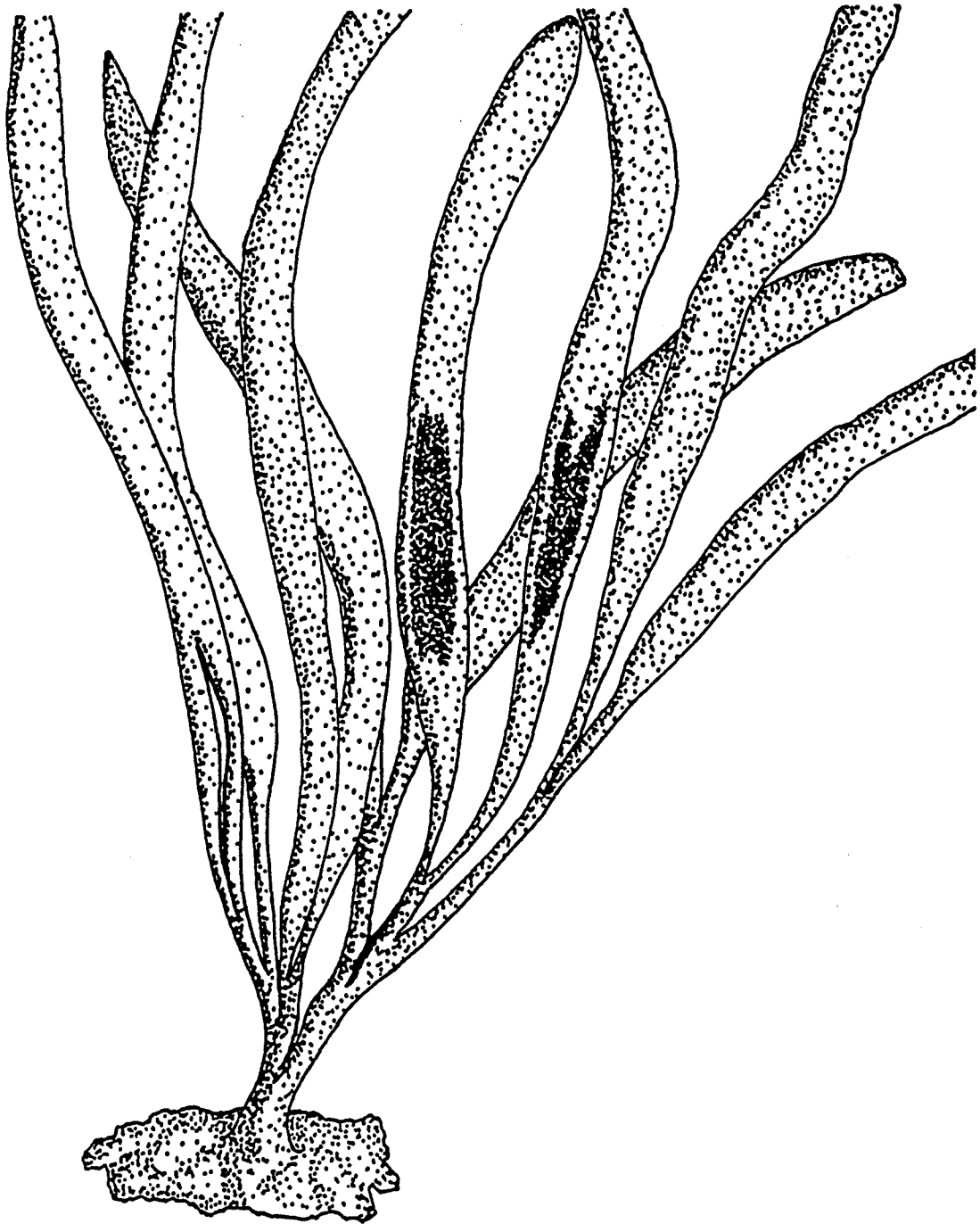


Fig. 2.

Fig. 3. Nereocystis leutkeana (Mertens) Postels & Ruprecht from Canada.

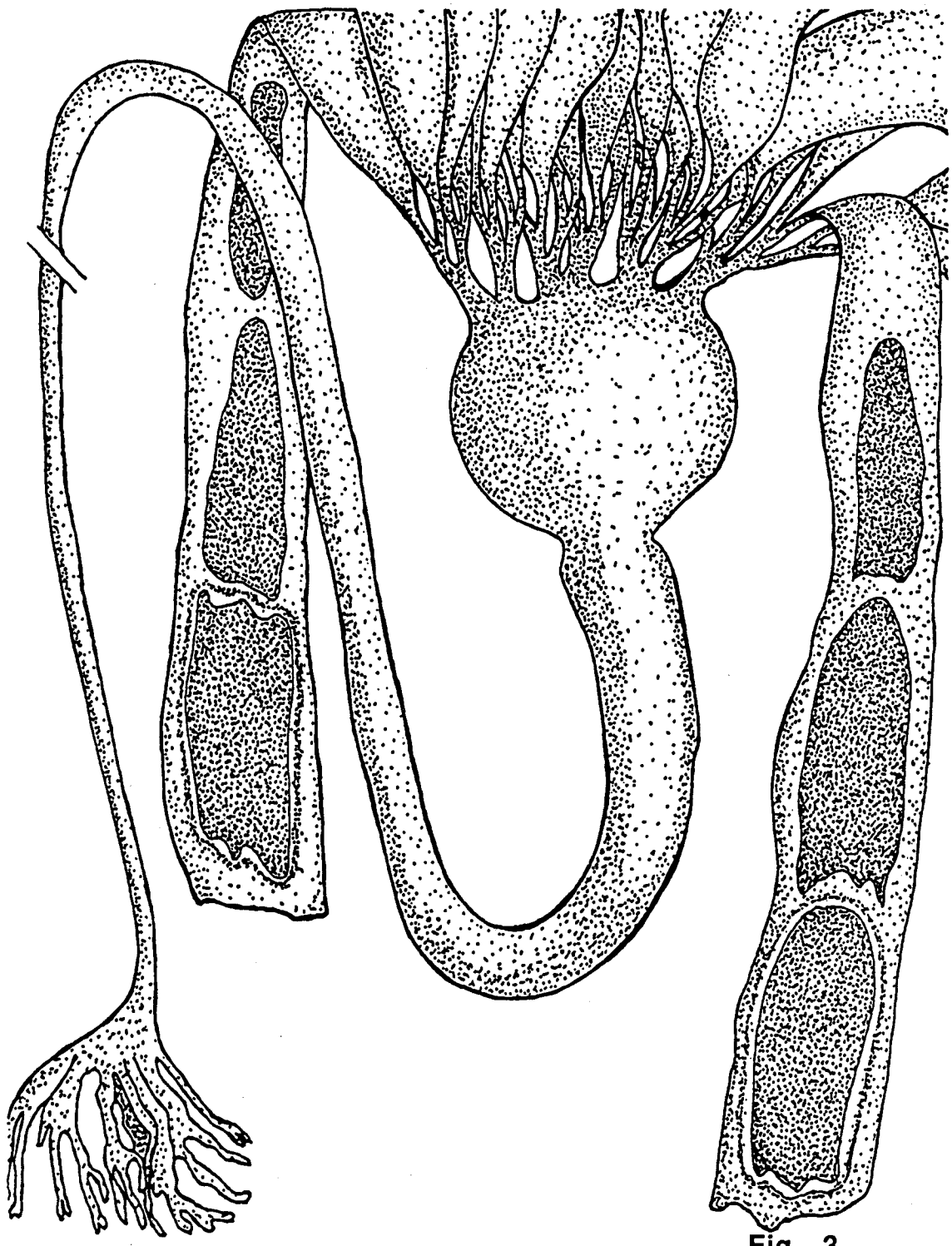


Fig. 3.

Fig. 4. Postelsia palmaeformis Ruprecht from Canada.

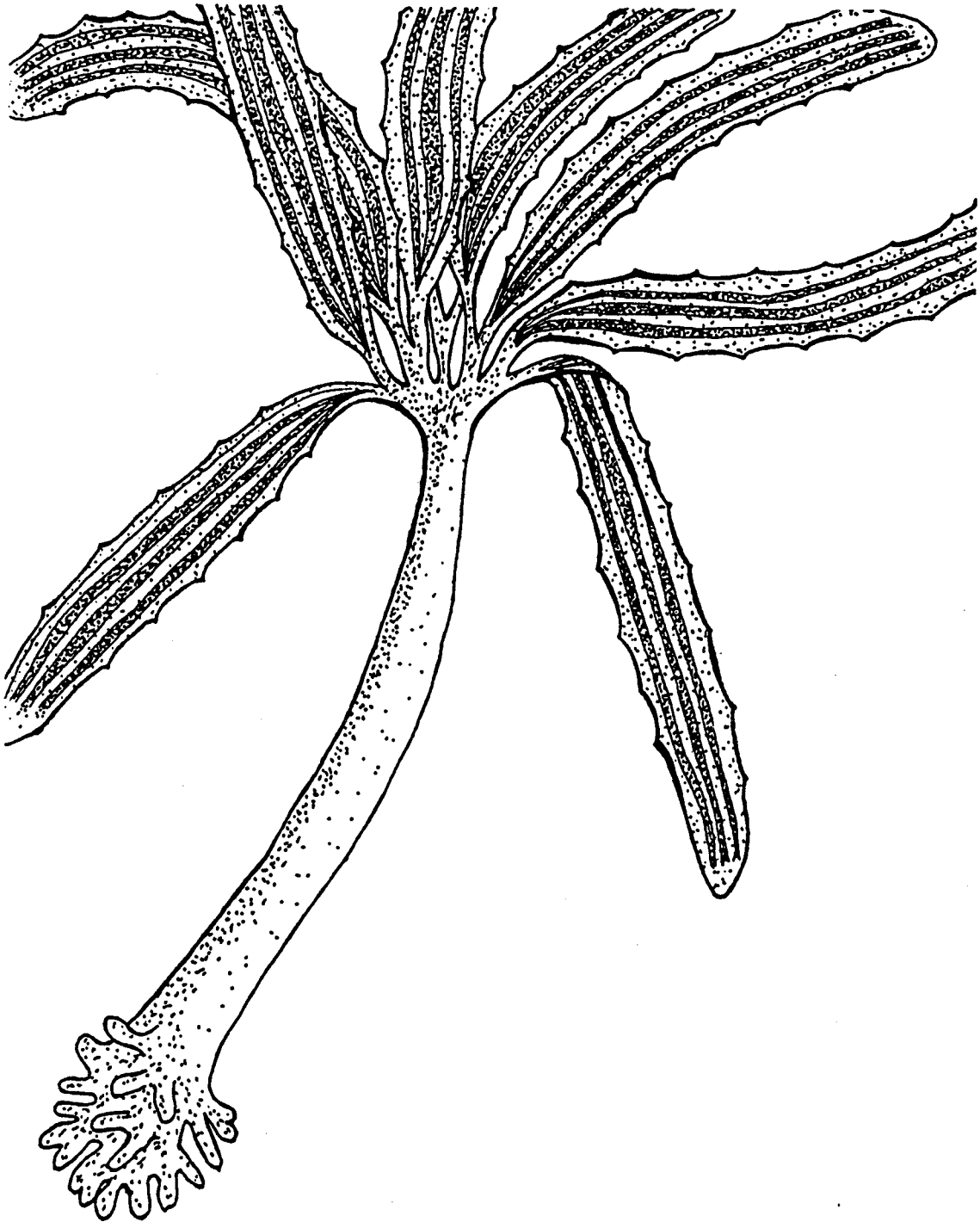


Fig. 4.

Fig. 5. Dictyoneurum californicum Ruprecht from the U.S.A.

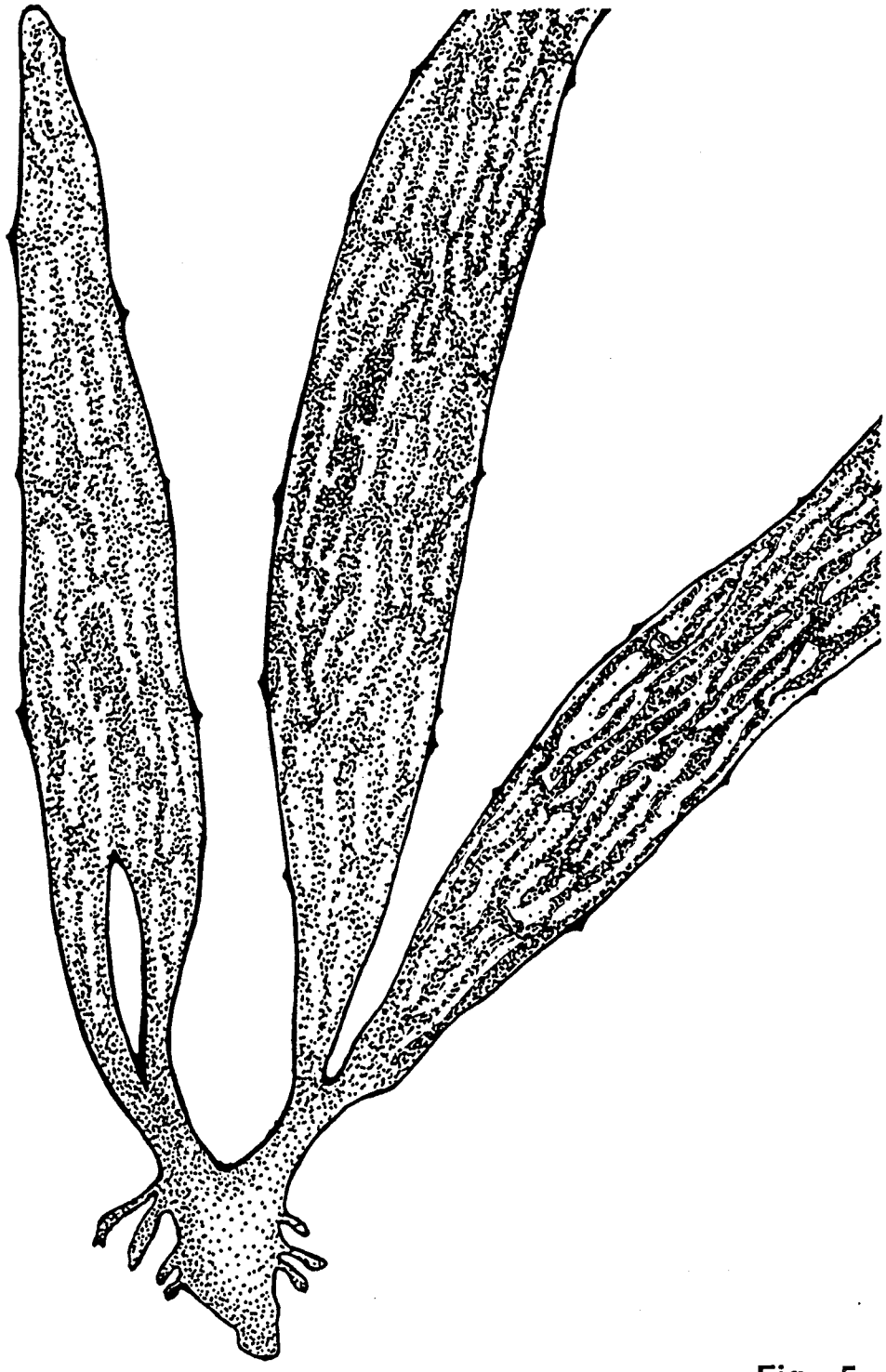


Fig. 5.

Fig. 6. Macrocystis integrifolia Bory from Canada.

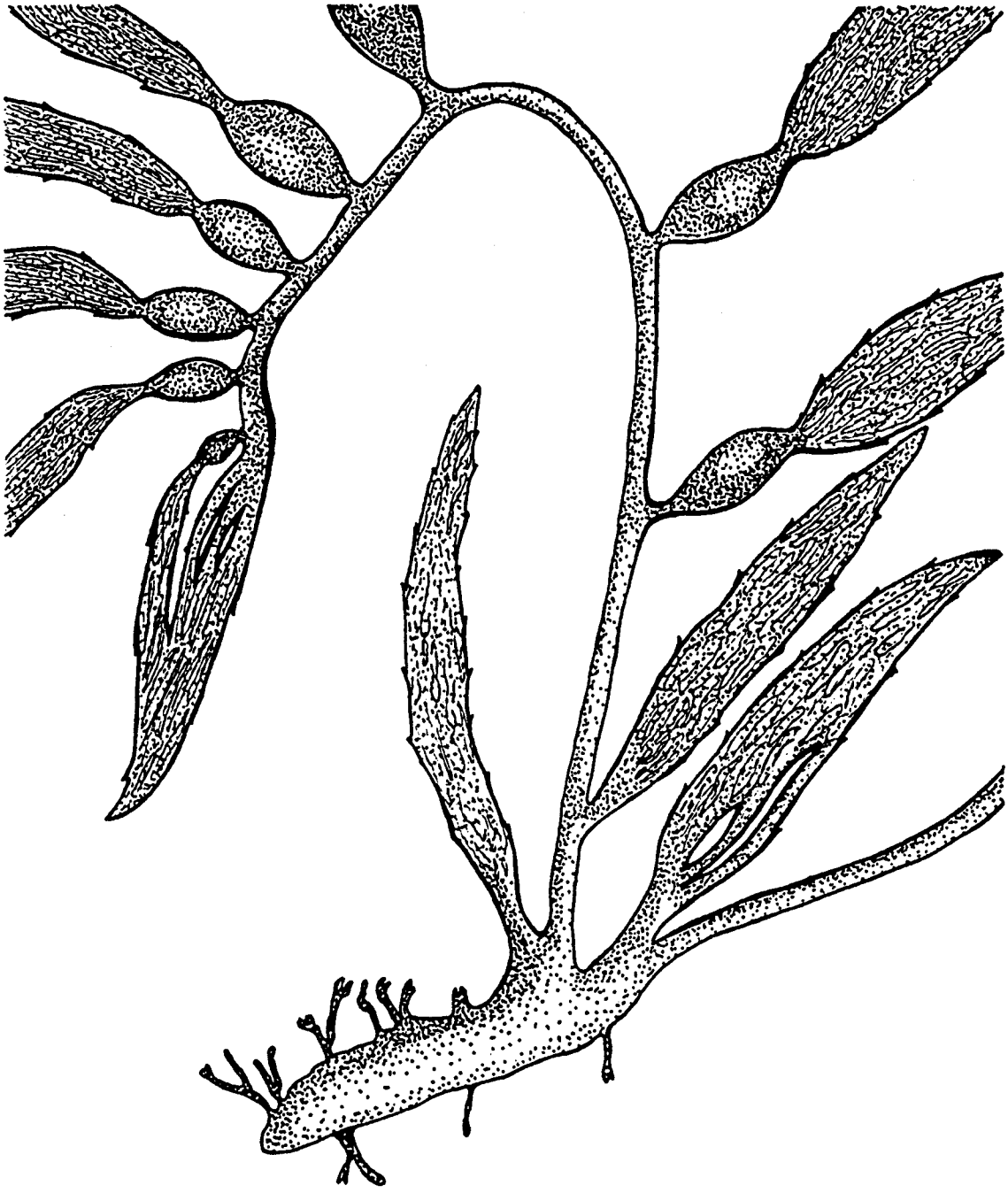


Fig. 6.

Fig. 7. Lessoniopsis littoralis (Tilden) Reinke from Canada.

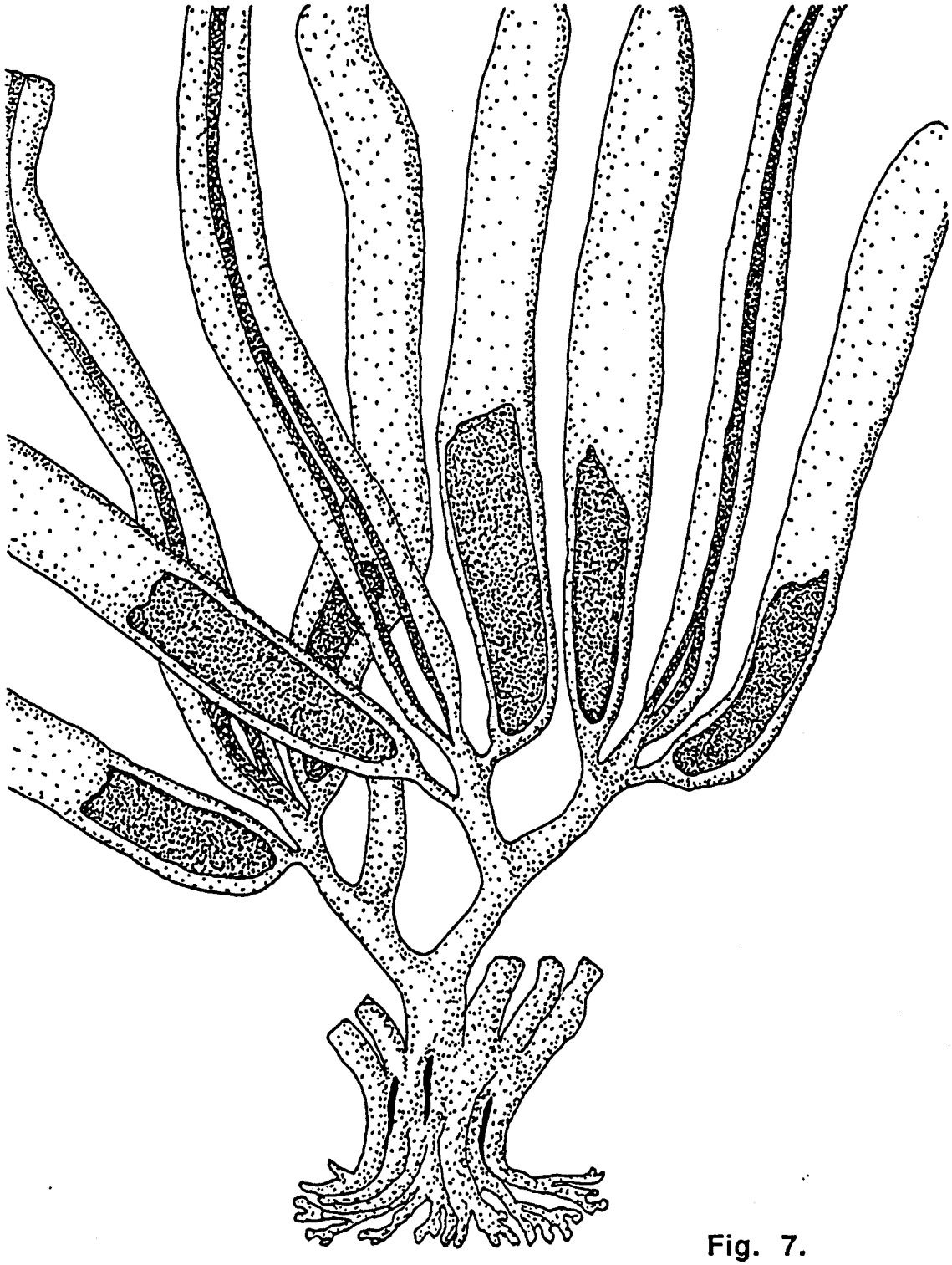


Fig. 7.

Fig. 8. Alaria marginata Postels & Ruprecht from Canada.

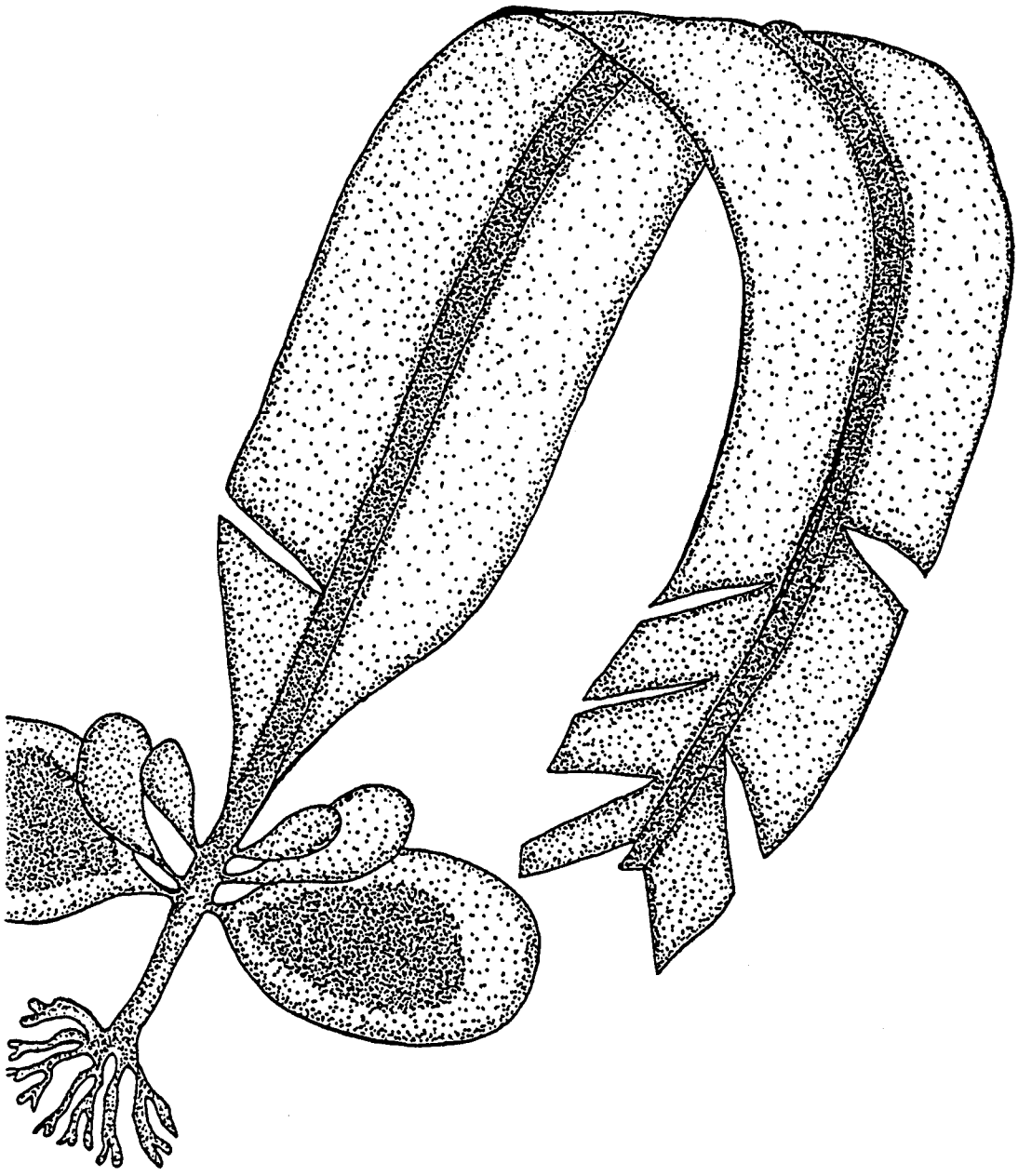


Fig. 8.

Fig. 9. Pterygophora californica Ruprecht from Canada.

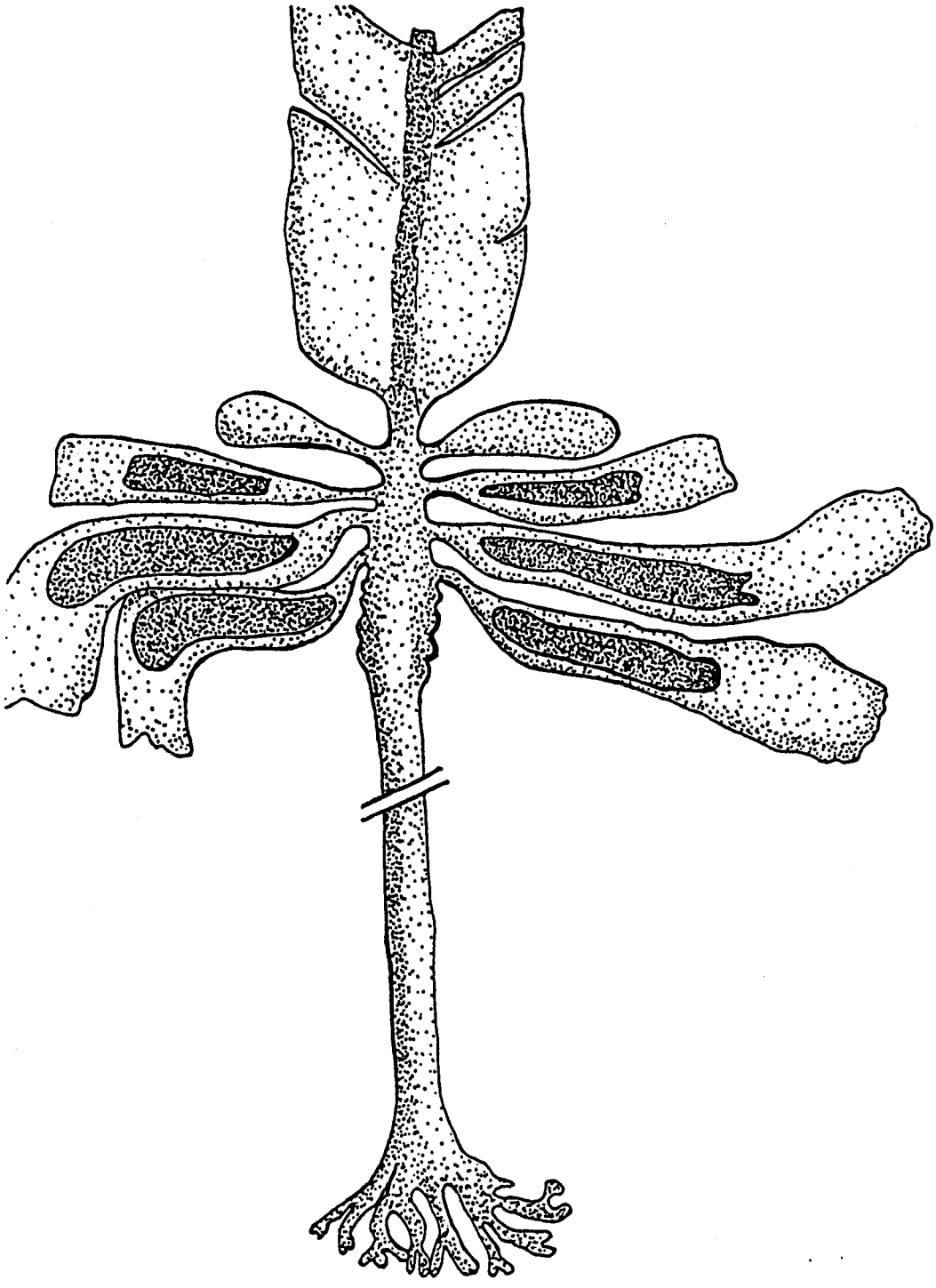


Fig. 9.

Fig. 10. Eisenia arborea Areschoug from Canada.

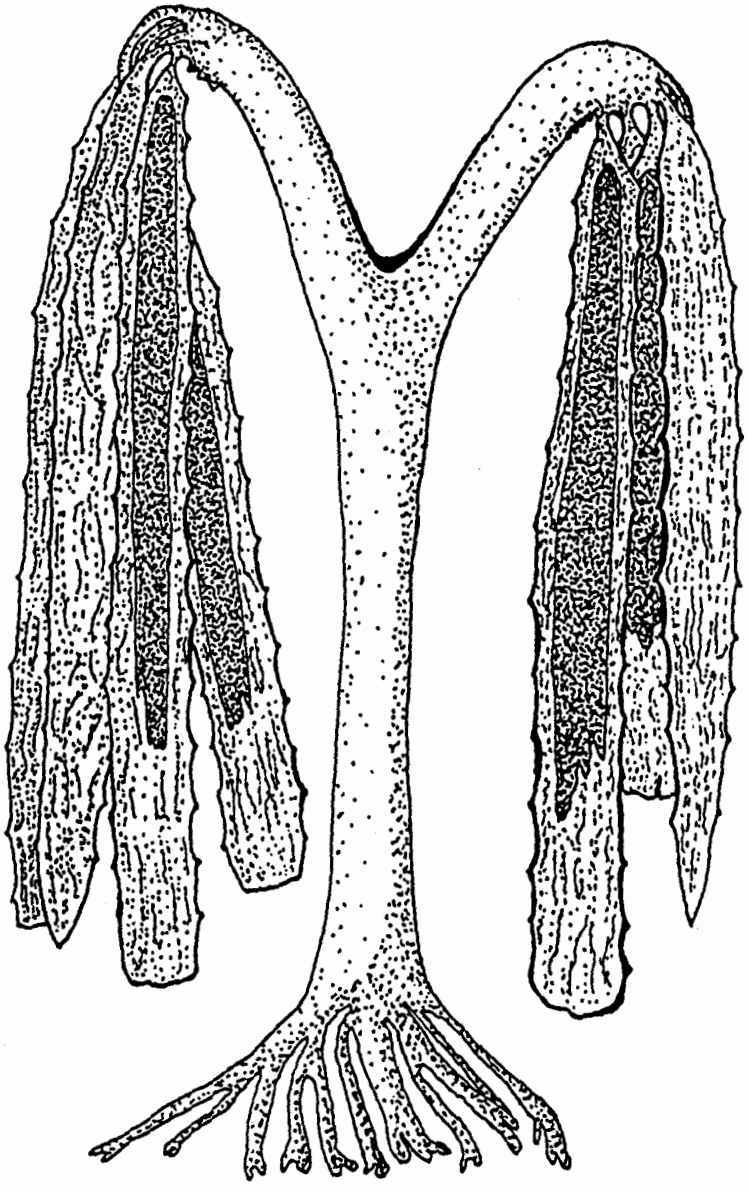


Fig. 10.

Fig. 11. Egregia menziesii (Turner) Areschoug from Canada.

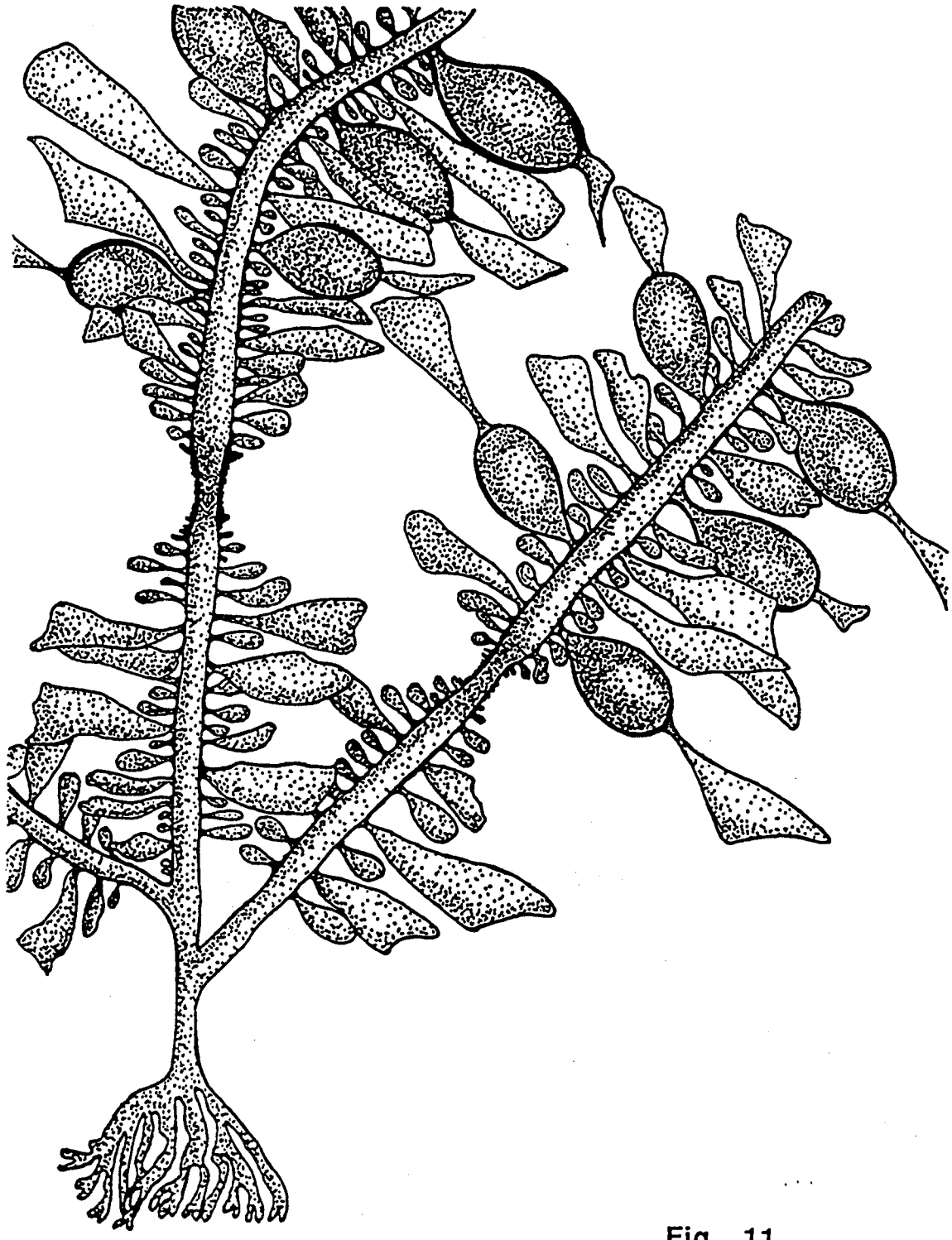


Fig. 11.

Fig. 12. Costaria costata (C. Agardh) Saunders from Canada.

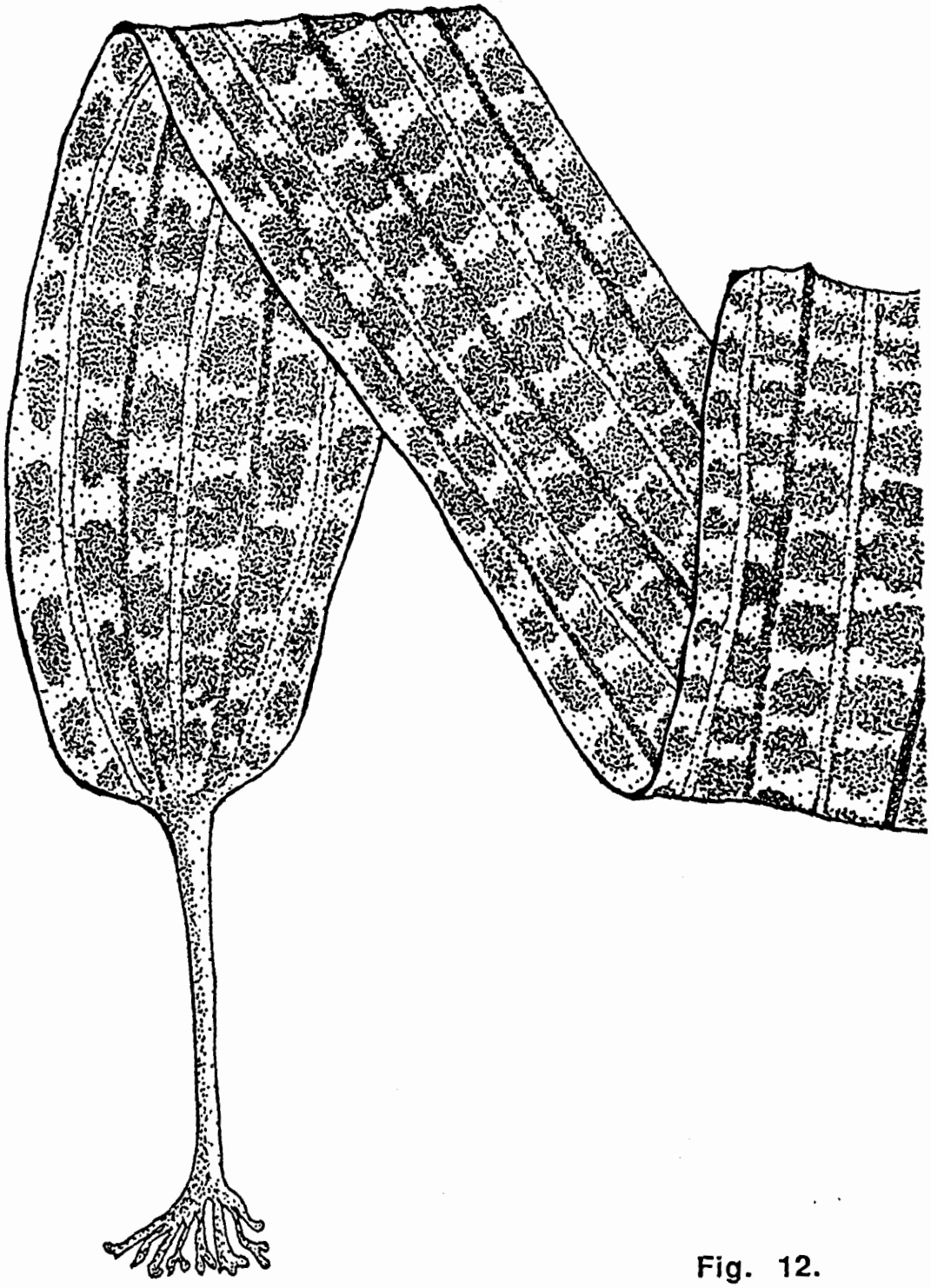


Fig. 12.

CHAPTER 2

RESTRICTION-ENZYME MAPPING OF THE NUCLEAR RIBOSOMAL CISTRON IN SELECTED LAMINARIALES (PHAEOPHYTA), A PHYLOGENETIC ASSESSMENT

Introduction

Classical taxonomy of the Laminariales separates the kelp into families on the basis of morphological features which result during development of the intercalary meristem at the stipe-blade transition (Setchell and Gardner 1925). My research was initiated to assess these traditional taxonomic divisions in view of recent molecular data on kelp chloroplast genomes (cpDNA). Specifically, restriction-fragment-length difference (RFLD) analysis of cpDNA led Fain et al. (1988) to propose that Nereocystis , morphologically in Lessoniaceae, had phylogenetic affinities with Laminaria Lamouroux, morphologically in Laminariaceae. Furthermore, they noted that Macrocystis and Lessoniopsis, both of Lessoniaceae, were more closely related to Alaria of Alariaceae than to Nereocystis. They concluded that Lessoniaceae was polyphyletic, and accordingly, they suggested that taxonomic systems based solely on morphological criteria may artificially define these taxa.

I have initiated investigations of the nuclear ribosomal cistron to determine if a nuclear-based molecular phylogeny would corroborate the chloroplast-derived phylogeny. This is important because chloroplast phylogenies trace matriarchal, not necessarily organismal, lineages owing to chloroplast introgression (see General Introduction). I have elected to study the nuclear ribosomal cistron because of its abundance in the genome and the ease with which it can be restriction-enzyme mapped (see General Introduction).

Restriction-enzyme mapping of the nuclear ribosomal cistron was completed for a variety of Laminariales. Taxa investigated were Alaria marginata Postels and Ruprecht, Egregia menziesii (Turner) Areschoug, Eisenia arborea Areschoug, Lessoniopsis littoralis

(Tilden) Reinke, Macrocystis integrifolia Bory, Nereocystis leutkeana (Mertens) Postels and Ruprecht, Postelsia palmaeformis Ruprecht and Pterygophora californica Ruprecht, with Sargassum muticum (Yendo) Fensholt (Fucales) as an outgroup. The restriction maps establish a foundation for future phylogenetic, as well as other molecular, investigations in the kelp. I also wanted to assess restriction-enzyme mapping of the nuclear ribosomal cistron for suitability in resolving intrafamilial and interfamilial taxonomic relationships in the Laminariales. Previously, for the kelp, restriction-enzyme mapping of the nuclear ribosomal DNA has been successfully employed to distinguish populations of the monotypic genus Costaria Greville (Bhattacharya et al. 1990a) and to define species in the morphologically plastic genera Alaria (Mroz 1989) and Laminaria (Bhattacharya and Druehl 1990, Bhattacharya et al. 1991). The intergenic spacer was too variable for phylogenetic comparisons at this level. Conversely, the gene regions were highly conserved with only three restriction-site differences observed among all the laminarialean taxa investigated.

Materials and Methods

Collection and DNA extraction

Seaweeds were collected from a variety of locations as summarized in Table 1. Algae were transported in plastic bags on ice to our laboratory, where plants were stored in a seawater tank (5^{o}C) or processed immediately. Blades were cleaned and nuclear DNA extracted as described by Fain et al. (1988) with the modifications provided in Bhattacharya and Druehl (1990).

Restriction digests and gel electrophoresis

One to two μg of nuclear DNA was digested with 10 to 20 units of one or more restriction endonucleases with six-base recognition sites, using the manufacturers', recommended procedures [Bethesda Research Laboratories (BRL), Boehinger Mannheim, Pharmacia]. BglII, ClaI, DraI, EcoRI, HindIII, PstI, SacI, SmaI, SphI and XbaI were the endonucleases employed. Digested DNA was size-fractionated by horizontal gel electrophoresis (0.7% agarose, 0.5 μg/mL ethidium bromide) at 19-24 v for 20-24 h in 1X TBE (Maniatis et al. 1982).

Southern transfer and hybridization

DNA was transferred unidirectionally, after limited acid hydrolysis, to nylon membrane (ZetaProbe) by an alkaline transfer method (Bio-Rad recommendations). After a 6-18 h transfer, filters were rinsed with 2X SSC (1X SSC= 15 mM NaCl and 1.5 mM sodium citrate), and washed in three consecutive rinses of 0.1X SSC and 0.1% SDS (sodium dodecyl sulfate) warmed to 42°C. The filters were blotted dry and stored in 5X SSPE, 5X BFP and 0.2% SDS (1X SSPE= 0.18 M NaCl, 10 mM sodium phosphate and 1 mM disodium EDTA, pH 7.0; 1X BFP= 0.02% w/v of each of bovine serum albumin, Ficoll 400000 and polyvinyl pyrrolidone) at 4°C. Filters were prehybridized at 65°C for 2-16 h. Hybridizations using probes pCc18 (clone from Costaria costata (C.A. Agardh) Saunders with most of the SSU and some upstream 5' sequence, Bhattacharya and Druehl 1988) and pCes370 (ribosomal repeat from the nematode Caenorhabiditis elegans) were from 6-20 h at 65°C in 5X SSPE, 0.2% SDS and 1X BFP. After hybridization, filters were washed: 10 min at 25°C in 1X SSC and 0.1% SDS followed by two 15 min washes at 65°C in 0.1X SSC and 0.1% SDS. Filters were blotted dry and sealed in plastic bags to

prevent desiccation. Probes were radiolabelled by a nick-translation procedure (Rigby et al. 1977), and unincorporated nucleotides were removed by Sephadex G-50 spin columns (Maniatis et al. 1982). Autoradiography was completed at -70°C for 16 h to 14 d with Kodak X-ray film.

Results

In this study, pCc18 was the main probe employed (Fig. 13). The repeat nature of the ribosomal cistron enables the mapping of restriction sites external to the sequence homologous to the probe. Problems arise when a restriction enzyme has two or more sites in the cistron outside the probe-homologous region. In this case any number of restriction sites could occur for an enzyme external to the sites that encompass the region homologous to the probe (Fig. 13, region C). When I encountered this situation and a conserved LSU site could not be elucidated for a taxon, I employed the probe pCes370 (Fig. 13) which is homologous to almost the entire ribosomal cistron in C. elegans. This probe is missing approximately 300 bp (base pairs) of the internal transcribed spacer (ITS) near the SSU. This probe acts effectively as a gene probe because the spacer regions are too divergent between C. elegans and kelp to allow hybridization. In contrast, the gene encoding regions are highly conserved among all eukaryotes. The use of these two probes also allowed me to position approximately the SSU and LSU onto my physical maps.

I employed a series of single and double digests with a variety of restriction endonucleases. Initially, seven enzymes were utilized for restriction mapping of the taxa. One such series of digests for six of the enzymes is presented for the alga Egregia menziesii (Fig. 14). Both pCc18 (Fig. 14a) and pCes370 (Fig. 14b) were probed against this series of digests to display my experimental approach. The fragments obtained for this series of digests as identified with the two probes are summarized in Table 2.

For Egregia, BglII, HindIII, PstI and XbaI all cut only once in the cistron giving a common band size of approximately 10.3 kb (kilobase pairs). The HindIII-PstI double digest (Fig. 14a, Table 2) allowed me to determine the distance between these two sites in the repeat unit. Additionally, because two bands were visible on the autoradiograph (Fig. 14a) when probed with pCc18 I knew that one of the two restriction sites was within the DNA region homologous to this probe. This process was continued until all of the fragments obtained in the digests were appropriately mapped. An example of the procedure of determining the physical map from the restriction-fragment data (Table 2) is provided for Sargassum muticum (Fig. 15). This same process was completed for all the algae investigated (fragment sizes summarized in Table 2, restriction maps in Figs 16 and 18).

In Egregia two extra DraI sites were inferred because the two DraI fragments summed up to a length of only 9.2 kb, falling about 1100 bp (base pairs) short of the expected cistron length (Table 2). One DraI site clearly maps in the SSU about 600 bp 3' from the conserved DraI site observed in all the kelp (Fig. 16). The other site was more difficult to map, but appears to be in the LSU because the large BglII-SmaI fragment encompassing the 3' end of the LSU and most of the IGS was only 100 bp shorter than the DraI-SmaI fragment instead of the expected 500 bp (Fig. 16). Similarly, the DraI fragments in Macrocystis added up to only 10.5 kb, falling about 500 bp short of the estimated cistron size. A BglII site in the spacer of Macrocystis precludes the same comparison used to confirm an additional DraI site in the LSU of Egregia. However, the large SmaI fragment (8.9 kb) when digested with DraI would be shortened 900 bp in the SSU and about 200 bp in the LSU, yielding a 7.8 kb DraI-SmaI fragment. The actual fragment observed was estimated at 7.3 kb, suggesting that an additional site about 500 bp 3' to the DraI site conserved in all the taxa investigated also occurs in Macrocystis. I considered 500 bp as the lower limit when comparing fragment estimates from different gels. Hence, Egregia

and Macrocystis appeared to share a common DraI site in the LSU not found among the other taxa investigated.

Restriction maps for Egregia, Macrocystis, Nereocystis and Alaria are provided (Fig. 16). I was unable to find restriction-site differences among Alaria, Eisenia and Pterygophora in the gene coding regions. I therefore present a complete cistron-restriction map only for Alaria (Fig. 16). Similarly, the invariant group of Nereocystis, Postelsia and Lessoniopsis (gene coding region) is represented by the Nereocystis restriction map (Fig. 16). This latter group is indistinguishable from Laminaria (Laminariaceae) on the basis of previously published results (Bhattacharya and Druehl 1990). Furthermore, Alaria (representing the Alariaceae) is divergent by only one restriction site (SmaI, LSU) from Nereocystis (representing the Laminariaceae/Lessoniaceae). Only when the outgroup Sargassum (Fig. 15) is compared to the Laminariales (Fig. 16) is extensive divergence evident. The alga Egregia menziesii displays divergence from other members of the Alariaceae, and in fact all of the taxa considered, as does Macrocystis, but to a lesser extent (Fig. 16).

I extended my analysis to ten restriction enzymes for a comparison of Lessoniopsis (Table 3) and Laminaria (Bhattacharya and Druehl 1990, Bhattacharya unpubl.). I was unable to locate a divergent site between these two taxa within the gene coding regions (Fig. 17). A single SphI site, within the kelp conserved EcoRI 5' to the start of the SSU, in Lessoniopsis was the only site that might prove taxonomically useful between these two genera.

In contrast to the gene coding regions of the ribosomal cistron, the IGS of the taxa are too divergent to be of value in relating taxa within and between the Alariaceae (Fig. 18a) and Laminariaceae/Lessoniaceae (Fig. 18b). An exception occurs when comparing Nereocystis and Postelsia, whose spacers are practically identical (Fig. 18b). The only observed difference between these two taxa is the presence of an additional PstI site in the

IGS of Nereocystis. DNAs from five plants each of Postelsia and Nereocystis were digested with the restriction endonucleases PstI and DraI. Plants of Nereocystis were collected from an exposed and a sheltered stand on the east side of San Juan I. The Postelsia was taken from two populations on Vancouver I (Cape Beale and Botanical Beach). The fragments observed from these digests (Table 4) support the restriction maps presented for these two taxa (Fig. 18b). All ten plants shared two common DraI sites, a common PstI site, and each had a genus specific second PstI fragment which accounted for the restriction-map difference.

Conservatively, I detected 38 different restriction sites for phylogenetic analysis; however, I was unable to score homologous sites within the IGS with certainty. This would limit a phylogenetic analysis to 15 sites from the EcoRI site conserved in all kelp 5' to the SSU through the gene encoding region to the HindIII site near the 3' end of the LSU in Sargassum (Fig. 15) inclusive. The limited restriction-site differences available are insufficient for phylogenetic analysis.

Discussion

This research was initiated to provide a foundation for future phylogenetic investigations within the Laminariales in light of recent chloroplast data provided by Fain et al. (1988). My restriction maps will facilitate the cloning of appropriate regions of rDNA for subsequent dideoxy sequence analysis in continued phylogenetic investigation. I also wanted to assess restriction-enzyme mapping of the nuclear ribosomal cistron for investigating intrafamilial and interfamilial relationships among the kelp. In previous taxonomic investigations at the kelp population (Bhattacharya et al. 1990a) and species (Bhattacharya and Druehl 1990, Mroz 1989) levels, restriction mapping of the nuclear ribosomal cistron proved valuable, particularly the IGS. In this study I found that the

spacer was too variable for use at the intrafamilial and interfamilial levels. This is an expected result because IGS sequence differs even between closely related species (Rogers and Bendich 1987). I am not yet certain what interpretation this observation will lead me to in view of the Nereocystis-Postelsia data. The single IGS restriction site distinguishing Nereocystis and Postelsia, is equivalent to divergence noted previously among species in the genus Laminaria (Bhattacharya and Druehl 1990) and species within Alaria (Mroz 1989), and between populations of Costaria (Bhattacharya et al. 1990a).

Restriction mapping of the gene coding regions also failed to resolve taxa within the Laminariales. In this case the maps of all the laminarialean taxa were too conserved. I tried to distinguish Lessoniopsis and Laminaria by digesting with three additional enzymes (Fig. 17), yielding 11 more restriction sites in Lessoniopsis, six of which were in the gene coding region considered potentially, taxonomically valuable in this study; however, when these additional sites were compared with published Laminaria maps, only one useful polymorphism was resolved. This site is actually slightly upstream of the SSU and not in a gene encoding region of the cistron (Chapter 3). Hence, I was unable to resolve phylogenies between the various genera. Even the families that are traditionally recognized within the Laminariales are separated by as few as one restriction site (LSU) change for the Alariaceae and Laminariaceae. My data demonstrate the absence of substantial genetic diversity among morphologically distinct families and genera of the Laminariales. In a similar study, DNA from morphologically defined stands of Zostera marina Linnaeus, displayed restriction-site divergence in the gene coding regions of the ribosomal cistron (Fain et al. 1991), equivalent to that noted in my interfamilial comparisons for the kelp. This suggests to me that the Laminariales may be a recently evolved group despite their considerable morphological diversity.

Table 1. Species investigated and collection sites.

SPECIES COLLECTED	LOCATION
FUCALES	
Sargassum muticum	Dixon Island, Barkley Sound, Canada
LAMINARIALES	
Alariaceae	
Alaria marginata	Kirby Point, Barkley Sound, Canada
Egregia menziesii	Dixon Island, Barkley Sound, Canada
Eisenia arborea	Kirby Point, Barkley Sound, Canada
Pterygophora californica	Cape Beale, Barkley Sound, Canada
Laminariaceae	
Costaria costata	Cape Beale, Barkley Sound, Canada
Lessoniaceae	
Dictyoneuropsis reticulata	Agassiz Beach, California, U.S.A.
Dictyoneurum californicum	DNA prep. S. R. Fain. California, U.S.A.
Lessonia nigrescens	Las Cruces, Chile.
Lessoniopsis littoralis	Kirby Point, Barkley Sound, Canada
Macrocystis integrifolia	Cape Beale, Barkley Sound, Canada
Nereocystis leutkeana	Turn Rock & Garbage Point, San Juan Island, USA. Cape Beale, Barkley Sound, Canada
Postelsia palmaeformis	Cape Beale, Barkley Sound, and Botanical Beach, Port Renfrew, Canada

Table 2. Summary of fragment lengths (kb) for single and double digests of the algae investigated in this study.

	Sa	Al	Eg	Ei†	Le	Ma	Ne	Po	Pt
X	12.2	7.1	10.3	9.4	10.0	11.0	8.9	9.2	9.2
		2.6							
X P	6.0	6.0	10.0	9.3	7.6	9.4	6.0	7.9	8.6
	0.5	1.6			2.6	1.2	0.3		
P	6.0	7.4	10.3	9.4	7.6	9.4	6.0	7.9	8.6
	1.9	2.0			2.6	1.6	1.5	1.4	2.4
P H	4.9	2.1	7.8	7.2	5.0*	2.4	2.2	2.0	2.4
	1.9	2.0	2.3	2.6	2.6	0.8	1.5	1.4	1.6
	1.1*				2.3				
H	12.2	6.0	10.3	9.5	10.0	3.6	8.9	9.2	4.2
H D	6.0	3.6	6.8	6.4	7.3	2.5	2.4	2.2	2.5
	2.6*	2.2	1.8	2.8	2.6	0.7	1.1	1.1	1.5
	1.4*								
D	6.0	6.3	6.8	6.4	7.3	7.4	3.0	3.0	7.8
	4.0*	2.7	2.4	3.4	3.1	3.1	1.1	1.1	3.2
D P	3.4	2.6	6.8	6.4	4.4*	3.1	2.9	2.7	3.1
	2.4*	2.0	2.4	3.3	3.0	1.5	1.1	1.1	2.3
	1.9				2.5				
D S	4.0*	6.3	4.1*	6.4	4.5*	7.3	1.1	1.1	2.2
	2.4	0.9	1.4	2.0	2.2	0.9	0.9	0.8	
	2.3*				0.9				
	1.2								

S	8.5	8.0	6.5*	7.8	6.7*	8.9	8.9	9.2	3.2
	2.3*		2.4	2.0	3.3				
	1.2								
S P	2.6*	2.0	6.5*	6.9	6.6*	1.6	1.5	1.6	2.3
	2.0	0.8	1.5	2.0	2.3	0.8	0.8	0.7	
	2.3*		0.9		0.8				
	1.2								
S B	2.8*	6.9	4.0*	7.2	3.7*	4.5	6.6	7.0	3.2
	1.5		2.4	2.0	3.3				
	2.3*								
	1.2								
	0.8*								
B	5.7	9.2	10.3	9.5	6.4	7.4	8.9	9.2	6.5
	2.8*				3.6*				
B P	4.1	3.0	6.9	6.3	3.6*	3.6	3.4	3.2	3.6
	2.0*	2.0	3.4	3.7	3.4	1.6	1.7	1.4	2.3
	1.5				2.6				
E	5.0	1.9	1.9	1.9	1.9	2.6	1.9	1.9	1.9
E P	1.9	1.1	1.1	1.1	1.1	1.8	1.1	1.1	1.1
	1.1	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8

Abbreviations for restriction enzymes; X= XbaI, P= PstI, H= HindIII, D= DraI, S= SmaI, B= BglII, E= EcoRI. Abbreviations for taxa; Sa= Sargassum, Al= Alaria, Eg= Egregia, Ei= Eisenia, Le= Lessoniopsis, Ma= Macrocystis, Ne= Nereocystis, Po= Postelsia, Pt= Pterygophora. * additional fragments observed with pCes370 but not pCc18. † Eisenia was probed with pCes370 only.

Table 3. Summary of fragment lengths (kb) for extended Lessoniopsis digests.

L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14
2.9	2.9	6.6	6.6		4.4	4.4	4.4	4.2	5.1	5.1	2.5	3.6	2.9
1.9	1.1	2.2	2.2		1.4	1.4		1.2	2.0	1.7	1.7	1.7	1.7
	0.8										1.2		

L1= EcoRI, L2= EcoRI and PstI, L3= PstI, L4= PstI and SalI, L5= SalI, L6= SalI and SphI, L7= SphI, L8= PstI and SphI, L9= SacI and SphI, L10= SacI, L11= SacI and PstI, L12= SacI and ClaI, L13= ClaI, L14= ClaI and PstI.

Table 4. Summary of restriction fragments observed (* = presence) in PstI and DraI restriction digests of DNA from Postelsia (Botanical Beach, BB. Cape Beale, CB) and Nereocystis (sheltered, GP. exposed, TR).

Restriction	Postelsia					Nereocystis				
Fragment	BB	BB	BB	BB	CB	GP	GP	GP	TR	TR
PstI 7.9 kb	*	*	*	*	*					
PstI 6.0 kb						*	*	*	*	*
DraI 3.0 kb	*	*	*	*	*	*	*	*	*	*
PstI 1.5 kb	*	*	*	*	*	*	*	*	*	*
DraI 1.1 kb	*	*	*	*	*	*	*	*	*	*

Fig. 13. Schematic of a ribosomal repeat. Below: regions homologous to inserts of pCc18 and pCes370 are indicated Above: Hypothetical DraI sites. Fragments A and B (shaded) would be observed in a DraI genomic digest probed with pCc18. This probe could not detect nor would it allow the mapping of any DraI sites in region C. pCes370 would hybridize to fragments A and B as well as region C allowing DraI sites in the LSU to be mapped.

Fig.13.

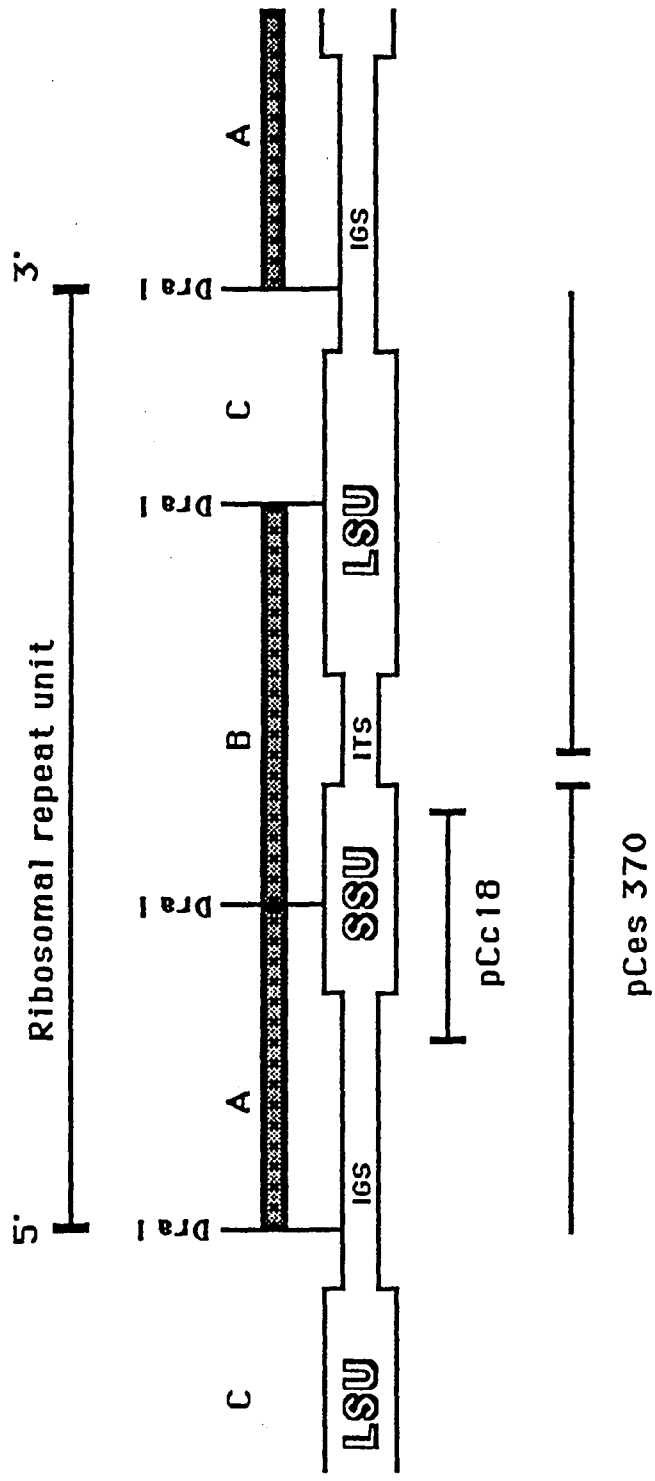

5'
3'
Ribosomal repeat unit
Dra I
Dra I
Dra I
Dra I
A
B
C
IGS
ITS
SSU
LSU
LSU
IGS
A
C
pCc18
pCes 370

Fig. 14. Autoradiographs of a series of digests of Egregia genomic DNA. Digests; Lane 1= XbaI, Lane 2= XbaI and PstI, Lane 3= PstI, Lane 4= PstI and HindIII, Lane 5= HindIII, Lane 6= HindIII and DraI, Lane 7= DraI, Lane 8= DraI and PstI, Lane 9= DraI and SmaI, Lane 10= SmaI, Lane 11= SmaI and PstI, Lane 12= SmaI and BglII, Lane 13= BglII, Lane 14= BglII and PstI. Values on the left indicate the sizes in kb of DNA fragments. A) Digest probed with pCc18. B) Digest probed with pCes370. Arrows indicate additional fragments homologous to this probe.

Fig. 14.

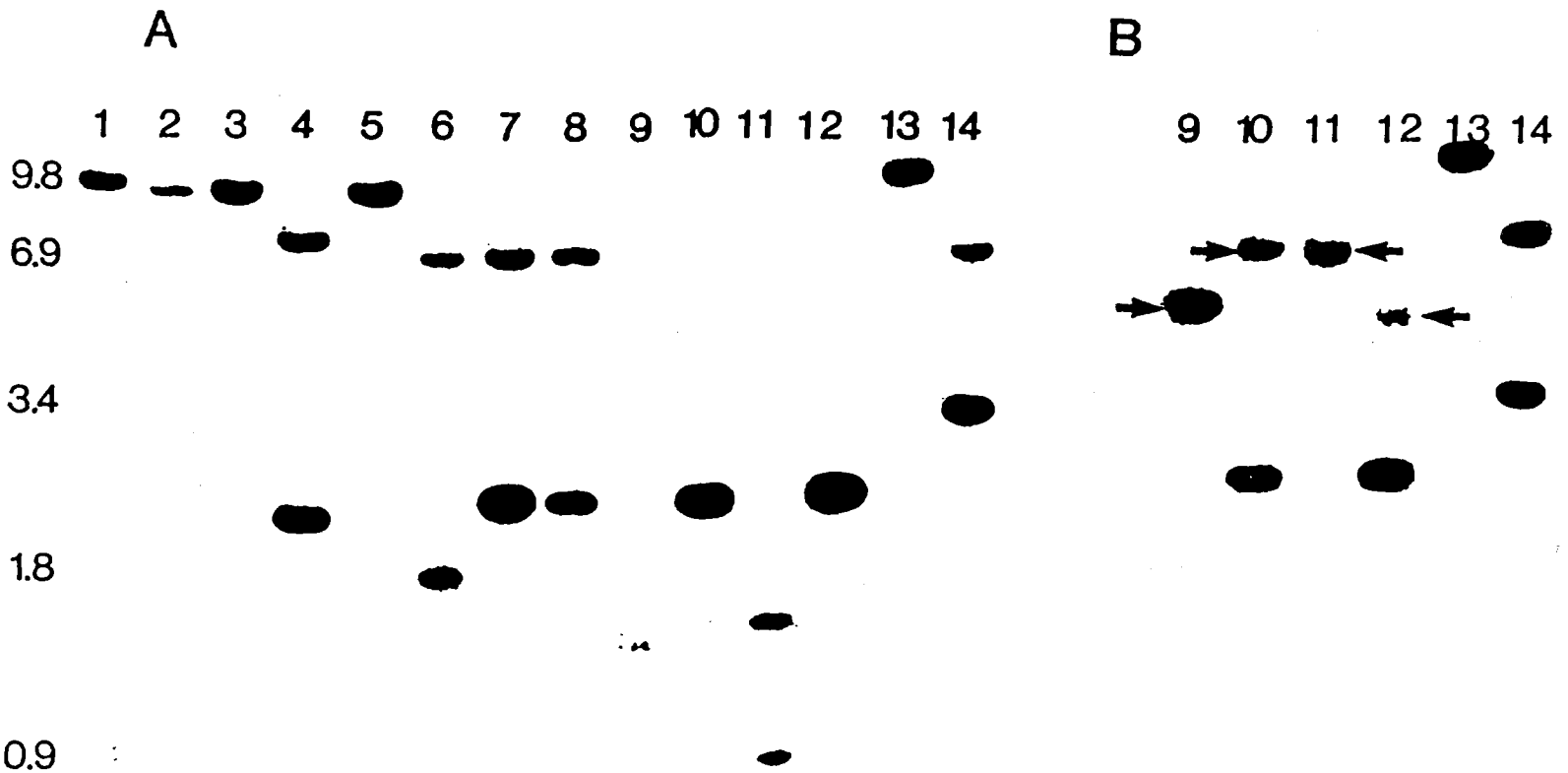


Fig. 15. Restriction map of ribosomal cistron for Sargassum as deduced from fragment lengths recorded in Table 2. Below: Fragments from each digest aligned with physical map (horizontal lines) with cut sites displayed (vertical lines). Solid lines represent fragments detected with pCc18 whereas dashed lines are additional fragments observed with pCes370. Blank regions are regions not detected by the probes for that digest. Fragment sizes on the lines are in kb whereas 'p' represents a partial fragment continuous with adjacent repeat units. The EcoRI and EcoRI- PstI digests were probed with pCc18 only.

Fig.15.

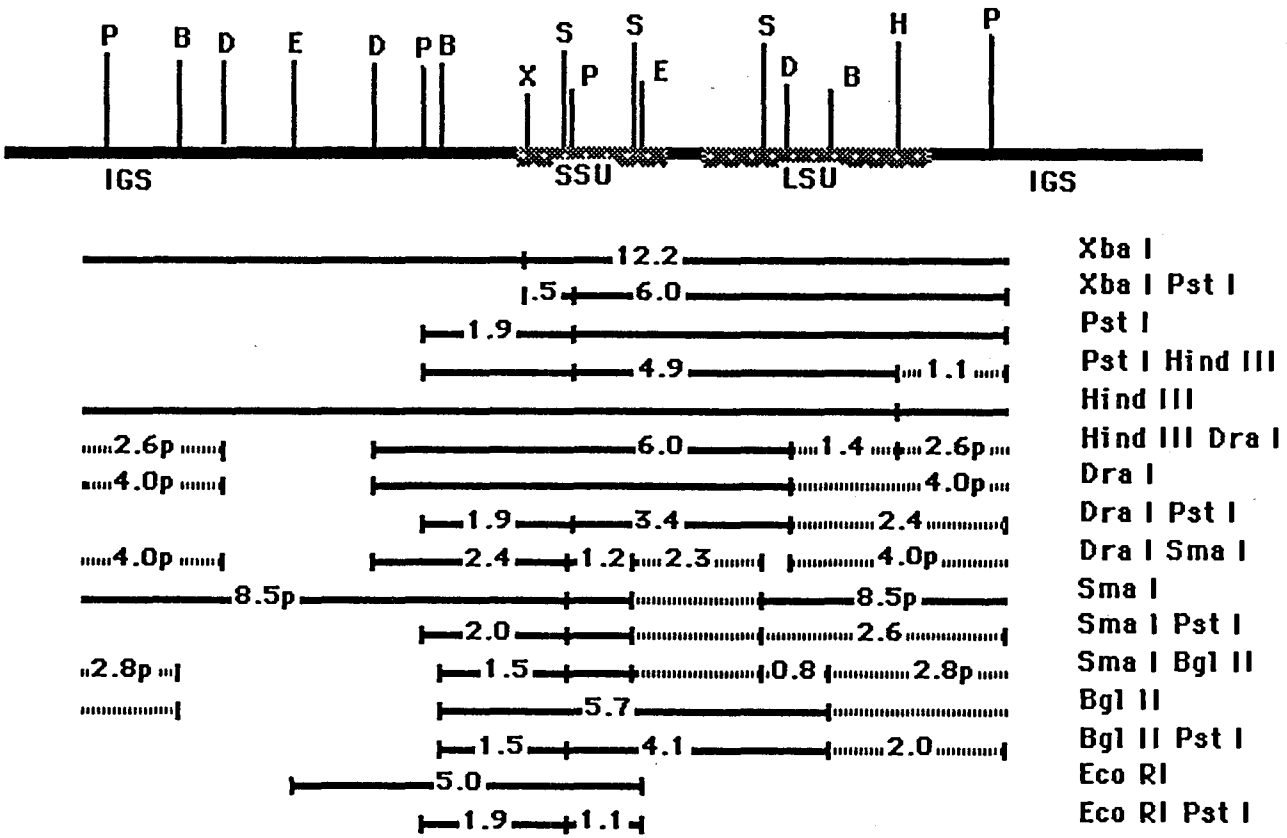

P B D E D P B X S P S E S D B H P
IGS SSU LSU IGS
12.2
1.5 6.0
1.9
4.9 1.1
2.6p 6.0 1.4 2.6p
4.0p 4.0p
1.9 3.4 2.4
4.0p 2.4 1.2 2.3 4.0p
8.5p 8.5p
2.0 2.6
2.8p 1.5 0.8 2.8p
5.7
1.5 4.1 2.0
5.0
1.9 1.1
Xba I
Xba I Pst I
Pst I
Pst I Hind III
Hind III
Hind III Dra I
Dra I
Dra I Pst I
Dra I Sma I
Sma I
Sma I Pst I
Sma I Bgl II
Bgl II
Bgl II Pst I
Eco RI
Eco RI Pst I

Fig. 16. Restriction maps for the algae Egregia, Macrocystis, Nereocystis and Alaria deduced from the data in Table 2. Letter abbreviations presented for restriction enzymes correspond with Table 2.

Fig. 16.

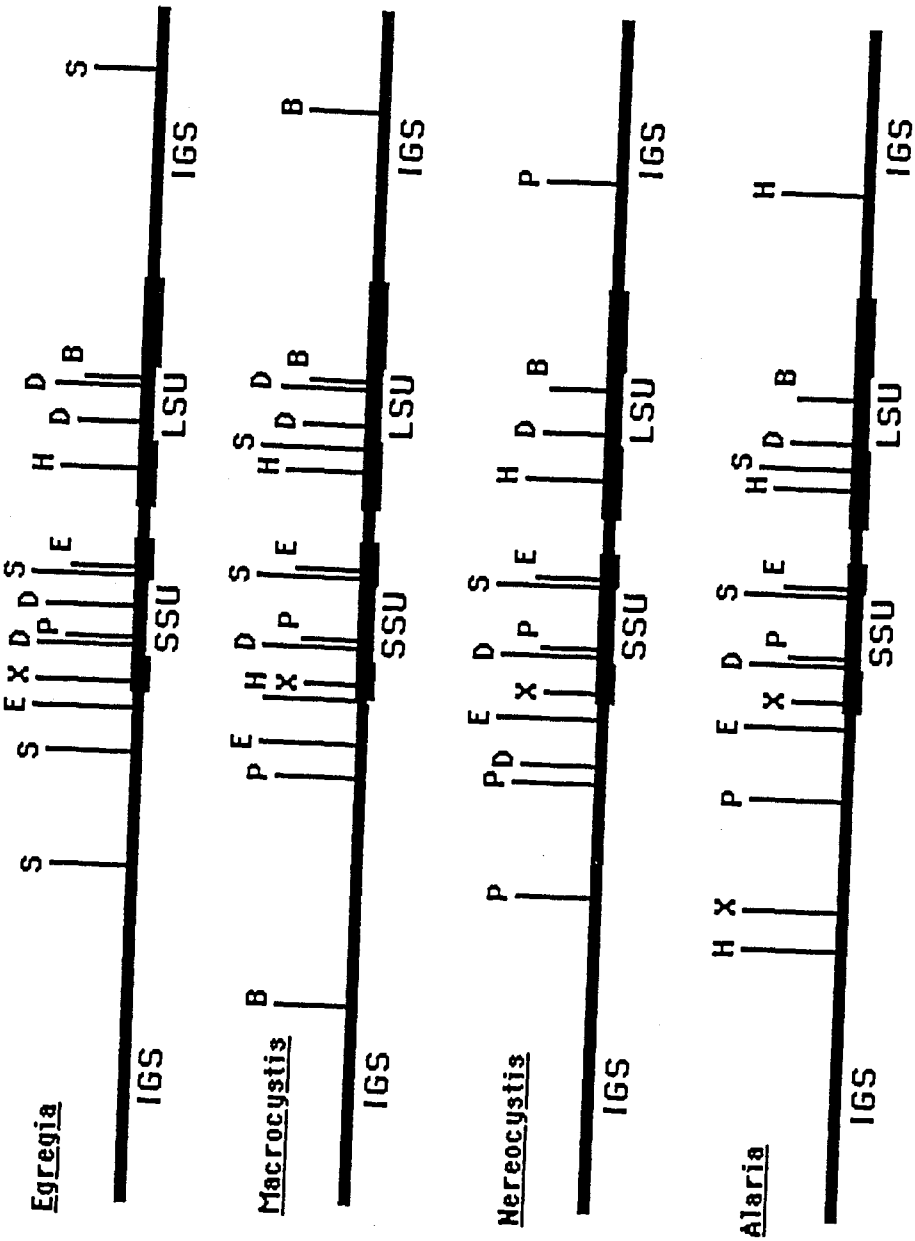
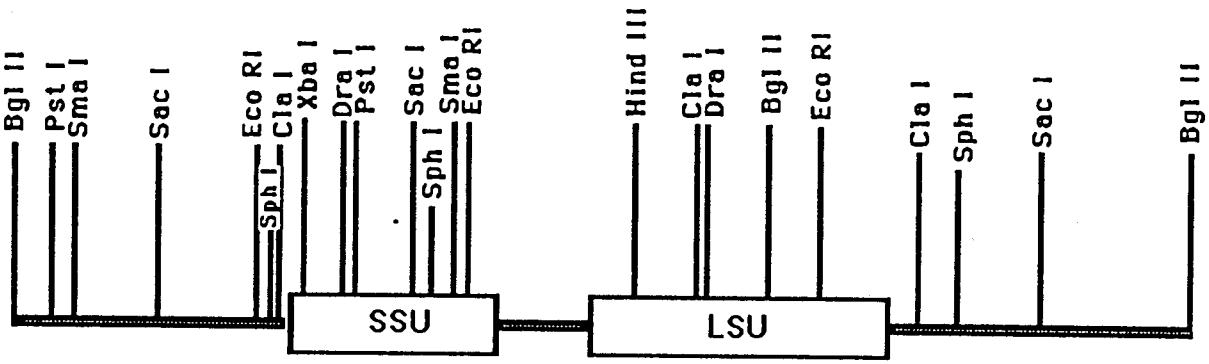
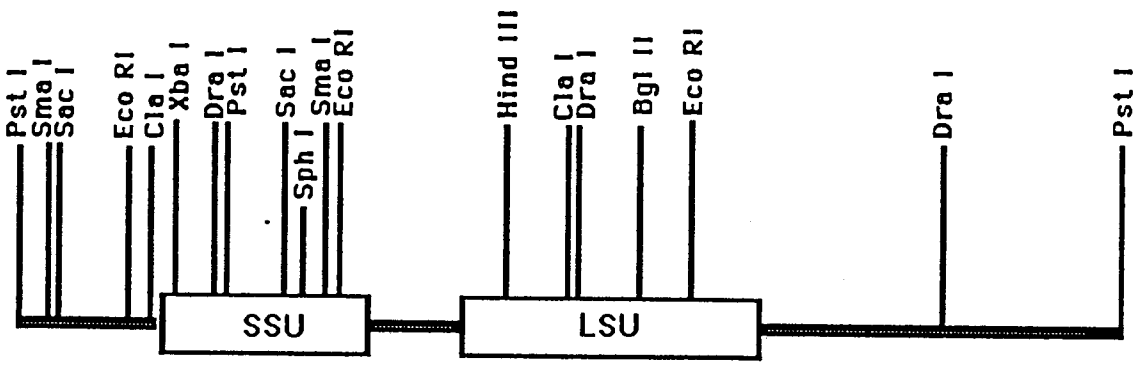

Egregia
Macrocystis
Nereocystis
Alaria
IGS
SSU
LSU
S
E
X
D
P
H
B
2 kb

Fig. 17. Restriction maps to ten enzymes for the algae Lessoniopsis and Laminaria agardhii Kjellman (after Bhattacharya & Druehl 1990, Bhattacharya unpubl.). Additional enzymes include ClaI, SacI and SphI.

Fig. 17.



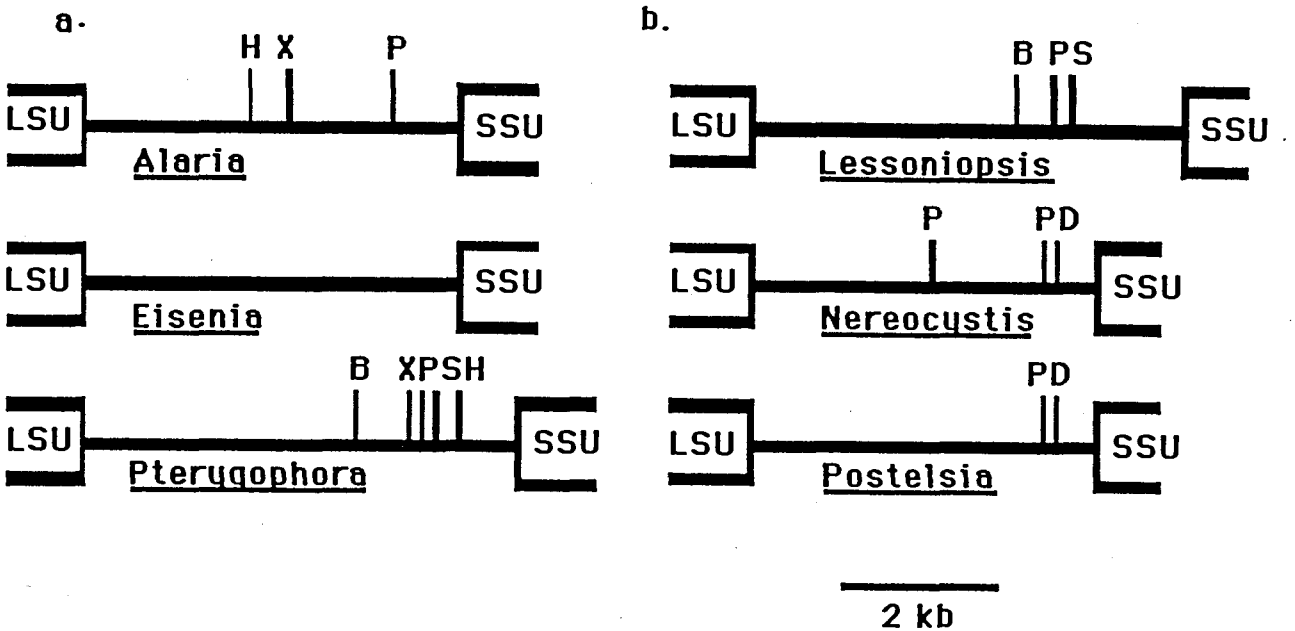
Lessoniopsis



Laminaria

Fig. 18. Restriction maps for the IGS regions of selected algae. a) Algae belonging in the Alariaceae including Alaria, Pterygophora and Eisenia. b) Algae belonging provisionally in the Laminariaceae/Lessoniaceae including Lessoniopsis, Nereocystis and Postelsia. Letter abbreviations used for restriction enzymes correspond with Table 2.

Fig. 18.


a.
H X
P
LSU
SSU
Alaria
LSU
SSU
Eisenia
B XPSH
LSU
SSU
Pterygophora
b.
B PS
LSU
SSU
Lessoniopsis
P
PD
LSU
SSU
Nereocystis
PD
LSU
SSU
Postelsia
2 kb

CHAPTER 3

NUCLEOTIDE SEQUENCES OF THE SMALL-SUBUNIT RIBOSOMAL RNA GENES FROM SELECTED LAMINARIALES (PHAEOPHYTA), IMPLICATIONS FOR KELP EVOLUTION

Introduction

To resolve the phylogenetic relationships of the kelp it is necessary to detect sufficient nucleotide divergence between the various taxa. The restriction-enzyme mapping approach did not allow for resolution of phylogenetic relationships (Chapter 2). Additional data could be sought by continuing restriction-enzyme mapping of the gene coding regions of the ribosomal cistron, resulting in some restriction-site differences among these diverse taxa. The work presented in the previous chapter has deterred me from continuing this approach and I have decided to assess some alternate methods. Dideoxy sequencing of the SSU has been successfully employed to resolve genera and species within the red algal order Gracilariales (Bird et al. 1990). Sequence data from the SSU and LSU have been used to establish phylogenetic relationships within the grass family Poaceae (Hamby & Zimmer 1988). This approach, sequencing of the SSU, has been applied to the kelp in this chapter.

The morphological diversity of the kelp suggests they are an ancient assemblage and this is reflected in interpretations of the fossil record (see Loeblich 1974, Clayton 1984). However, there has been increasing evidence for a recent radiation (10-15 mya, see General Introduction). In restriction mapping of the kelp nuclear ribosomal cistron, I noted similar divergence amongst families of kelp (Chapter 2) as noted between morphologically distinct stands of Zostera marina Linnaeus (Fain et al. 1991). These varied observations suggest rapid morphological evolution over a short evolutionary time.

Molecular data can be employed to infer the time since two organisms shared a common ancestor using a molecular clock (see General Introduction). Hence, these data can provide an indication of the age of extant kelp genera in the absence of a complete fossil record.

The entire small-subunit (SSU) ribosomal RNA (rRNA) sequence was inferred for kelp representing seven genera: Alaria marginata (1824 base pairs, bp), Egregia menziesii (1825 bp), Lessoniopsis littoralis (1825 bp), Macrocystis integrifolia (1825 bp), Nereocystis leutkeana (1824 bp), Postelsia palmaeformis (1826 bp) and Pterygophora californica (1825 bp). I obtained a partial sequence for Eisenia arborea (1669 bp), from a single clone of amplified PCR product. The SSU sequence was too conserved amongst all of these morphologically distinct taxa to permit phylogenetic analysis. The divergence between the most distant taxa was only 0.66%. This value was used in an SSU molecular clock to suggest that the most distantly related kelp investigated in this study diverged between 16-30 (more probably 16-20) mya. As I present the first entire kelp SSU sequences I reassess phylogenetic relationships among the heterokonts including a chrysophyte, a water mold and my Alaria. A single most-parsimonious tree supports earlier molecularly derived hypotheses of heterokont relationships.

Materials and Methods

Sample collection and DNA extraction

Specimens investigated in this study and their collection sites are presented in Table 1. Plants were packaged in plastic bags and transported to the laboratory on ice. Plants were stored in seawater tanks (5°C) or immediately processed for DNA extraction. Blades

were cleaned and nuclear DNA extracted as described previously (Fain et al. 1988) with modifications described in Bhattacharya & Druehl (1990).

Polymerase chain reaction

The SSU coding sequence was amplified for the taxa using 100-200 ng of nuclear DNA. The Gene-Amp Kit (Perkin-Elmer Cetus) was used following manufacturer's recommendations with the exceptions noted below. Two strategies were employed: 1) Double-strand (DS) amplification of the SSU gene for subsequent cloning and sequencing; 2) Single-strand (SS) and DS amplification of 300-900 bp subfragments of the gene for direct sequencing protocols. Amplifications were completed in an automated cycler as follows. For the entire gene, an initial cycle of (denature 2 min at 94°C, anneal 1 min 40-55°C, extension 7 min 72°C) followed by 36-40 cycles of (denature 1 min 93°C, anneal 1 min 40-55°C, extension 7 min 72°C) and a final cycle (denature 2 min 93°C, anneal 2 min 40-55°C, extension 12 min 72°C). Primers LD1 and LDF (Fig. 19) were modified from Elwood et al. (1985) and used at a final concentration of 1.0 μM.

To amplify subfragments, an upstream primer (5' by the rRNA, see Fig. 19a) complementary to the coding strand and a downstream primer (3') complementary to the noncoding strand yielding PCR products of 300-900 bp were used. Primers were modified from Elwood et al. (1985) with some novel phaeophycean-specific primers developed (Fig. 19). In DS amplifications primers had a working concentration of 1 μM. For SS amplifications one of the primers was limiting, 0.01 μM. Reaction profiles were (denature 4 min 94°C, anneal 30 sec 60°C, extension 1 min 72°C), 36-38 cycles of (denature 30 sec 94°C, anneal 30 sec 60°C, extension 1 min 72°C), and a final cycle (denature 30 sec 94°C, anneal 30 sec 60°C, extension 10 min 72°C).

Cloning and subsequent sequencing

An aliquot of PCR total gene product was double-digested with XbaI, a site conserved within all kelp 18S genes (Fig. 19b), and HindIII, a site in the polylinker of primer LDF (Fig. 19c). Digests were completed following manufacturer's recommended procedures (Bethesda Research Laboratories, BRL). The plasmid pVZ1 was similarly digested, and directional ligation completed using the manufacturer's protocol (BRL). Subcloning-efficiency competent cells were transformed (BRL) and positive colonies selected from AMP-Xgal plates. Plasmids were isolated and prepared for sequencing using the Miniprep Kit Plus (Pharmacia), and sequenced on both strands using my primers (Fig. 19) according to a modified Sequenase® (United States Biochemicals) protocol that reduces secondary structure artifacts (T. Snutch pers. comm.).

Direct sequence from SS and DS PCR product

SS template was prepared in Centricon®-30 centrifugal concentrators (Micon) and used in a Taq Trak® (Promega) sequencing protocol employing the two-step extension/termination with [^{35}S] dATP and deaza mixes. I made one change to the manufacturer's protocol, annealing the template and primers by heating to 70°C then cooling them to 37°C over 30 min. Sequencing employed the limiting primer for the PCR reaction or primers along the strand removed from the PCR primer by 600 bp.

DS template was prepared for sequencing using Miniprep Spun Columns and sequenced according to a manufacturer-provided protocol using a T7 polymerase (Pharmacia, Analacts 8, 1990). In this protocol both primers used in the initial PCR reaction, as well as any internal primers, gave readable sequence. Sequence was obtained from both strands.

Sequence alignment and phylogenetic analyses

I added the Alaria and a Chlorella (Huss & Sogin 1989) SSU sequence to an alignment for the chromophyte Ochromonas danica, a water mold Achlya bisexualis and the chlorophyte Chlamydomonas reinhardtii (Gunderson et al. 1987). Sequences were acquired from GenBank using the FASTA program (Pearson & Lipman 1988). I made four minor realignments involving about 20 nucleotides. I used only those regions that I felt were unambiguously aligned to maximize homology of the aligned nucleotides (1615 bp). I used the parsimony analysis program DNAPARS, as well as a bootstrapping procedure, DNABOOT, of the PHYLIP package (version 3.3, by Joseph Felsenstein 1990, compiled for the MacIntosh by Willem Ellis). I was limited to 1500 nucleotide positions for the distance programs and removed 115 bp of conserved sequence from the 5' and 3' ends of the gene with some intervening sequence. This procedure affects the distance estimates for pairwise comparisons (Felsenstein 1990). I have used these distances in treeing algorithms to establish congruence with the parsimony results. Distances were calculated with the Jukes & Cantor (1969) option of DNADIST (PHYLIP). FITCH, KITCH (PHYLIP), modifications of the Fitch & Margoliash (1967) algorithm, UPGMA (Nei 1987) and Neighbor-joining (Saitou & Nei 1987) were also utilized in this study.

Results

Sequence data

The SSU rRNA sequence for Alaria marginata, as inferred from PCR-amplified gene product, is presented (Fig. 20). The 5' and 3' termini were defined by alignment with other chromophyte sequences (Gunderson et al. 1987). The A. marginata SSU is 1824

nucleotides in length. The initial 20 nucleotides were not determined and were assigned on the basis of identity with the primer LD1. In a comparison with the Costaria SSU sequence (Bhattacharya & Druehl 1988), for which this region was determined, nucleotide 13 is different, being a U rather than a C.

The entire SSU rRNA was inferred for six other kelp genera (Table 5). In addition, a partial SSU sequence from a single PCR clone of Eisenia, with the terminal 263 nucleotides confirmed by direct sequencing (P1 to the 3' end), was determined (Table 5). I noted 24 variable sites for the eight genera, encompassing the two families of kelp. The variable sites are presented, displaying the nucleotide at that site for all of the kelp in the study (Table 5). All numbering is from the Alaria sequence (Fig. 20). Of these changes, seven are confined to Nereocystis. This latter genus was markedly different from others employed in this study. To confirm this result I sequenced 517 nucleotides, from LDA to LD6, for seven individuals from three populations (two each from Cape Beale and Turn Rock, three from Garbage Point, Table 1), This region of the SSU includes 16 of the 24 variable sites including five of the seven changes unique to Nereocystis. All the results were consistent with the data in Table 5, indicating that these changes were not the result of PCR artifact.

Direct sequencing versus cloning

SS PCR was difficult to perform consistently, and product was not obtainable for certain primer combinations. When SS PCR product was obtained, excellent sequence, yielding 300-350 bp, was common. Unfortunately, only the limiting primer used in the PCR reaction could be employed in sequence reactions. Primers of the same strand could be used only when removed 500-600 bp from the limiting PCR primer. This required the SS amplification of long PCR products or numerous PCR reactions to obtain adequate

sequence data. DS PCR was consistent for most primer combinations with our template. Although not as readable as the previous method (250-300 bp) this was the preferred approach. DS PCR product was easy to obtain and purify, and the ability to use forward, reverse and internal primers allowed more sequence information to be obtained per PCR reaction.

When I initiated this research, DS sequencing of PCR product was not well developed. I elected to clone my PCR products into plasmid vectors and employ plasmid-sequencing protocols. Initially, I amplified with a modified LD1 and LDF (Fig. 19), both of which contain polylinkers. I had poor success with cloning from the primer polylinkers, and predicted that the restriction-enzyme site that I wanted to use in the modified LD1 was too near the 5' end of the primer for adequate endonuclease activity. To circumvent this problem I cloned from digests with XbaI, an enzyme with a recognition site near the 5' end of the SSU in all kelp (Figs 16 & 19b) and a HindIII site in the LDF polylinker (Fig. 19c) with 10 bp of 5' flanking sequence. This latter combination yielded six to 15 positive clones for each alga in my study. I sequenced one clone from each plant to determine the phylogenetic potential of this approach and to avoid problems encountered when I attempted to sequence mixed populations of clones.

I completed direct sequencing of PCR products to determine the accuracy of data obtained from single PCR clones. Ambiguities were common between sequence determined by these two methods (Fig. 21). Differences probably result from errors introduced during later PCR cycles, that are accentuated by single-clone selection. PCR errors were template- and reaction-dependent with from zero to seven errors noted per 1668 nucleotides (Fig. 21). This suggests that no general value can be applied to the expected number of errors in PCR. I observed about twice as many transitions as transversions, with A-to-G errors being the most common (Fig. 21).

Chromophyte phylogeny

Previously, a partial SSU sequence from Costaria was employed to determine the relationships of the Phaeophyta amongst other heterokonts (Bhattacharya & Druehl 1988). I have now determined the entire SSU sequence for a variety of kelp and have decided to reevaluate heterokont relationships. The sequence for Alaria was compared with two heterokonts, Achlya bisexualis and Ochromonas danica, and two chlorophytes, Chlamydomonas reinhardtii (Gunderson et al. 1987) and Chlorella vulgaris (Huss & Sogin 1989). Two parsimony trees are presented (Fig. 22). The most-parsimonious tree (Fig. 22a) requires 426 steps with an additional seven steps required for an alternate hypothesis (Fig. 22b). Bootstrap values are presented for the most-parsimonious tree, with the node for an Alaria-Achlya clade being supported 75 times out of 100 replicates. Distance methods that do not constrain branch lengths by invoking a molecular clock (FITCH, Neighbor-joining, Fig. 22c) support the most-parsimonious tree (Fig. 22a). Methods that do constrain branch lengths (KITCH, UPGMA; Nei 1987) support the alternate tree (Fig. 22b). The discrepancy in the distance methods is probably the result of the accelerated evolution in the water mold lineage since it last shared an ancestor with the Phaeophyta, compounded by the short branch length separating the Alaria-Achlya group from the Ochromonas branch (Fig. 22c).

Discussion

Choice of method

I preferred direct sequencing of DS PCR product. This approach was the simplest to perform consistently, and is quicker than SS methods by yielding more sequence per PCR

reaction. Direct sequencing of DS product gave consistent results for successive amplification of the same target DNA. Although cloned PCR product was the easiest to sequence and gave the best sequence results (readability), obtaining clones was difficult and inconsistent. I have recently tried the TA Cloning system (Invitrogen) with substantially improved results. My major concern with the cloning approach was the unpredictable error rate of the PCR reactions. Caution should be exercised when results are obtained by this approach.

Phylogeny and time

SSU sequence comparisons have resolved genera and species in the red algal order Gracilariales (Bird et al. 1990), interfamilial phylogeny in the Chlorophyta (Zechman et al. 1990) and, in conjunction with the 26S-like rRNA, intrafamilial relationships of angiosperms (Hamby & Zimmer 1988). It was because of these previous reports that I was surprised by the conservative nature of the SSU amongst all the diverse kelp I considered. The situation in Nereocystis requires further investigation. I am not certain why this one species appears so different from the other kelp. Based on previous restriction mapping of the ribosomal cistron (Chapter 2) and subsequent investigations of the internal transcribed spacer of the ribosomal cistron (Chapter 5) Nereocystis and Postelsia appear closely related. Here they differ by 13 sites (Table 5). For now I exclude Nereocystis from further discussion pending additional investigation. I noted 23 differences between Alaria and 1595 bp of SSU sequence previously published for Costaria (Bhattacharya & Druehl 1988). This variation was equivalent to that noted between the eight SSU sequences in this report. Further, 18 of the changes were deletion/insertion changes contrasting the rare occurrence of these events amongst my taxa (two sites). This plant is also excluded from further discussion pending additional investigation.

Morphologically kelp span, and dwarf, all the other algal groups. I investigated forms from the simple winged kelp Alaria, to the complex Macrocystis and Egregia. Unfortunately, the data are insufficient for phylogenetic analyses, but I should note one interesting similarity. The SSU gene for the lessoniacean alga Lessoniopsis differs at one position from Alaria marginata (U versus X at position 694, respectively). This site was extremely variable amongst the kelp (Table 5) and is a U residue for the alga Alaria nana Schrader (Saunders unpubl.). It was noted that Lessoniopsis had close affinities with the Alariaceae on the basis of chloroplast-DNA restriction data (Fain et al. 1988). The current results suggest that Lessoniopsis is deep within the alariacean lineage, possibly more closely related to Alaria than this latter genus is to the morphologically similar genus Pterygophora. This issue is discussed elsewhere (Druehl & Saunders 1991) and is the subject of continuing investigation.

The extent of morphological divergence within the Laminariales suggests an underlying and equal amount of genetic divergence. This is suggested in some interpretations of the fossil record that date putative kelp fossils at 200-300 mya (see Loeblich 1974). A variety of morphological, culture, and transplant investigations (see Mathieson et al. 1981) as well as recent molecular investigations (Bhattacharya & Druehl 1990, Bhattacharya et al. 1990a, Bhattacharya et al. 1991, Druehl & Saunders 1991, Fain et al. 1988, Fain & Druehl unpubl, Mroz 1989, Chapter 2) support the contrary notion. In other words, the extensive morphological variation observed amongst the kelp comes from genetically similar plants. These observations, as well as the occurrence of intergeneric hybrids in the wild and culture (Neushul 1971, Sanbonsuga & Neushul 1978), have prompted some phycologists to suggest a recent radiation for the kelp.

Estes & Steinberg (1988) have proposed a middle-to-late-Miocene radiation for the kelp, with some suggestions of even more recent dates, 3-5 mya. They discussed kelp biogeography and distribution, habitat and ecological requirements, and the fossil record of

kelp associates. In another investigation paleooceanographic constraints (thermal and physical), thermal tolerances of extant species, and hybridization investigations were employed to suggest dates for kelp evolution (Lüning & tom Dieck 1990). The latter authors predicted the Laminariales evolved during climatic deterioration in the Tertiary, possibly at the Eocene/Oligocene boundary (40 mya) and during the Miocene (10-15 mya). Fossil evidence supporting these ideas comes from the 7-10 mya fossil kelp Julescrania, hypothesized to be an ancestor of the morphologically distinct genera Nereocystis and Pelagophycus (Parker & Dawson 1965).

Setting a molecular clock for the kelp provides an alternate method for dating divergence among the kelp. I have applied a cpDNA divergence rate of approximately 0.3% per 1 million years (Fain & Druehl unpubl) to previously published chloroplast-DNA divergence estimates for a variety of kelp (Fain et al. 1988). These data support a 15-22 mya divergence for the kelp.

I continued this speculative pursuit and applied a SSU molecular clock to divergence estimates for the kelp. I acknowledge that such data are estimates and the previously coined phrase "quasi-clock-like process" (Wilson et al. 1987) probably best describes the exercise. However, in the absence of an extensive fossil record, this approach can provide useful insights into aspects of kelp evolution.

SSU-divergence estimates vary from 1% per 60 my (vertebrates) to 1% in 25 my (green plants) for eukaryotes and 1% per 50 my in prokaryotes (Ochman & Wilson 1987). I have assumed a divergence rate for the kelp SSU of 1% per 25-50 my. With this rate, the deepest divergence in the kelp (approximately 0.66%) would have occurred 16 to 30 mya. Both these values are recent, but the lower limit, 16 mya, is in agreement with previous divergence estimates based on the more recent Miocene climatic deterioration (10-15 mya; Lüning & tom Dieck 1990), biogeographic and habitat interpretations (Estes & Steinberg 1988), and divergence times I predicted from the cpDNA data (Fain et al. 1988, Fain &

Druehl unpubl.). Another supporting feature is the occurrence of the fossil kelp, Julescrania, postulated to be ancestral to two extant yet morphologically distinct genera, Nereocystis and Pelagophycus, that was isolated from 7-10 mya sediment (Parker & Dawson 1965). Further, morphologically similar taxa (for example, Alaria and Pterygophora) would have radiated only 3 to 6 mya. This latter observation is supported by the fossil record of kelp associates, the abundance of monotypic genera (Estes & Steinberg 1988), biogeographical distribution of species/genera, and hybrid studies among a variety of kelp (Neushul 1971, Sanbonsuga & Neushul 1978, Lüning & tom Dieck 1990).

If the lower limit (1% per 25-30 my) is the correct rate for kelp 18S-gene divergence, this suggests that the kelp nuclear genome is evolving at the same rate as in green plants. Fain & Druehl (unpubl.) reported that the chloroplast genome for kelp is evolving at the same rate as those of green plants. My divergence times contrast slightly with those of Stam et al. (1988) where species of the genus Laminaria alone were estimated to have diverged 15-19 mya. This is almost as deep as the earliest lineage I propose for the kelp. However, if the outer bound for kelp 18S-gene divergence is used (1% per 50 my), and I assume that Laminaria is an old assemblage (relative to other kelp genera), then my values would support those of Stam et al. (1988). Regardless, all the data point to a recent divergence of the kelp, but more work is required before the upper and lower limits of these estimates can be established.

My data lead me to suggest the kelp as a group diverged 16-20 mya, or at most 30 mya based on divergence of 0.66%. In a recent publication, the SSU sequences for two species of the rhodophycean genus Gracilaria were approximately 0.84% divergent (Bird et al. 1990). This is more divergence then I noted among all the kelp in my study. Molecular investigations employing the 5S gene had similar results (Lim et al. 1983a, 1983b, 1986). In these latter investigations, two species of Porphyra (Rhodophyta) had divergence (2%)

equivalent to that observed among 5 orders of Phaeophyta (1-4%). These brown orders were dwarfed by the divergence observed amongst red algal orders (19-37%). Divergence between diatoms and the Phaeophyta was estimated at 20%, this value barely entering the realm of Rhodophyta ordinal divergence. Chrysophytes were divergent from the browns by 27%. From the 5S data it was estimated that the Phaeophyta diverged approximately 200 mya. My data are not surprising in retrospect. Evolutionary divergence time is clearly at odds with taxonomic hierarchy based on morphological diversity in the Rhodophyta and Phaeophyta, with taxonomic systems under- and overinflated respectively. This is reflected in contemporary phycology by the proliferation of red algal families and orders (see Hommersand 1990) and by the opposite trend for species of the Phaeophyta (Mathieson et al. 1981).

Phaeophyta and the heterokont lineage

That the heterokont water molds and the chlorophyll a & c chrysophytes, phaeophytes and diatoms form an evolutionary lineage is firmly established in the molecular literature (Gunderson et al. 1987, Bhattacharya & Druehl 1988, Medlin et al. 1988, Bhattacharya et al. 1990b). This relationship had been proposed based on flagellar-state (heterokont) and other ultrastructural features (see Cavalier-Smith 1986). The molecular phylogenies in the former publications suggest the Oomycete lineage occurs within the chromophyte (I use chromophyte to refer exclusively to chlorophyll a & c heterokonts) lineage, rendering the latter paraphyletic. These phylogenies therefore invoke a gain-loss of the chloroplast in the evolution of the water molds plus the parallel gain of a heterotrophic mode of nutrition. The parsimony and distance analyses support these proported relationships. However, an alternate parsimony tree, requiring only seven additional steps, places the water molds as a sister group to the chromophytes. This would invoke a simple

gain of a chloroplast leading to a monophyletic chromophyte lineage. Soon after this endosymbiotic event, lineages leading to the extant chrysophytes and phaeophytes would have diverged. I am not claiming the alternate tree is correct; I only suggest that the molecular results should not be interpreted without reference to the organisms themselves. We should challenge phylogenies based on a single gene sequence by considering all the available information. In a recent publication employing the SSU, the Oomycetes were positioned as a sister group to the chromophytes, however, the branches were not considered to be resolved by the data (Ariztia et al. 1991). More and better data are required before the relationships among the heterokonts are resolved.

Table 5. Summary of nucleotide differences in the SSU genes for taxa in this study.

	NUCLEOTIDE POSITION																								
ALGA	176	193	194	497	685	694	702	720	722	723	724	728	735	740	744	745	746	747	841	1077	1080	1393	1727	1737
Alaria	U	C	G	C	U	X	U	X	U	C	G	G	G	C	U	U	C	U	A	U	C	G	C	U
Egregia	C	C	G	C	U	U	U	X	U	U	G	G	G	C	U	U	C	C	G	U	A	G	C	U
Eisenia	C	C	G	C	U	U	U	X	U	A	G	G	G	C	U	U	C	U	A	U	C	G	C	C
Lessoniopsis	U	C	G	C	U	U	U	X	U	C	G	G	G	C	U	U	C	U	A	U	C	G	C	U
Macrocystis	U	A	C	C	C	U	C	X	U	C	U	G	G	C	G	U	C	U	A	U	C	G	C	C
Nereocystis	U	C	G	U	U	X	U	X	C	G	U	A	C	U	C	G	U	U	A	A	C	A	C	C
Postelsia	U	C	G	C	U	U	C	C	U	G	G	G	A	U	U	U	C	U	A	U	C	G	U	C
Pterygophora	U	C	G	C	U	U	U	X	U	C	G	G	U	U	G	U	C	U	A	U	C	G	C	U

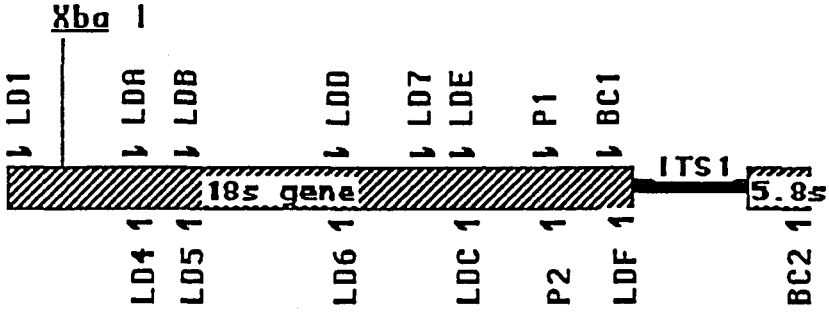
X = deletion.

Fig. 19. Schematic of the kelp SSU, ITS1 and 5.8S gene of the ribosomal cistron. a) rRNA of the SSU indicating the 5' (upstream) and 3' (downstream) orientation as referred to in the text. b) Location and orientation of synthetic primers. XbaI is a restriction-endonuclease site conserved in all kelp. c) Sequence and exact position (Alaria numbering) of synthetic primers complementary to the coding and noncoding strands.

Fig. 19.

a) 5' rRNA 3'

b)



c) CODING STRAND COMPLEMENT

5' 3'

LD1	AAT CTG GTT GAT CCT GCC AG	1-20
LDA	CGA TTC CGG AGA GGG AGC CTG	378-398
LDB	GTC TGG TGC CAG CAG CCG CGG	559-579
LDD	CAG AGG TGA AAT TCT TGG AT	915-934
LD7	CTG AAA CTT AAA GAA ATT GAC GG	1147-1169
LDE	GGT GGT GGT GCA TGG CCG TTC	1283-1303
P1	TAA TCT GTT GAA CGT GCA TCG	1542-1562
BC1	GAT TCC GGA CTG TGG CTC GCG TG	1701-1723

NONCODING STRAND COMPLEMENT

5' 3'

BC2	CGA GTG GTG TCA ACA GAC ACT CC	5.8s gene-3'
LDF	CCCGGGATCCAAGCTT → (SMA I / BAM HI; HIND III)	polylinker→
	GAT CCT TCT GCA GGT TCA CCT AC	1800
P2	CTA TCA CGA TGC ACG TTC AAC AG	1568-1546
LDC	GAA CGG CCA TGC ACC ACC ACC	1303-1283
LD6	ATC CAA GAA TTT CAC CTC TG	934-915
LD5	CCG CGG CAG CTG GCA CCA GAC	579-559
LD4	TCA GGC TCC CTC TCC GG	399-383

Fig. 20. Inferred rRNA sequence for the SSU from Alaria marginata. Lower-case letters indicate nucleotides assigned on basis of identity with a primer. X at positions 694 and 720 represent gaps in the Alaria sequence when compared to the other kelp in this study.

Fig. 20.

```
   1  aaucugguug auccugccag UAGUCAUACG CUUGUCUCAA AGAUUAAGCC
  51  AUGCAUGUCU AAGUAUAAGC GCUUUAUACU GUGAAACUGC GAAUGGCUCA
 101  UUAUAUCAGU CAUAGUUUAU UUGAAAGUCC CUUACUACAU GGAUAACCGU
 151  AGUAAUUCUA GAGCUAAUAC AUGCAUGAAG GCCCAACUGC UUCGGCGGAC
 201  GGGCUGCAUU GAUUAGACCG AAACCAAUGC GUCUUCGGAC GGUUUUGUGG
 251  UGAAUCAUAA UCACUUGCGG AUCGCACGCU UCGGCGGCGA CGUUUCAUUC
 301  AAGUUUCUGC CCUAUCAGCU UUGGAUGGUA GGGUAUUGGC CUACCAUGGC
 351  UUUAACGGGU AACGGGGAAU UGGGGUUCGA UUCCGGAGAG GGAGCCUGAG
 401  AAACGGCUAC CACAUCCAAG GAAGGCAGCA GGCGCGUAAA UUACCCAAUC
 451  CUGACACAGG GAGGUAGUGA CAAUAAAUAA CAAUGCCGGG CUUAUACAAG
 501  UCUGGCAAUU GGAAUGAGAG CAAUUUAAAU CCAUCAUCGA GGAUCAAUUG
 551  GAGGGCAAGU CUGGUGCCAG CAGCCGCGGU AAUUCCAGCU CCAAUAGCGU
 601  AUAUUAAAGU UGCUGCAGUU AAAAAGCUCG UAGUUGGAUU UGUGGCGCGG
 651  CCGUCCGGCG GGGCUCUUUC AUUAGGGCCG UUUGUCCGGU UUUXCGGCCG
 701  CUCCAUUCUC GGGUAGCGUX GUCGCUGGCA UUAGGUUGUC GGCUUCUUCG
 751  CGCCCGUCGU UUACUGUGAA AAAAUUAGAG UGUUCAAAGC AGGCUUAGGC
 801  CGUUGGAUAC AUUAGCAUGG AAUAAUGAGA UAGGGCCACG ACGGUCUAUU
 851  UUGUUGGUUU GCACGUUGUG GUAAUGAUUA ACAGGAACGG UUGGGGGUAU
 901  UCGUAUUCAA UUGUCAGAGG UGAAAUUCUU GGAUUUAUGG AAGACGAACU
 951  ACUGCGAAAG CAUUUACCAA GGAUGUUUUC AUUAAUCAAG AACGAAAGUU
1001  AGGGGAUCGA AGAUGAUUAG AUACCAUCGU AGUCUUAACC AUAAACUAUG
1051  CCGACUAGGG AUUGGCGGUC GUUAAUUUAC AGGACUCCGU CAGCACCUUC
1101  CGAGAAAUCA AAGUCUUUGG GUUCCGGGGG GAGUAUGGUC GCAAGGCUGA
1151  AACUUAAAGA AAUUGACGGA AGGGCACCAC CAGGAGUGGA GCCUGCGGCU
1201  UAAUUUGACU CAACACGGGG AAACUUACCA GGUCCGGACA UAGUGAGGAU
1251  UGACAGAUUG AGAGCUCUUU CUUGAUUCUA UGGGUGGUGG UGCAUGGCCG
1301  UUCUUAGUUG GUGGAGUGAU UUGUCUGGUU AAUUCCGUUA ACGAACGAGA
1351  CCCCCGCCUG CUAAAUAGUG UGGCUUACGC UUCUGUGUAG GUGCUCGCUU
1401  CUUAGAGGGA CUUUCGGUGA CUAACCGAAG GAAGUUGGGG GCAAUAACAG
1451  GUCUGUGAUG CCCUUAGAUG UCCUGGGCCG CACGCGCGCU ACACUGAUGC
1501  AUGCAACGAG UUCUUUUUUU UCCUGGGUCG AGAGGCCCGG GUAAUCUGUU
1551  GAACGUGCAU CGUGAUAGGG AUAGAUCAUU GCAAUUAUUG AUCUUGAACG
1601  AGGAAUUCCU AGUAAACGCG AGUCAUCAGC UCGCAUUGAU UACGUCCCUG
1651  CCCUUUGUAC ACACCGCCCG UCGCACCUAC CGAUUGAAUG UUUCGGUGAA
1701  GAUUCCGGAC UGUGGCUCGC GUGCUUCACG GCGCUCUUGC CGUGGGAAGU
1751  UAUCUAAACC UCAACAUUUA GAGGAAGGUG AAGUCGUAAC AAGGUUUCCG
1801  UAGGUGAACC UGCGGAAGGA UCAUUA
```

Fig. 21. Summary of differences noted from direct sequencing to determination of sequence for a single PCR-amplified clone. Differences presented by recording their position in the SSU. * Indicates a change in the opposite direction.

Fig. 21.

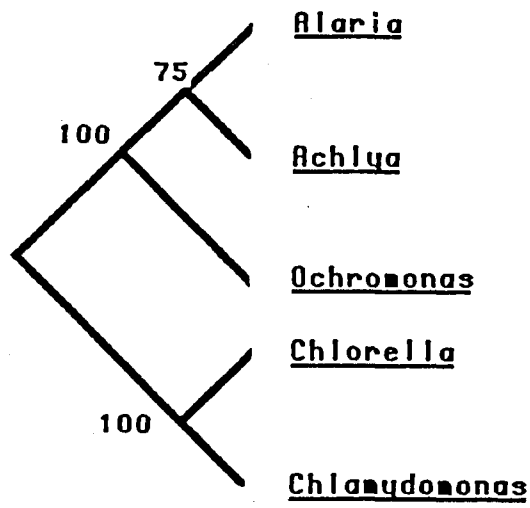
PCR SINGLE CLONE COMPARED TO DIRECT SEQUENCING

ALGA	TRANSITION		TRANSVERSION	TOTAL CHANGE	TOTAL NUCLEOTIDE
	A-G	T-C	A-T		
Alaria	392*	243	1571	3	1668
Egregia		1386		1	1669
Eisenia		1727*		1	263
Lessoniopsis				0	1669
Macrocystis	1807			1	1669
Nereocystis				0	1668
Postelsia	297 414		462 985		
	876*		1524*		
	1628			7	1670
Pterygophora		1658		1	1669
TOTAL	6	4	4	14	

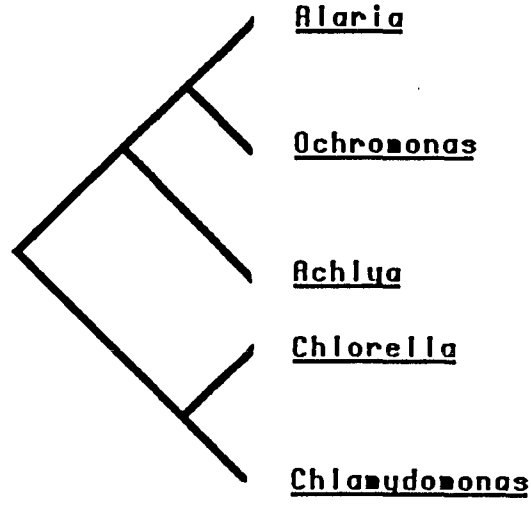
Fig. 22. Phylogenetic trees inferred for relationships among three heterokont organisms and two chlorophytes. a) Most-parsimonious tree with bootstrap confidence limits at the nodes (100 replicates). b) An alternate parsimony tree requiring seven additional steps. c) Neighbor-joining tree derived using distance estimates.

Fig. 22.

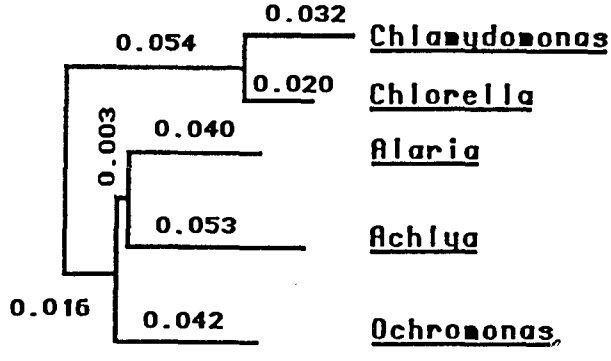
a.


Alaria
75
100
Achlya
Ochromonas
Chlorella
100
Chlamydomonas

b.


Alaria
Ochromonas
Achlya
Chlorella
Chlamydomonas

c.


0.032
Chlamydomonas
0.054
0.020
Chlorella
0.003
0.040
Alaria
0.053
Achlya
0.016
0.042
Ochromonas

CHAPTER 4

NUCLEOTIDE SEQUENCES OF THE INTERNAL TRANSCRIBED SPACERS AND 5.8S GENES FROM ALARIA MARGINATA AND POSTELSIA PALMAEFORMIS (PHAEOPHYTA, LAMINARIALES)

Introduction

Regions of the ribosomal cistron are under varying degrees of functional constraint and hence provide a universal spectrum of divergence. The spacer regions are the least conserved and resolve intra- and interpopulation (ETS and nontrancribed spacer NTS) as well as interspecies and intergeneric relationships (ITS1 and ITS2) (Appels & Honeycutt 1986). The most evolutionary conserved regions are the rRNA genes which are suited for more distant phylogenetic comparisons. In this chapter I completed investigations of the ITS1 and ITS2 to assess their potential in resolving kelp phylogeny.

Outside the realm of phylogeny, ribosomal spacer regions are investigated to define post-transcriptional processing sites. It was proposed that processing-sequence motifs should be detectable in the primary RNA structure near the processing sites themselves (see Torres et al. 1990). In the quest for consensus sequence patterns, the ITS regions have been sequenced for a select variety of eukaryotes, mostly animals and fungi (Torres et al. 1990). It is not certain if a universal processing system occurs among eukaryotes and recent views suggest this is probably not the case (Gerbi 1984, Nazar et al. 1987, Torres et al. 1990). During attempts to understand rRNA processing, the phenomenon of G+C balance was noted between the ITS1 and ITS2 regions, for a wide variety of eukaryotes.

Nucleotide sequences have been determined for the 5.8S gene, and the first and second internal transcribed spacers (ITS1 and ITS2), for representatives of two phaeophycean genera Alaria and Postelsia. The ITS regions were similar in length to those

of green plants and fungi but only similar to the former in G+C content. G+C balance was exhibited for the ITS1 and ITS2 of Postelsia but less so for Alaria. A putative post-transcriptional processing secondary structure has been identified. This stem-loop structure occurred in a conserved region shared by these taxa just 5' the 5.8S gene. This is the first report of 5.8S gene sequence for a phaeophycean alga and I add this algal division to a recent 5.8S universal phylogenetic tree (Yokota et al. 1989). Phylogenetic relationships for the Phaeophyta amongst animals, plants and fungi were inferred on the basis of the 5.8S gene sequence.

Materials and Methods

Sample locations and DNA extraction

Algal material was collected from Kirby Point (Alaria marginata) and Cape Beale (Postelsia palmaeformis), British Columbia, Canada (Table 1). Plants were packaged in plastic bags and transported on ice. In the laboratory plants were cleaned and nuclear DNA extracted according to the protocol of Fain et al. (1988) with modifications (Bhattacharya & Druehl 1990).

Polymerase chain reaction

Double strand (DS) DNA amplification was carried out with 100-200 ng of nuclear DNA according to the manufacturer's protocol (Perkin Elmer Cetus, Gene-Amp Kit). Synthetic primers (Fig. 23) were used at a working concentration of 1 μM. Amplifications employed primers P1 (5' TAA TCT GTT GAA CGT GCA TCG 3') complementary to the coding strand of the kelp SSU (positions 1542-1562, Chapter 3) and GITS4 (5' CTT TTC

CTC CGC TTA TTG ATA TG 3') complementary to the LSU noncoding strand of Saccharomyces in a conserved region near the 5' end of this gene (positions 64-42, LSU alignment Baroin et al. 1988).

Amplification reactions were completed in an automated cycler as follows. Reaction profiles were (denature 4 min 94°C, anneal 30 sec 60°C, extension 1 min 72°C), 36-38 cycles of (denature 30 sec 94°C, anneal 30 sec 60°C, extension 1 min 72°C), with a final cycle (denature 30 sec 94°C, anneal 30 sec 60°C, extension 10 min 72°C).

Cleaning and sequencing DS amplification product

Amplification products were agarose gel purified according to the manufacturer's protocol using the Prep-A-Gene DNA purification Kit (BIO-RAD). DNA cleaned by this method was sequenced following alkaline denaturing (1 M NaOH) and subsequent neutralization with 1 M HCl (Pharmacia, Analects 8). Denatured DNA was sequenced using the Sequenase sequencing system (Manufacturer's directions, United States Biochemicals). Amplification primers P1 and GITS4 were used as well as additional synthetic primers (Fig. 23): BC1 (5' GAT TCC GGA CTG TGG CTC CGG TG 3') complementary to kelp SSU coding strand (positions 1701-1723, Chapter 3), P5 (5' GCA TCG ATG AAG AAC GCA G 3') complementary to 5.8S gene coding strand (positions 530-548, Fig. 24), and GITS2 (5' GCT GCG TTC TTC ATC GAT G 3') complementary to the 5.8S gene noncoding strand (positions 549-531, Fig. 24). The latter two primers were modified from White et al. (1990) to remove GC termini that enabled primers to anneal to themselves causing primer-dimer artifacts. An additional primer, BC2 (5' CGA GTG GTG TCA ACA GAC ACT CC 3'), was complementary to the ITS2 and 5.8S gene noncoding strand (positions 669-647, Fig. 24).

Sequence alignment and phylogenetic analysis

I aligned my 5.8S gene sequence for Alaria with a previous alignment for seven other eukaryotes (Fig. 26): a mammal (Rattus rattus), a sea urchin (Lytechinus variegatus), an insect (Drosophila melanogaster), an ascomycete (Saccharomyces cerevisiae), a monocot plant (Triticum aestivum), a dicot plant (Daucus carota) and a slime mold (Dictyostelium discoideum). The sequences and the alignment were modified from Yokota et al. (1989). Only unambiguously aligned sequence was used in phylogenetic analysis. Distance and parsimony methods were employed. DNADIST (PHYLIP, Felsenstein 1990) was used to construct a distance matrix for the data, with corrected distances calculated using Kimura's two parameter model (Kimura 1980). The distance matrix was converted to a phylogenetic tree by the method of Fitch & Margoliash (1967) in the FITCH program of PHYLIP. Parsimony analysis employed the DNAPARS and DNABOOT programs of PHYLIP that complete a slightly modified version of Wagner parsimony (Felsenstein 1990). An option of DNAPARS, a modified log likelihood test (Templeton 1983), was used to determine if user defined trees differed significantly from the most-parsimonious trees computed for the 5.8S data (Felsenstein 1990).

Results and Discussion

Kelp ITSs compared to those of other eukaryotes

Figure 24 presents the primary nucleotide structure for the region spanning the 3' end of the SSU to the 5' start of the LSU, including the ITS1, 5.8S gene and ITS2, for representatives of the morphologically diverse genera Alaria and Postelsia. The 3' end of the SSU/start of the ITS1, was assigned by alignment to other SSU sequences (Gunderson et al. 1987). The 5' start and the 3' terminus of the 5.8S gene were defined on the basis of conservation for a variety of eukaryotes (Yokota et al. 1989). The 3' terminus of the ITS2/start of LSU, was defined by alignment to other eukaryote LSU sequences (Baroin et al. 1988).

The ITS1 regions for both taxa were 246 bp with G+C content of 52% and 60% for Alaria and Postelsia respectively (Table 6). The ITS2 region was slightly longer, 259 bp, in Alaria than the ITS1, while in Postelsia it was substantially longer, 309 bp, due mainly to a large insert near the 3' end of the ITS2 (Fig. 24, Table 6). Generally, kelp ITS regions were longer than those observed for green plants, but they were similar in length to those recorded for yeasts (Torres et al. 1990). As with the fungi and green plants, the kelp ITSs were substantially shorter than those observed for animals (Torres et al. 1990). In Alaria the G+C content was 52% and 67% for the ITS1 and ITS2 respectively. These values are similar to those for most green plants but differ from those noted for most animals and sharply contrast those of fungi (Torres et al. 1990). The Alaria ITS regions only weakly display the G+C balance phenomenon noted for most other eukaryotes (Torres et al. 1990). The Postelsia ITS1 and ITS2 regions had similar G+C content to these regions in Alaria, 60% and 67% respectively (Table 6), but better fit the universal G+C balance model.

Comparison of kelp ITS1, 5.8S and ITS2 regions

The two kelp ITS1 regions had an initial conserved region (position #234-242, Fig. 24), followed by a divergent region, D1 (#243-284). The next region was 83% conserved between the two species (#285-371), followed by a short divergent region, D2 (#372-388). D2 was flanked by two short compression artifacts that could not be fully resolved with my method and were tentatively assigned (Fig. 24). The remainder of ITS1 (#389-518) was 94% conserved except for a gap in the Postelsia alignment (#463-469) that occurred in the center of a sequencing artifact (discussed below). The 5.8S gene was estimated to be 160 nucleotides long with only one change noted between the two kelp genera. The kelp ITS2s had an initial region of 11 conserved nucleotides (#658-669) followed by a variable stretch of T and C nucleotides (#670-701). This area, D3, was the most variable among an extended survey of eight kelp genera (data not shown) and was difficult to resolve with my method. Sequence could be read with primers from both sides of this region with some overlap, but rarely through this region, as a multitude of background bands were observed. Two factors probably account for this problem. I have some evidence that the Taq polymerase cannot faithfully amplify through such runs of nucleotides. This results in a heterogenous population of frameshift amplification products after this point that cause the background sequencing artifacts. Secondly, the organisms themselves probably have inherent heterogeneity in these variable regions. Microheterogeneity was noted for the ITS regions within individuals of Xenopus (Stewart et al. 1983). The next region of the kelp ITS2s (#702-883) was 92% conserved except for two small gaps in Alaria. D4 (884-938) included a large insert for Postelsia. D4 accounted for most of the size difference between the ITS2s of the two kelp. The terminal ITS2 region (#939-966) was 78% conserved leading into the LSU. This pattern of conserved and variable regions noted for my taxa is similar to that observed in green plants, particularly in the ITS2. The pattern of conserved

and divergent regions noted for these former organisms is different from the interspersion of conserved and variable nucleotide tracts observed for animals (Torres et al. 1990).

The order Laminariales contains the most morphologically diverse and developed algae. The genera in this order are divided into families on the basis of morphological diversity that spans and dwarfs that observed among all the algal divisions. Despite the separation of Alaria and Postelsia into distinct families on the basis of extensive morphological variation, the similarity noted here at the molecular level, suggests an underlying level of genetic similarity. This view of a recent divergence in the kelp is becoming strongly supported by traditional as well as molecular evidence (Chapters 2 & 3).

rRNA processing

The intention of the current chapter was to present the primary structure for the SSU-LSU region strictly for phylogenetic purposes. As such, I did not determine if consensus sequence or secondary structure processing sites noted for other eukaryotes occurred in the kelp. However, one observation I made may have some implications for such investigations. Sequencing of the ITS1 produced a strong secondary structure artifact (Fig. 24, #448-482). I used deaza reagents in my sequencing reactions and successfully resolved this region for both taxa. This compression artifact was noted for eight other genera of kelp (data not shown). This region can be folded into a secondary stem-loop structure 15 nucleotides upstream of the 5.8S gene (Fig. 25). Torres et al. (1990) noted a stem-loop for green plants that was similar in position, and structure, to that presented here for the kelp, and they suggest that this structure is involved in some, as yet undetermined, aspect of post-transcriptional processing.

5.8S eukaryote phylogeny

The 5.8S gene sequence for Alaria was estimated to be 160 bp. This sequence was placed (Fig. 26) with 5.8S sequences for Rattus, Lytechinus, Saccharomyces, Triticum, Daucus and Dictyostelium using the data and alignment presented by Yokota et al. (1989). Only regions that were unambiguously aligned, 142 bp, were used in phylogenetic anlayses (Fig. 26). A distance matrix was prepared for pairwise comparisons of the taxa (Table 7). This matrix was transformed to a phylogenetic tree using the method of Fitch & Margoliash (1967) as provided in the PHYLIP computer package (Felsenstein 1990). The distance tree acquired by this method (Fig. 27a) agrees with one of the two most-parsimonious trees (168 steps, Fig. 27b) obtained by Wagner parsimony (DNAPARS, PHYLIP). An equally parsimonious tree suggests that Drosophila diverged after, rather than before, Lytechinus in the animal clade. Bootstrap analysis (50 replicates) of the parsimony tree, as expected, suggested that the data only weakly support the nodes in the animal portion of the tree (Fig. 27b). My distance and parsimony trees support a group containing the green plants and the fungi, as suggested by Yokota et al. (1989). The chromophytes appear more closely related to the fungi than either of these groups does to the green plants or animals (Fig. 27a & b). The node supporting the green plants and the fungi-chromophytes is supported by the bootstrap analysis but the relationship within this group between the fungi and chromophytes is weak.

The relationships inferred here for the fungi, green plants and animals contrast with SSU and LSU derived trees (Sogin et al. 1986, 1989, Baroin et al. 1988). The current 5.8S phylogeny additionally contrasts SSU determined chromophyte relationships (Bhattacharya & Druehl 1988, Bhattacharya et al. 1990b, Gunderson et al. 1987). SSU trees suggest a radiation of most eukaryote groups in a short evolutionary time (Sogin et al. 1989). Of the taxa considered here, SSU trees suggest the chromophytes branched first

followed by the green plants with the fungi and the animals being last to diverge. I tested the SSU derived tree against the 5.8S data by invoking the user tree option of DNAPARS. The SSU tree required an additional 9 steps and was significantly different than my tree for the 5.8S data. I tested several user defined trees and one requiring 7 additional steps (Fig. 27c), that was not considered significantly worse from the most-parsimonious trees, supported the LSU derived tree with the fungi branching off before the animals and green plants diverged (Baroin et al. 1988).

The chromophytes were added to the 5' LSU phylogeny (Perasso et al. 1989). This tree was the same as my Figure 27c, except that the chromophytes and Saccharomyces grouped together on a common branch separate from a green plant-animal branch. I tested this hypothesis against the 5.8S data and eight additional steps were required, but this tree was not considered significantly worse than my most-parsimonious trees. However, it was noted that the LSU derived relationship between fungi and chromophytes was only weakly supported by the LSU data (Perasso et al. 1989). My tree presented in Figure 27c is, therefore, not ruled out by the 5.8S or LSU data and requires the swapping of only one node in the SSU derived tree.

The 5.8S gene has been considered too small to provide statistically valid determinations of phylogeny (McCarrol et al. 1983). The SSU tree predicts a nearly simultaneous divergence of animals, green plants, fungi and chromophyte algae as well as other groups of eukaryotes. It was acknowledged that the precise branching orders for these lineages could not be determined from the SSU data (Sogin et al. 1989). Based on the phylogenies proposed for the SSU, LSU and my current observations, I contend that the tree in Figure 27c best reflects the branching orders of the eukaryote lineages considered. It is evident that phylogeny among the eukaryotes still remains to be resolved. I feel that ultimately trees should be constructed from all the available sequence data rather

than from single genes. Possibly such analyses will enable us to determine the branching order of the proposed eukaryotic radiation.

Table 6. Length and G+C comparisons for kelp ITS regions.

Taxon and ITS	G	C	A	T	TOTAL	% G+C
Alaria ITS1	60	68	64	54	246	52
Alaria ITS2	76	97	33	53	259	67
Postelsia ITS1	62	86	55	43	246	60
Postelsia ITS2	93	114	41	61	309	67

Table 7. Distance matrix for the taxa compared in this study.

	AL	RA	DR	LY	SA	TR	DA
AL							
RA	0.3923						
DR	0.5269	0.2563					
LY	0.5446	0.1535	0.3508				
SA	0.1871	0.3309	0.5188	0.4092			
TR	0.3036	0.3350	0.4967	0.4173	0.2042		
DA	0.2991	0.3677	0.5229	0.4223	0.2219	0.0519	
DI	0.7959	0.7984	0.7984	0.7980	0.7277	0.7669	0.7750

Abbreviations for taxa. AL= Alaria. RA= Rattus. DR= Drosophila. LY= Lytechinus. SA= Saccharomyces. TR= Triticum. DA= Daucus. DI= Dictyostelium.

Fig. 23. Schematic of a portion of the kelp ribosomal cistron displaying the approximate location of amplification and sequencing primers employed in this study. See text for abbreviations.

Fig. 23.

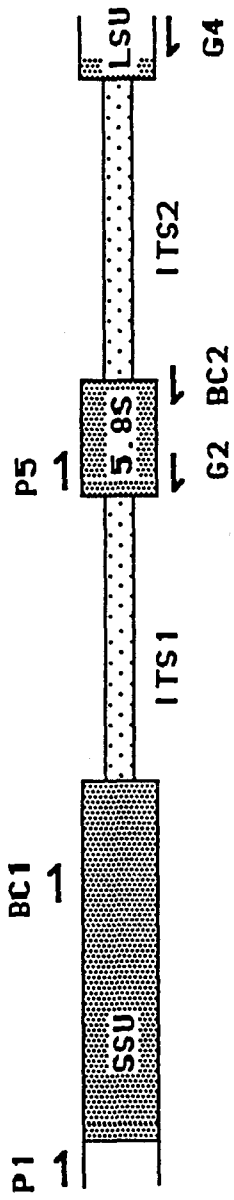


Fig. 24. Alignment of 233 bp of 3' SSU, ITS1, 5.8S gene, ITS2 and 22 bp of 5' LSU sequence for the kelp Alaria and Postelsia. Gene coding regions are boxed and labelled accordingly. Dashed underline indicates the divergent regions D1, D2, D3 and D4. Thin double arrow connected by a loop indicates a putative stem-loop structure. Solid underline indicates strong sequence compressions that were not fully resolved with my method.

Fig. 24.

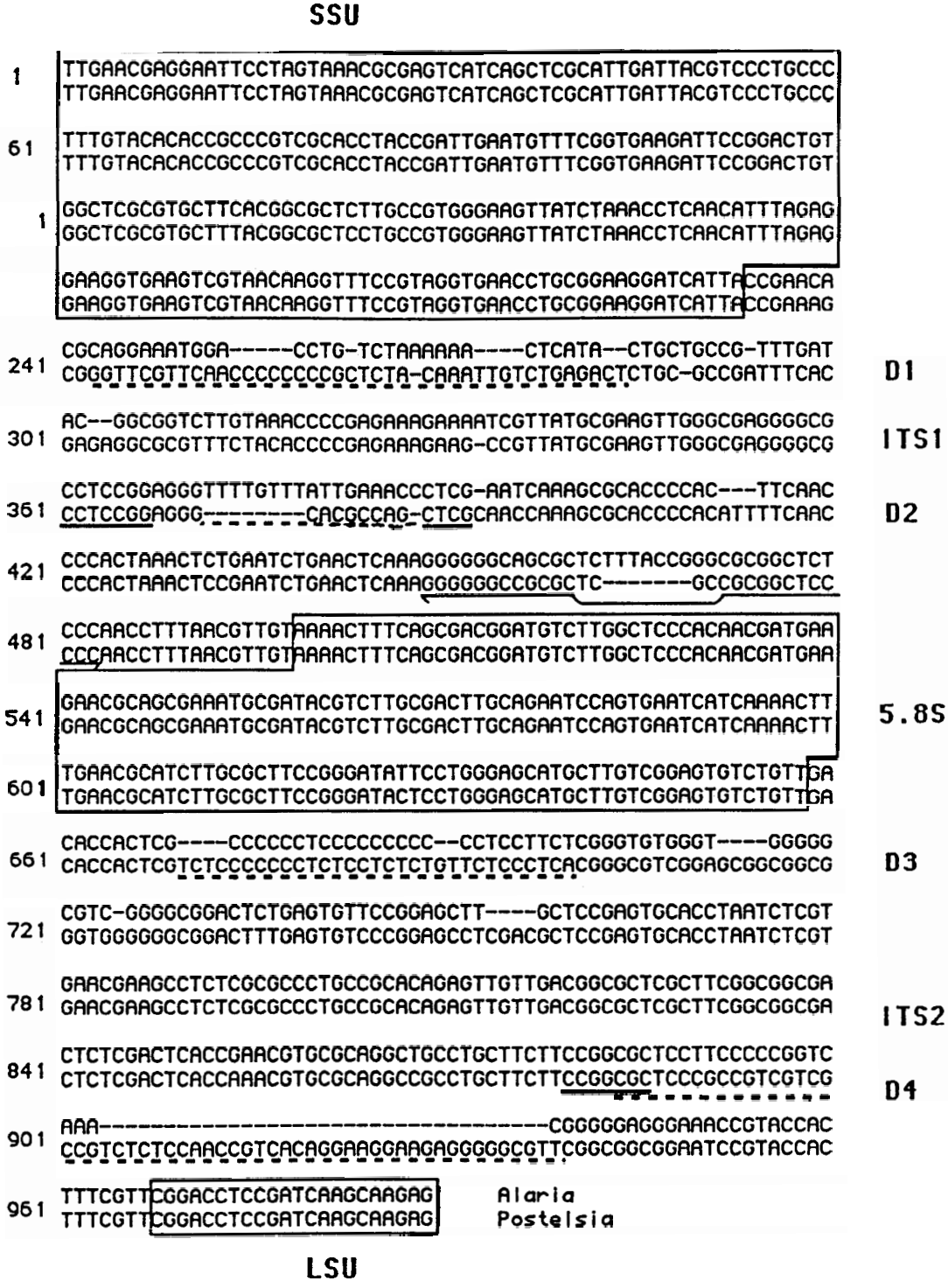

SSU
1 TTGAACGAGGAATTCCTAGTAAACGCGAGTCATCAGCTCGCATTGATTACGTCCCTGCCC
TTGAACGAGGAATTCCTAGTAAACGCGAGTCATCAGCTCGCATTGATTACGTCCCTGCCC
61 TTTGTACACACCGCCCGTCGCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGT
TTTGTACACACCGCCCGTCGCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGT
1 GGCTCGCGTGCTTCACGGCGCTCTTGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAG
GGCTCGCGTGCTTTACGGCGCTCCTGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAG
GAAGGTGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAACA
GAAGGTGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAAAG
241 CGCAGGAAATGGA-----CCTG-TCTAAAAAA----CTCATA--CTGCTGCCG-TTTGAT
CGGGTTCGTTCAACCCCCCCCGCTCTA-CAAATTGTCTGAGACTCTGC-GCCGATTTCAC
D1
301 AC--GGCGGTCTTGTAAACCCCGAGAAAGAAAATCGTTATGCGAAGTTGGGCGAGGGGCG
GAGAGGCGCGTTTCTACACCCCGAGAAAGAAG-CCGTTATGCGAAGTTGGGCGAGGGGCG
ITS1
361 CCTCCGGAGGGTTTTGTTTATTGAAACCCTCG-AATCAAAGCGCACCCCAC---TTCAAC
CCTCCGGAGGG--------CACGCCAG-CTCGCAACCAAAGCGCACCCCACATTTTCAAC
D2
421 CCCACTAAACTCTGAATCTGAACTCAAAGGGGGGCAGCGCTCTTTACCGGGCGCGGCTCT
CCCACTAAACTCCGAATCTGAACTCAAAGGGGGGCCGCGCTC-------GCCGCGGCTCC
481 CCCAACCTTTAACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAA
CCCAACCTTTAACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCCACAACGATGAA
541 GAACGCAGCGAAATGCGATACGTCTTGCGACTTGCAGAATCCAGTGAATCATCAAAACTT
GAACGCAGCGAAATGCGATACGTCTTGCGACTTGCAGAATCCAGTGAATCATCAAAACTT
5.8S
601 TGAACGCATCTTGCGCTTCCGGGATATTCCTGGGAGCATGCTTGTCGGAGTGTCTGTTGA
TGAACGCATCTTGCGCTTCCGGGATACTCCTGGGAGCATGCTTGTCGGAGTGTCTGTTGA
661 CACCACTCG----CCCCCCTCCCCCCCCC--CCTCCTTCTCGGGTGTGGGT----GGGGG
CACCACTCGTCTCCCCCCCTCTCCTCTCTGTTCTCCCTCACGGGCGTCGGAGCGGCGGCG
D3
721 CGTC-GGGGCGGACTCTGAGTGTTCCGGAGCTT----GCTCCGAGTGCACCTAATCTCGT
GGTGGGGGGCGGACTTTGAGTGTCCCGGAGCCTCGACGCTCCGAGTGCACCTAATCTCGT
781 GAACGAAGCCTCTCGCGCCCTGCCGCACAGAGTTGTTGACGGCGCTCGCTTCGGCGGCGA
GAACGAAGCCTCTCGCGCCCTGCCGCACAGAGTTGTTGACGGCGCTCGCTTCGGCGGCGA
ITS2
841 CTCTCGACTCACCGAACGTGCGCAGGCTGCCTGCTTCTTCCGGCGCTCCTTCCCCCGGTC
CTCTCGACTCACCAAACGTGCGCAGGCCGCCTGCTTCTTCCGGCGCTCCCGCCGTCGTCG
D4
901 AAA----------CGGGGGAGGGAAACCGTACCAC
CCGTCTCTCCAACCGTCACAGGAAGGAAGAGGGGGCGTTCGGCGGCGGAATCCGTACCAC
951 TTTCGTTCGGACCTCCGATCAAGCAAGAG
TTTCGTTCGGACCTCCGATCAAGCAAGAG
Alaria
Postelsia
LSU

Fig. 25. Putative stem-loop secondary structure in ITS1 of Postelsia. Lower case letters indicate nucleotide changes (3) and an insert for the same region in Alaria. Numbering of nucleotides refers to Fig. 24.

Fig. 25.

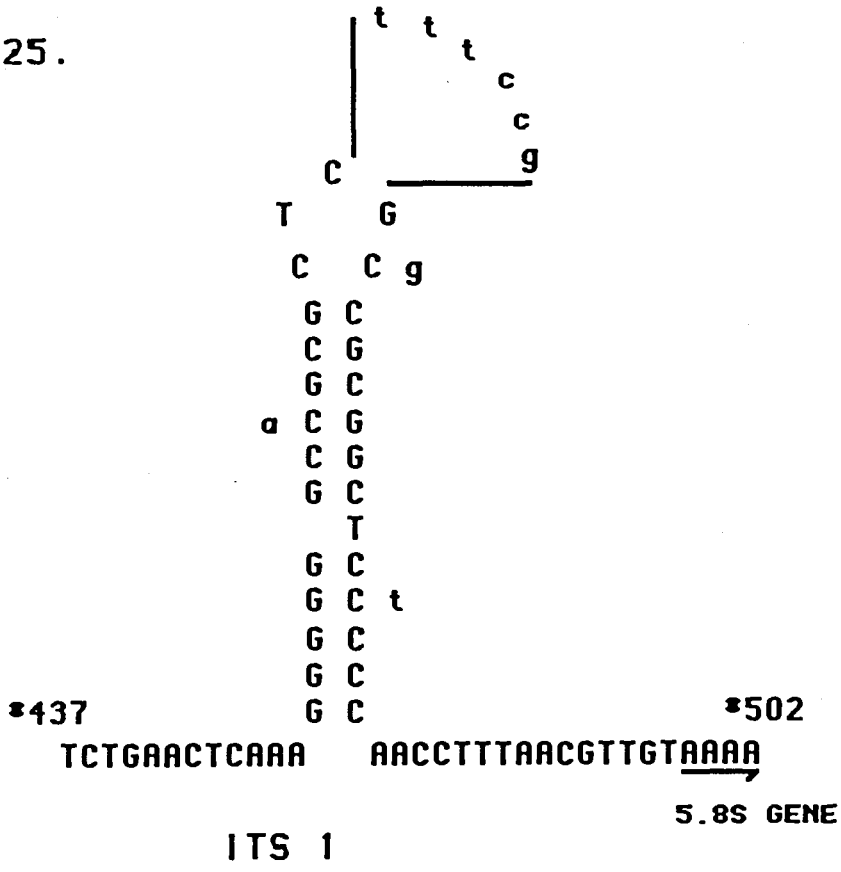

t t t
c
c
g
C
T G
C C g
G C
C G
G C
a C G
C G
G C
T
G C
G C t
G C
G C
G C
*437
*502
TCTGAACTCAAA AACCTTTAACGTTGTAAAA
5.8S GENE
ITS 1

Fig. 26. Alignment of 5.8S sequence for taxa employed in my phylogenetic analyses. Boxed regions could not be unambiguously aligned and were not used for phylogenetic comparisons. * indicates gaps invoked in the alignment.

Fig. 26.

5.8S rDNA gene sequnces

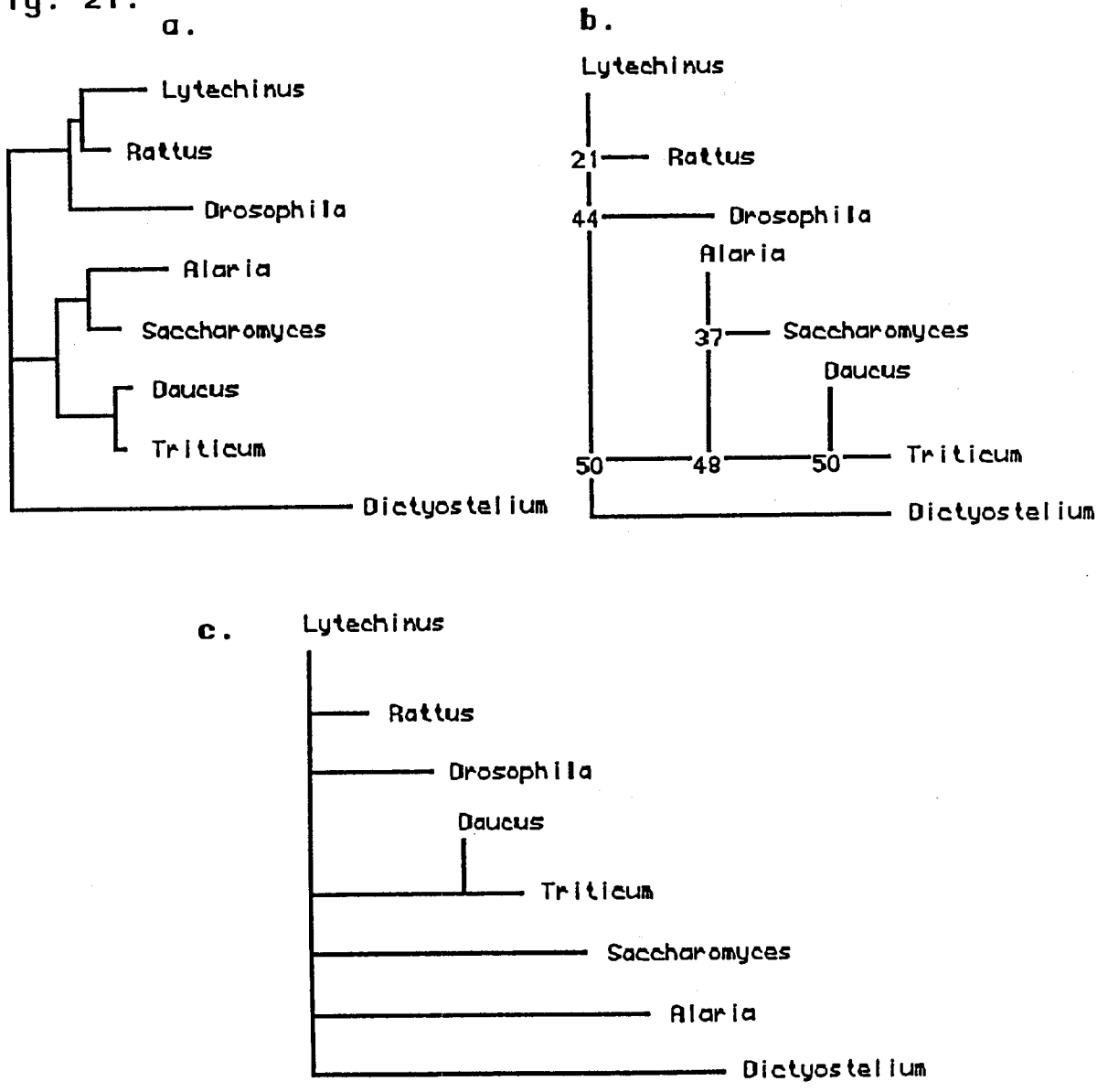
```
AA******AAC TTTCAGCGAC GGATGTCTTG GCTCCCACAA CGATGAAGAA CGCAGCGAAA
**....CG.. .C.T....GT ....CA..C. ....GTG.GT .......... ......T.GC
.*.....*.. .C.A....GT ....CA..C. ....ATGGGT .......... ......A..C
**....C..T .C.TG...GT ....CA..C. ....GTG.GT .......... ......C.GC
.*........ .....A.A.. ....C..... .T..T.G..T .......... ..........
C.C..ACG.. .C..G..A.. ....A...C. ....T.G..T .......... ..T.......
..A..ATG.. .C..G..A.. ....A..CC. ....T.G..T .......... ..T.......
**...TT..G CA.A.A..GT .A..AC..C. A......A.T T........C ..T...A..C

TGCGATACGT CTTGCGACTT GCAGAATCCA GTGAATCATC AAAACTTTGA ACGCATCTTG
.....G.AT. AA..T..A.. ....G.CA.. T*TG...... G.C....C.. .....*....
..T.CGT.A. .G..T..AC. ....G.CA.. **TG.A.... G.C.T..... ......A.C.
.....G.AT. AA..T..A.. ......CT.C T*TG...... G.C.T..C.. .....CA.G.
.......... AA..T..A.. .......T.C .......... G..T...... .....CA...
........C. GG..T..A.. .........C .C...C.... G.GT...... .....AG...
........T. GG..T..A.. .........C .....C.... G.GT...... .....AG...
.......AT. .ACTT..A.. ....CC.A.T .G..*.AG.T G...TG.... .....CA.GA

CGCTTCCGGG ******ATATT CCTGGGAGCA TGCTTGTCGG AGTGTCT*GT T*   Alaria
..GCC..**. G...GT.CC. ..C...GCT. C..C....T. ..C...G.C* *T   Rattus
.AG**T.CAT G.CTGT.CC. G..T...CT. CATA..GTT. ..G..TG.** .A   Drosophila
..GCC.G**. G.CCTTCGCG G.CC.AGC.. C..CC...C. ..G...G... *.   Lytechinus
...CC.TT.. .....*.... ..A...G... ...C...TT. ..C...*.A. .T   Saccharomyces
..**C...A* GCCA.C.C*G ..GA..GC*. C..C..C.T. G.C.*TCACG C.   Triticum
..**C...AA GCCA.T..GG ..CA..GC*. C.TC..C.T. G...*TCACG C.   Daucus
T.ACAT...T CCTTTCGGA. TAG.T.TTAT ACT.G.G*T. *AGAGTGG** .C   Dictyostelium
```

Fig. 27. Phylogenetic trees derived for taxa investigated in this study and other eukaryotes. a) Distance matrix tree from data in Table 7. b) Maximum parsimony tree with bootstrap confidence intervals (50 replicates) indicated at the branch nodes. c) An alternate parsimony tree considered as not significantly different from the most parsimonious tree.

Fig. 27.


a.
Lytechinus
Rattus
Drosophila
Alaria
Saccharomyces
Daucus
Triticum
Dictyostelium
b.
Lytechinus
21
Rattus
44
Drosophila
Alaria
37
Saccharomyces
Daucus
50
48
50
Triticum
Dictyostelium
c.
Lytechinus
Rattus
Drosophila
Daucus
Triticum
Saccharomyces
Alaria
Dictyostelium

CHAPTER 5

A PHYLOGENY OF THE LAMINARIALES CONSTRUCTED FROM SEQUENCE DATA

Introduction

Laminarialean taxonomy has been firmly established in the brown algal literature (Setchell & Gardner 1925). The advanced members of this order were separated into three families on the basis of developmental features exhibited at the stipe-blade transition zone. In summary, the Alariaceae are characterized by having their reproductive sori on sporophylls, the Lessoniaceae by splitting and the Laminariaceae by an undifferentiated transition zone (Setchell & Gardner 1925). However, there are several inconsistencies in this taxonomic system as noted by the originators (Setchell & Gardner 1925). Further, recent cpDNA restriction analyses challenge the current system of taxonomy, in particular, suggesting polyphyly for the Lessoniaceae. As noted in the General Introduction to this thesis, the cpDNA derived phylogenies may not necessarily equate to organismal phylogenies. In this section of my research I have addressed the proposed polyphyly of the Lessoniaceae and investigated the relationships of these taxa with those of alariacean and laminariacean affinity. This was done by completing phylogenetic analyses of 3' SSU (113 nucleotides), ITS1, 5.8S and 5' ITS2 (12 nucleotides) sequence data from a variety of taxa.

Materials and Methods

All materials and methods for DNA isolation, PCR amplification and direct dideoxy sequencing in the current Chapter are as presented in Chapter 4. The sample locations for

the taxa investigated are presented in Table 1. Sequences were aligned by eye and employed in subsequent phylogenetic analysis with the PHYLIP package (Felsenstein 1990). DNAPARS and DNABOOT were used for parsimony analyses and FITCH was employed in distance calculations.

Results

Amplification and direct sequencing of PCR product was difficult for the kelp ITS regions. For the ITS1, the stem-loop structure noted previously (Chapter 4), was a problem and accurate sequence was not obtained for this region in all the taxa. Further, this structure rendered the acquisition of sequence information from both strands difficult for most taxa. Because I could only obtain sequence from one strand, several regions of compression artifact could not be resolved in the ITS1 regions for my taxa. Tentative sequences are presented for this region from representatives of 12 laminarialean genera from the three morphologically advanced families (Fig. 28). The 3' SSU and 5.8S gene sequence was highly conserved among all the taxa and hence easily aligned. Unfortunately these genes offered few phylogenetically informative sites. The ITS1 consisted of conserved, semiconserved and variable tracts of nucleotides. Variable regions were characterized by large insertion/deletion events and were difficult to align with certainty.

Because of the previous constraints, I have limited phylogenetic analyses to 490 bp of unambiguously determined sequence that I felt was confidently aligned (Fig. 28). Dictyoneurum and Dictyoneuropsis were identical in nucleotide sequence for the phylogenetically informative region used in this study and were considered as one taxon for subsequent analyses.

I initially calculated a rooted distance tree (Fig. 29) using the Fitch & Margoliash (1967) algorithm of PHYLIP (FITCH with outgroup option). This tree sharply contrasted

with traditional views on kelp taxonomy. Three major groups of taxa were defined (Fig. 29). The data were then subjected to parsimony analysis for which four equally parsimonious trees were derived. All four trees were similar to the distance tree (Fig. 29) except for minor rearrangements within Group 3. Three of the parsimony trees presented all pairwise combinations among Macrocystis, Nereocystis and Postelsia. The fourth tree had Eisenia branching off as a distinct lineage before Lessonia. Further, in all four trees, parsimony analysis supported a clade of Alaria and Lessoniopsis with Pterygophora as a sister taxon contrasting the arrangement for Group 1 in the distance tree. The data were subjected to 100 replicates of bootstrap, parsimony analysis (DNABOOT) to determine the confidence of the nodes in the tree. The three major Groups were each supported by 100 replicates, suggesting that these three Groups (Fig. 29) were strongly supported by the data. Within Group 1 relationships among the taxa could not be firmly established using this data set. Four lineages were resolved in Group 3 arising from a polytomy; Egregia, Eisenia, Lessonia and Macrocystis-Nereocystis-Postelsia. The relationships among taxa in the latter lineage were not resolved by the data.

Discussion

Current results versus previous observations

The tree presented here (Fig. 29) clearly contrasts laminarialean taxonomy based on traditional interpretations of development at the stipe-blade transition zone. My data suggest that both the Alariaceae and Lessoniaceae are polyphyletic while the Laminariaceae may prove paraphyletic in excluding Dictyoneurum. Sporophylls, considered characteristic of the Alariaceae, were noted for members of my Groups 1 & 3; while splitting, a lessoniacean trait, occurred for some representatives of all three groups. CpDNA derived

phylogenies were largely congruent with the current observations (Fain 1986, Fain et al. 1988). My data support the suggestion that Nereocystis is more closely related to laminariacean taxa, in this case Costaria, than to Alaria or Lessoniopsis (Fain et al. 1988). These latter two taxa were also noted to be closely related (Fain et al. 1988) as presented in the current results. Dictyoneurum/Dictyoneuropsis were indistinguishable based on cpDNA investigations and were the furthest removed from all other lessoniacean genera (Fain 1986) as noted in the current study. Further, Nereocystis and Postelsia were the two most closely related lessoniacean taxa (Fain 1986) as supported in my Chapter 2 and the current results. One notable incongruency between the chloroplast study (Fain et al. 1988) and the current research concerns the positioning of Macrocystis. The cpDNA derived phylogeny placed Macrocystis with my Group 1 plants. This inconsistency is most easily explained as a technical error owing to the use of the fragment method of analysis in the cpDNA investigation (see General Introduction). Alternatively, chloroplast introgression may have given rise to the differences in phylogenies (see General Introduction), although I feel this is less likely, considering the divergence between Macrocystis and Alaria in the current phylogeny. Regardless, the observation of Macrocystis-Nereocystis hybrids in culture (Neushul 1971, Sanbonsuga & Neushul 1978), lend support to the current nuclear derived phylogeny.

Nuclear derived relationships

Group 1, consisting of Alaria, Lessoniopsis and Pterygophora seems a reasonable phylogenetic assemblage. These algae all have paired sporophylls that arise from the primary stipe and vegetative blades characterized by midribs. Although sporophylls occur for other laminarialean taxa I consider them analogous rather than homologous structures. Within Group 1 relationships amongst the taxa were not fully resolved with the current

data. I prefer to consider Alaria and Pterygophora as the more closely related for reasons of morphological similarity. However, as my data show, morphology can be very misleading in the Laminariales.

If sporophylls are an ancestral feature of the Laminariales, then they must have been lost four times leading to Group 2 and 3 taxa, based on the current molecular phylogeny (Fig. 29). Alternatively, sporophylls may be a derived trait that evolved independently in Group 1 & 3 taxa. I believe a number of observations support the latter interpretation. First, when the sporophylls are removed from an individual of Alaria it frequently will produce sori on the vegetative blade (C. Pfister pers. comm.). This potential for blade tissue to produce sori leads me to suggest that blade-borne sori represent the ancestral state. Second, the sporophylls of Group 1 taxa are highly specialized relative to those of Group 3. In Group 1 sporophylls are paired blades that arise on the primary stipe. For the Group 3 taxon Eisenia, sporophylls are derived as extensions of the vegetative blade and not on the primary stipe. I consider the frond of Macrocystis to be a blade consisting of vegetative and reproductive bladelets; hence, these sporophylls are derived from the blade and not the stipe as in Group 1. Egregia, in Group 3, has a unique arrangement with rowed pinnae derived from the stipe and blade. However, in Egregia, sporophylls develop randomly among the vegetative bladelets (Setchell & Gardner 1925) and not in the regular and exclusive pattern of the Group 1 specialized sporophylls. This suggests to me that sporophylls have evolved independently even among Group 3 plants. The Group 1 sporophylls are clearly the most specialized and are referred to in the remainder of this text as specialized sporophylls. Hence Group 1 taxa, despite their morphological diversity (Figs 7-9), share the feature of specialized sporophylls.

The assemblage of taxa in Group 3 is not as easily defined. The relationships proposed among Macrocystis, Nereocystis and Postelsia are not to difficult to accept in view of previous cpDNA and hybrid cross observations (Fain 1986, Fain et al. 1988,

Neushul 1971, Sanbonsuga & Neushul 1978). My data could not fully resolve relationships among these taxa and three most-parsimonious trees support all pairwise combinations. I believe that Nereocystis and Postelsia are more closely related, a view first presented by Setchell & Gardner (1925) on the basis of morphology and subsequently supported by molecular evidence (Fain 1986, Chapter 2 this thesis).

Within Group 3, four lineages were noted arising from an unresolved polytomy. One lineage is the alga Egregia. Considering the morphological diversity of Group 3, and the lack of specialized sporophylls in Egregia, as noted for Group 1 taxa, I can see no other place that this taxon might better fit into the current phylogeny.

Eisenia and Lessonia were weakly allied and are more correctly considered as two lineages arising from the unresolved polytomy in Group 3. Eisenia was originally classified in the Alariaceae by possessing sporophylls. These structures in Eisenia, however, are derived from the blade and not like the specialized sporophylls described here for Group 1 plants. Eisenia has a biogeography unique among the studied taxa by occurring in the Northwest, Northeast and Southeast Pacific. It is generally accepted that the Laminariales evolved in the North Pacific (Estes & Steinberg 1988, Lüning & tom Dieck 1990). Most taxa occur in the Northeast Pacific with a number occurring exclusively there. Within Group 3 it appears that the lineages Egregia and Macrocystis-Nereocystis-Postelsia evolved in the Northeast Pacific with only Macrocystis subsequently and recently (Fain & Druehl unpubl.) migrating to the Southern Hemisphere. The genera Eisenia and Ecklonia, which has similar sporophyll development to the former, appear to have evolved from the Northwest Pacific. I suggest this because both Eisenia and Ecklonia have geographical distributions uncharacteristic of Group 3 taxa by occurring in the Northwest Pacific. For the genus Eisenia, E. bicyclis (Kjellman) Setchell occurs in the Asian Pacific. Since its divergence from an ancestor common with Ecklonia in the Northwest Pacific, Eisenia probably invaded the Northeast Pacific via currents to California (see Lüning 1990,

p. 139). Since the invasion of North American waters, Eisenia has probably, recently entered cold water corridors to the South Pacific, along with Macrocystis (Fain & Druehl unpubl.), giving rise to E. cokeri Howe, a species very similar to the Northeast Pacific E. arborea. Three additional species of Eisenia have been recognized from the East Pacific (E. desmarestiodea Setchell & Gardner, E. masonii Setchell & Gardner and E. galapagoensis Taylor): however, Lüning (1990) did not recognize these entities. While Eisenia was traversing the North Pacific and subsequently the equator, Ecklonia was radiating and diverging throughout the Northwest Pacific. Ecklonia has a variety of morphologically distinct species spanning generic diversity of the Northeast Pacific taxa. Ecklonia has also radiated and diverged throughout the Southwest Pacific, presumably along Indo-asian cold water corridors similar to those noted between North and South America (Fain & Druehl unpubl., Lüning 1990). Since its introduction, Ecklonia has migrated and diverged extensively throughout the Southwest Pacific, around, and into the South Atlantic and finally to deep water populations in the Northeast Atlantic (Lüning 1990).

Lessonia represents another lineage of the Group 3 polytomy. Lessonia is very similar in habit to Lessoniopsis but lacks the specialized sporophylls and midribs noted for the latter taxon. Traditional thought considers that Lessonia evolved in the Southeast Pacific, after migration via a cold water corridor along the Americas of a common ancestor with the morphologically similar Lessoniopsis (Lüning 1990). This view was not supported by the current molecular data. My data indicated that Lessonia shared a distant ancestor in common to the Group 3 taxa while Lessoniopsis evolved from Group 1 ancestors. At some point, probably a much earlier cooling event than lead to the introduction of Macrocystis and Eisenia to South America, the ancestor of the modern species of Lessonia entered the south Pacific via a cold water corridor along the Americas. From here the ancestor migrated and diversified throughout the South Pacific while its sister lineages were evolving in the North Pacific. Similarity between Lessonia and

Lessoniopsis represents a strong case for convergent evolution and not divergence from a recent common ancestor. The kelp as a group display incredible morphological diversity and frequent examples of convergent evolution have occurred. Examples include the stipe morphology of Nereocystis and Ecklonia maxima (Osbeck) Papenfuss and the stoloniferous development in a variety of genera including Laminaria and Ecklonia.

The positioning of Dictyoneurum/Dictyoneuropsis with Costaria in the Laminariaceae is not such a difficult concept considering that splitting in the transition zone, diagnostic of the Lessoniaceae, was clearly not a phylogenetically valid character in the current tree (Fig. 29). Saunders (1895) originally described Dictyoneuropsis reticulata as Costaria reticulata Saunders. The Laminariaceae are generally characterized by simple blades lacking sporophylls. This latter character, lacking sporophylls, has held in placing Dictyoneurum in association with Costaria. Dictyoneurum has a prostrate stipe decumbent along the substratum with haptera formed along the stipe as it develops. Splitting occurs at the stipe-blade transition to yield two new blades. Devlopment in this plant seems similar to that noted in Arthrothamnus bifidus (Gmel.) Ruprecht and Streptophyllopsis kuroshioensis (Segawa) Kajimura, plants traditionally placed in the Laminariaceae. The similarity of these taxa may have resulted from convergent evolution or possibly these plants will prove to be related to Dictyoneurum within Group 2.

Fig. 28. Alignment of 3' SSU, ITS1, 5.8S gene and 5' ITS2 sequence data for representatives of 12 kelp genera. Boxed in regions were those data used for subsequent phylogenetic analysis. Underline text represent regions of secondary structure artifact not unambiguously resolved in this study. Lower case indicates nucleotide state at sites for Egregia that were difficult to assign with certainty. '.' positions where the nucleotide state for a taxon could not be determined.

Fig. 28.

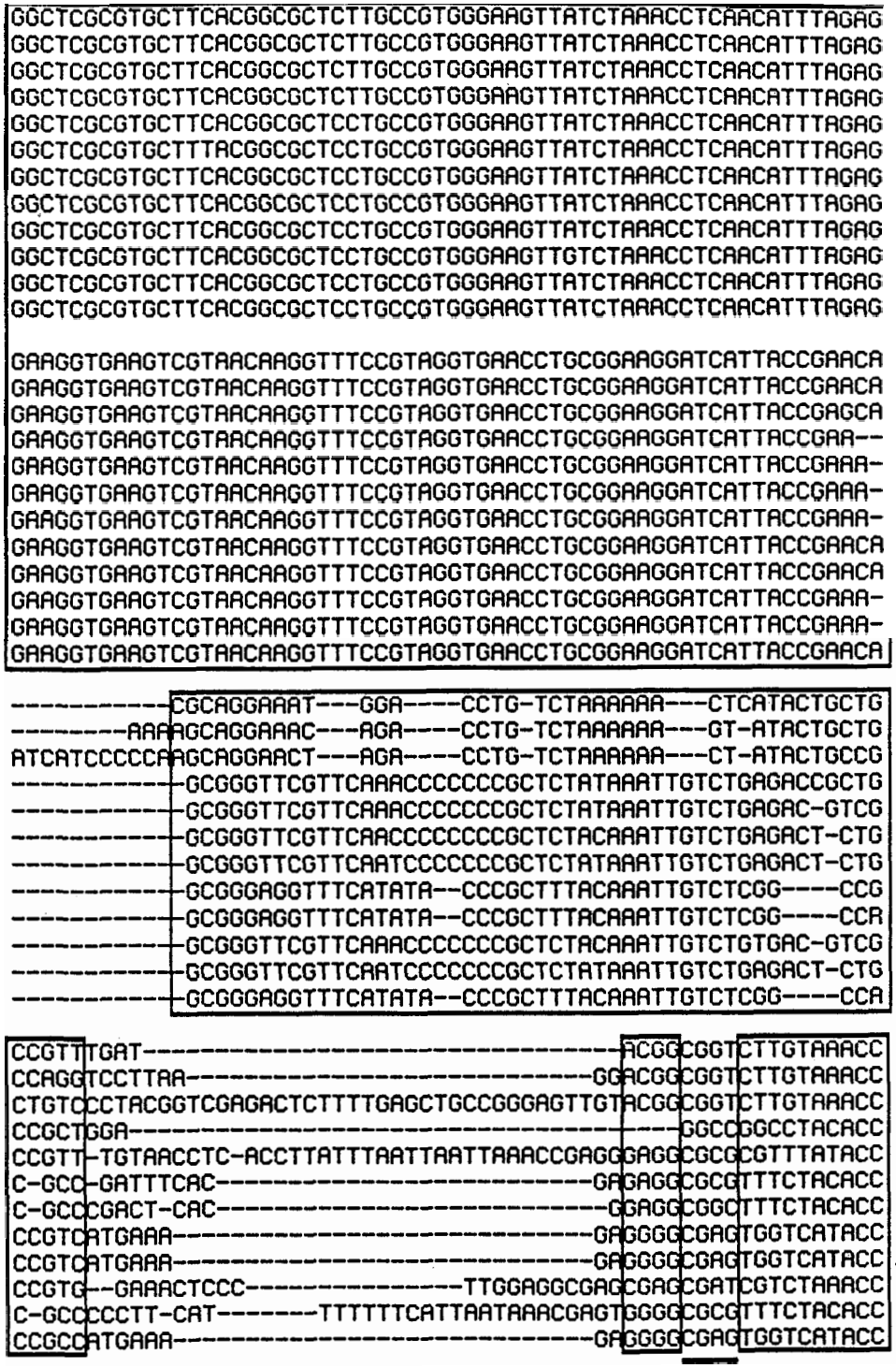


Fig. 28. cont.

Fig. 28. cont.

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GCGCGGCTCTCCCAACCTTT-AACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC
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TCACTGCTCTCCCAACCTTTTAACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC
CGCGCGCCTCCCCAACCTTTTAACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC
GCGGGGCTCCCCCAACCATTTAACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC
CCGCGGCTCCCCCAACCTTT-AACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC
..GCGGCTCCCCCAACCTTTTAACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC
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TTTCCTTCCCCCCAACCTTTTAACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC
CCGCGGCTCCCCCAACCTTT-AACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC
CCGCGGCTCCCCCAACCTTTTAACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC
CCGCGGCCCCCCCAACCTTT-AACGTTGTAAAACTTTCAGCGACGGATGTCTTGGCTCCC

ACAACGATGAAGAACGCAGCGAAATGCGATACGTCTTGCGACTTGCAGAATCCAGTGAAT
ACAACGATGAAGAACGCAGCGAAATGCGATACGTCTTGCGACTTGCAGAATCCAGTGAAT
ACAACGATGAAGAACGCAGCGAAATGCGATACGTCTTGCGACTTGCAGAATCCAGTGAAT
ACAACGATGAAGAACGCAGCGAAATGCGATACGTCTTGCGACTTGCAGAATCCAGTGAAT
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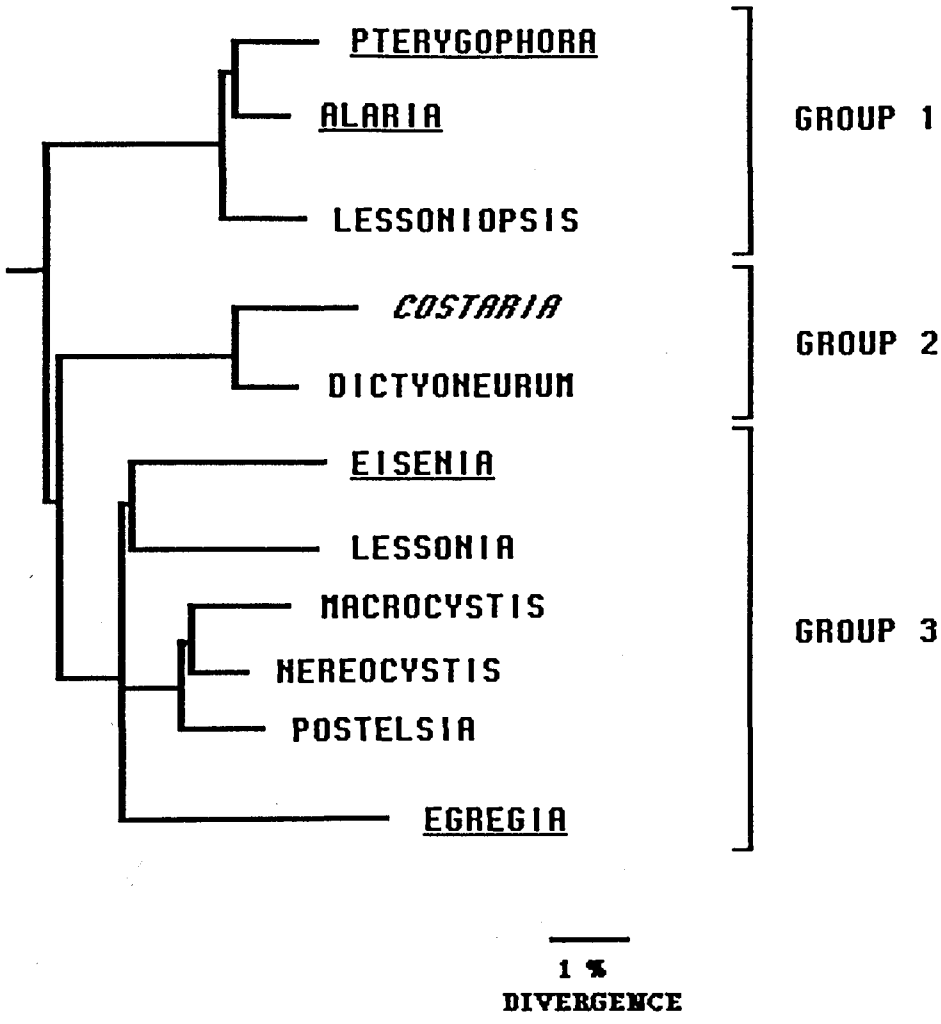
ACAACGATGAAGAACGCAGCGAAATGCGATACGTCTTGCGACTTGCAGAATCCAGTGAAT
ACAACGATGAAGAACGCAGCGAAATGCGATACGTCTTGCGACTTGCAGAATCCAGTGAAT

CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATATTCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATATTCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCACCTTGCGCTTCCGGGATATTCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATAcgCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATACTCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATACTCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATACTCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATACTCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATACTCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCACCTTGCGCTTCCGGGTTACTCCTGGGAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATACTCCTGGAAGCATGCTTGTCGGA
CATCAAAACTTTGAACGCATCTTGCGCTTCCGGGATACTCCTGGGAGCATGCTTGTCGGA

GTGTCTGTTGACACCACTCGC      ALARIA
GTGTCTGTTGACACCACTCGC      LESSONIOPSIS
GTGTCTGTTGACACCACTCGC      PTERYGOPHORA
GTGTCTGTTGACACCACTCGC      EGREGIA
GTGTCTGTTGACACCACTCGC      EISENIA
GTGTCTGTTGACACCACTCGT      POSTELSIA
GTGTCTGTTGACACCACTCGC      NEREOCYSTIS
GTGTCTGTTGACACCACTCGC      DICTYONEURUM
GTGTCTGTTGACACCACTCGC      DICTYONEUROPSIS
GTGTCTGTTGACACCACTCGC      LESSONIA
GTGTCTGTTGACACCACTCGC      MACROCYSTIS
GTGTCTGTTGACACCACTCGC      COSTARIA
```

Fig. 29. Phylogenetic tree presenting relationships among representatives of 11 kelp genera. Underlined generic epithets were traditionally in the Alariaceae. Plain generic epithets were originally in Lessoniaceae. Italicized generic epithet was in Laminariaceae

Fig. 29.


PTERYGOPHORA
ALARIA
LESSONIOPSIS
COSTARIA
DICTYONEURUM
EISENIA
LESSONIA
MACROCYSTIS
NEREOCYSTIS
POSTELSIA
EGREGIA
GROUP 1
GROUP 2
GROUP 3
1 %
DIVERGENCE

CHAPTER 6

CONCLUSIONS

In this thesis I have applied and developed techniques for the phylogenetic analyses of all levels of phaeophycean taxonomy above the species level. This was probably the most significant accomplishment of my endeavours. These techniques and modifications of them are currently being used in our Laboratory to address a variety of aspects concerning phaeophycean phylogeny and evolution. The current and future efforts will contribute much to the knowledge of this exciting and diverse group of eukaryotes.

In developing these techniques I have also provided some new and potentially controversial insights into laminarialean phylogeny and evolution. Based on the SSU sequence data I propose a recent origin for the advanced Laminariales, 16-20 mya, rather than the 200-300 mya suggested in some interpretations of the fossil record (see Loeblich 1974). This suggests that this morphologically complex assemblage of algae has acquired its magnificent diversity in a very short evolutionary time span. The inherent phenotypic plasticity displayed by this assemblage of algae, probably provided the evolutionary framework for such incredible and rapid diversity. The molecular basis of both phenotypic plasticity and morphological diversity in the kelp has yet to be explored. This may prove to be one of the most exciting features of kelp molecular biology.

By investigating the ITS1 region for a variety of kelp, I have also provided a new and potentially controversial hypothesis on advanced kelp relationships. I am not certain if the relationships presented in this thesis represent the phylogenetic order of things or an artifact of technique or nature. I am hopeful however, that they will encourage and stimulate new discussions on the long closed story of kelp phylogeny. I believe that Setchell & Gardner (1925) themselves, based on their insightful commentaries, would be less shocked by my

findings than will some of my contemporaries. I only ask that the reader consider the ideas I present with an open mind.

I feel this research has set the stage for a new and exciting chapter on phaeophycean phylogeny and evolution. I hope that I will be fortunate enough to continue to be involved with this aspect of evolutionary research. The Phaeophyta, in particular the Laminariales, are unquestionably some of the most magnificent eukaryotes to study and observe.

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