

**MOLECULAR PHYLOGENY OF THE KELP GENUS LAMINARIA
(LAMINARIACEAE: PHAEOPHYTA)**

by

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B.Sc., Simon Fraser University, 1984

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in the Department
of
Biological Sciences

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SIMON FRASER UNIVERSITY

November 1993

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(LAMINARIACEAE: PHAEOPHYTA)**

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(LAMINARIACEAE: PHAEOPHYTA

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ABSTRACT

Evolutionary and taxonomic relationships among kelp species (Laminariales: Phaeophyta) are currently established on the basis of morphological characters. Extensive phenotypic plasticity and the occurrence of hybridization between putative species, genera and families, along with a poor fossil record, limit the utility of morphological similarity for inferring phylogenetic and taxonomic relationships among extant kelp species. To overcome this limitation, evolutionary relationships may be inferred from DNA sequences which provide characters independent of morphology.

Phylogenetic relationships among nine species of the kelp genus Laminaria Lamouroux [L. angustata Kjellman, L. digitata (Huds.) Lamouroux, L. ephemera Setchell, L. groenlandica (Rosenvinge) sensu Druehl, L. japonica Areshoug, L. longipes Bory, L. saccharina (L.) Lamouroux, L. setchellii Silva, and L. sinclairii (Harv.) Farlow, Anderson & Eaton] were inferred from rDNA sequences. Approximately 900 bp were sequenced for each of the study taxa which included the 3' end of the Small Subunit gene, the Internal Transcribed Spacers (ITS1 & ITS2) and the 5.8S gene. Two familial species, Cymathere triplicata (Post. & Rupr.) J.G. Agardh and Costaria costata (C. Ag.) Saunders, with morphological similarity to species within Laminaria were included in the phylogenetic analyses. Results indicate that the morphological characters of holdfast and blade type, upon which current Laminaria taxonomic and phylogenetic relationships are

based, may be invalid indicators of evolutionary relatedness.

The inferred molecular phylogenies better reflect biogeographic distributions and other characteristics of these algae.

An additional outcome of this work has been the development of fast and simple DNA extraction techniques using the alternate life history stages of kelp species. Total cellular DNA of sufficient quantity and purity for a variety of molecular applications was isolated from dried sporophyte blades, fresh gametophytes, and meiospores. In addition, DNA from herbarium specimens (up to 22 years of age) was isolated and amplified using the polymerase chain reaction and specific oligonucleotide primers. This approach may provide an alternate method for comparing the identity of type material with field collected specimens and allow access to rare herbarium material for molecular analyses.

ACKNOWLEDGEMENTS

The work of this thesis could not have been undertaken or completed without the unfailing encouragement, kindness and generosity of my senior supervisor, Dr. Louis Druehl. This thesis is dedicated to you, Louis.

I wish to express my gratitude and affection to my fellow kelpings (Gary Saunders, Ian Tan, Michael Liptack, Larry Mroz and Debashish Bhattacharya) and to adopted kelpings (Karen Beckenbach, Vic Bourne, Jackie Schein, Irene Barriga, Cam Muir, Al Arndt, Rob Linning, Marco Marra - my apologies to anyone left out). My sojourn at S.F.U. was enriched by the many friendships made here.

I also wish to acknowledge the kindness of the Bamfield Marine Station staff during my many collection trips to the West Coast of Vancouver Island. Bamfield trips always included sumptuous repasts and glorious sunsets at Maison Druehl-Hopkins a la Port Desire. This was without a doubt the icing on the cake and my stomach salutes you, Rae.

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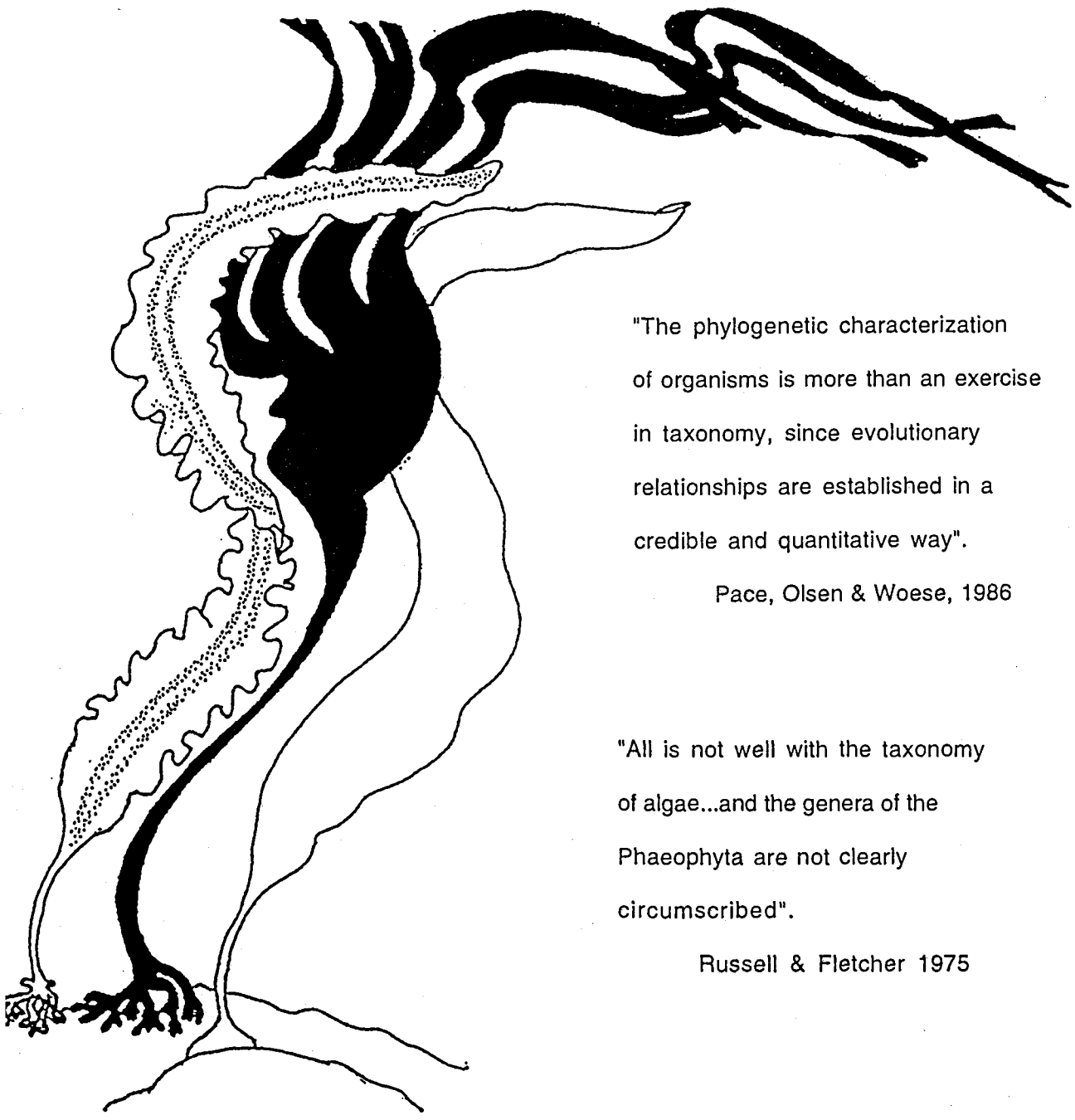
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"The phylogenetic characterization of organisms is more than an exercise in taxonomy, since evolutionary relationships are established in a credible and quantitative way".

Pace, Olsen & Woese, 1986

"All is not well with the taxonomy of algae...and the genera of the Phaeophyta are not clearly circumscribed".

Russell & Fletcher 1975

INTRODUCTION

Kelp species (Laminariales: Phaeophyta) are the most morphologically and anatomically specialized brown algal members of temperate, seaweed floras worldwide. Evolutionary and taxonomic relationships within kelp families, such as the Laminariaceae, are currently established on the basis of morphological characters. However, relationships based on morphological similarity are problematic due to the considerable phenotypic plasticity displayed by kelp species (Svendsen & Kain 1971; Mathieson *et al.* 1981; Druehl & Kemp 1982) and the demonstration of hybridization between species, genera and families (Sundene 1958; Luning 1975, Sanbonsuga & Neushel 1978; Cosson & Olivari 1982; Bolton *et al.* 1983; Coyer *et al.* 1992; Liptack & Druehl 1992). In addition, the phylogenetic utility of the traditional morphological characters chosen to establish relationships among kelp species remains uncorroborated by the present insubstantial fossil record (Druehl & Saunders 1992). Laminaria (Lamouroux), the largest and most complex genus of kelp, is fraught with taxonomic confusion and poor differentiation among species (Kain 1979; Tokida *et al.* 1980; Bhattacharya *et al.* 1991). Phenotypic plasticity and disagreement among authors as to what constitutes a species contribute to the taxonomic confusion (ex. Druehl 1979 versus Nicholson 1976). The problem of poor species differentiation is further compounded because some of the morphological features used to delineate species within Laminaria subtaxa are also characteristic of other kelp

species [ex.: Cymathere triplicata (Post. & Rupr.) J.G. Agardh, Ecklonia stolonifera Okamura and Streptophyllopsis kuroshioensis (Segawa) Kajimura].

Given the current problematic morphology-based taxonomy and phylogeny, the objective of this study was to obtain characters independent of morphology that could be used to infer phylogenetic relationships among representative species of Laminaria. These characters were obtained from sequence analysis of the nuclear encoded ribosomal cistron (rDNA) including the 3' end of the Small Subunit (SSU) gene, internally transcribed spacers (ITS1 & ITS2), and 5.8S gene. Nine Laminaria species [L. angustata, Kjellman, L. digitata (Huds.) Lamouroux, L. ephemera Setchell, L. groenlandica (Rosenvinge) sensu Druehl, L. japonica Areshoug, L. longipes Bory, L. saccharina (L.) Lamouroux, L. setchellii Silva and L. sinclairii (Harv.) Farlow, Anderson & Eaton] and two additional species, representing genera also within the kelp family Laminariaceae [Cymathere triplicata (Post. & Rupr.) J.G. Agardh and Costaria costata (Turn.) Saunders] were included in the phylogenetic analyses. Published sequences from two species representing the kelp families Alariaceae (Alaria marginata Postels & Ruprecht) and Lessoniaceae (Postelsia palmaeformis Ruprecht) (Saunders & Druehl 1993a), were included as outgroups.

Laminaria Life History, Taxonomy and Distribution

Laminaria species display the typical, heteromorphic life cycle of the kelp in which a macroscopic, diploid sporophyte alternates with microscopic, haploid, dioecious gametophytes. Sporophytes release biflagellated meiospores which settle and germinate into filamentous gametophytes responsible for the production of gametes. Following fertilization, a diploid zygote develops, giving rise to the parenchymatous sporophyte phase. Laminaria sporophytes have a tripartite morphology (Fig. 1a). Sporophytes attach to hard substrates by a root-like or disc-shaped holdfast, from which a stem-like stipe and simple terminal blade arise. Between the stipe and blade is the intercalary meristem or transition zone (Fig. 1a).

The kelp family, Laminariaceae Reichenbach, to which the genus Laminaria belongs, is one of six families in the order Laminariales. Three kelp families, the Laminariaceae, Lessoniaceae and the Alariaceae are considered evolutionarily advanced because of their morphological and anatomical specialization and the absence of eyespots as observed in the other three families (Druehl & Saunders 1992). The Laminariaceae contains the greatest number of putative kelp species (47) and genera (10) (Druehl & Saunders 1992). Members of this family lack a differentiated transition zone. True splitting resulting in branching of the transition zone (as found in the kelp family Lessoniaceae), does not occur in the Laminariaceae. In

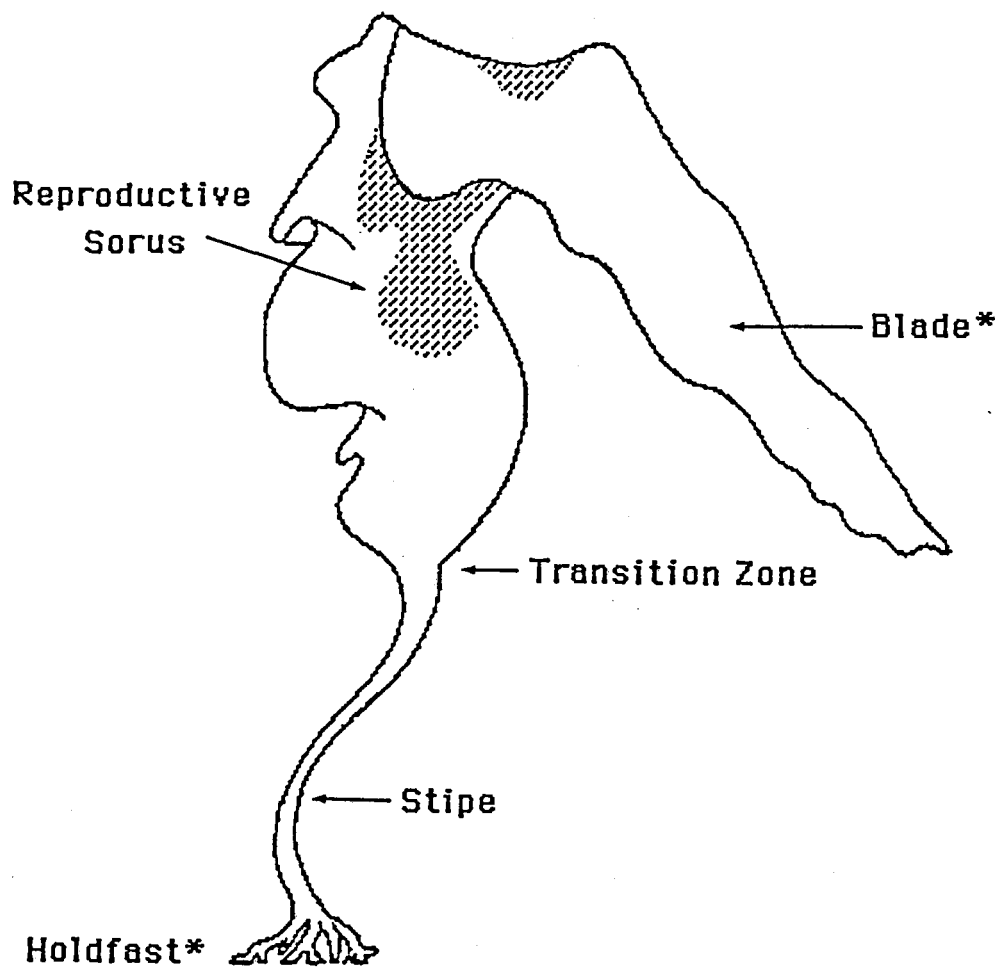


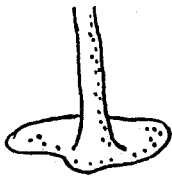
Fig. 1a. Generalized Sporophyte Morphology of Laminaria.

*Conspicuous morphological variation occurs in the holdfast type and blade features among species. Holdfasts may be either discoid, rhizomatous or hapteral while blades may have longitudinal folds (fasciate), several segments or digits (digitate) or may be undivided (simple). See Fig. 1b for examples.

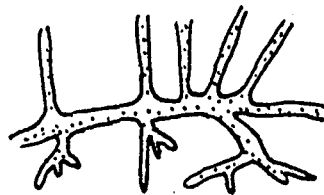
Fig. 1b Morphological variation in Laminaria:
Holdfast and blade types

HOLDFASTS

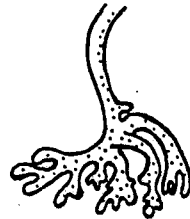
Discoid



Rhizomatous

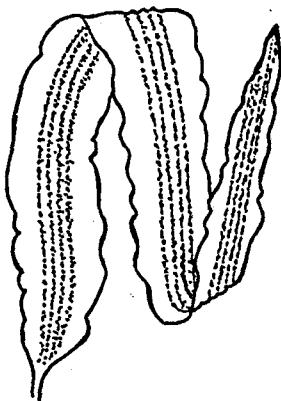


Hapteral



BLADES

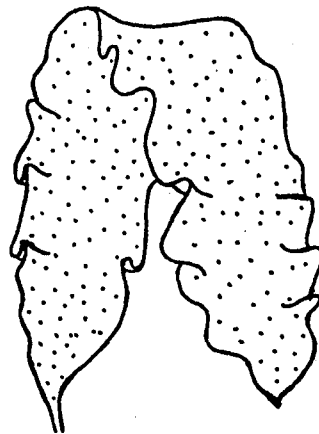
Fasciate



Digitate



Simple



contrast to the Alariaceae, reproductive sori form on the vegetative blade in members of the Laminariaceae, rather than on specialized blades (sporophylls) arranged along the stipe (Fig. 1a).

Up to 100 species have been attributed to Laminaria since Lamouroux established the genus in 1813. Environmentally-influenced characters and an underestimation of the phenotypically plastic responses of these algae, contributed to the plethora of species included by early phycologists (Kain 1979). Currently, 32 species of Laminaria are recognized, however, the inclusion of at least seven species remains controversial (Kain 1979). Present species are partitioned into three subgenera on the basis of holdfast type (Petrov 1974). Subgenus Solearia includes all species with a discoid holdfast (round, attachment disc), Rhizomaria contains species with a rhizomatous holdfast (horizontal stolons supporting multiple stipes with blades) and subgenus Laminaria includes species with a hapteral holdfast (rootlike projections) (Fig. 1b). Species within the subgenus Laminaria are further divided into sections on the basis of blade morphology: species within section Fasciatae produce a single blade with parallel, longitudinal folds (which constitute a median thickened region or fascia) (Druehl *et al.* 1988c). Section Digitatae species produce a blade with ontogenetically-induced splits or digits (Kain 1979; Druehl 1968) while section Simplices species grow a plain, undivided blade (Kain 1979) (Fig. 1b). However, species of the Simplices may display digitate blades that result from environmental damage (Druehl 1968).

Membership in the section *Digitatae* is not universally agreed upon among phycologists. Some authors consider the presence of a divided blade sufficient for inclusion in this section regardless of the origin of blade splitting (environmentally-induced versus ontogenetic) (Luning & tom Dieck 1990; tom Dieck 1992). Druehl (1968), however, includes only those Laminaria species with ontogenetically-induced blade digitation in section *Digitatae*. I have adopted the distinction of the latter author.

Species from each of the subgenera and sections of Laminaria have been included in the present study (Table 1).

Laminaria species are distributed predominantly along cold-temperate, rocky coasts of the northern hemisphere, where they inhabit the lower intertidal and subtidal zones. Six Laminaria species are known from warm temperate/tropical regions, including the coasts of Brazil, west and south Africa, the Philippines and the southern Mediterranean. However, these plants have been found only in cooler water, at depths exceeding 40 m (Joly & Oliveira Filho 1967; Kain 1979). The greatest species diversity of the genus is found in the North Pacific where approximately 22 species exist; at least 20 of these species are exclusively North Pacific in distribution (Druehl 1968; Kain 1979).

Table 1: Taxonomic synopsis of species included in the phylogenetic analyses

Laminariaceae Reichenbach (plant simple, no branching of the transition zone)

Laminaria Lamouroux

Subgenus **Solearia** Ju. Petrov (**discoid holdfast**)

L. ephemera Setchell (ephemeral, mucilage ducts absent)

Subgenus **Rhizomaria** Ju. Petrov (**rhizomatous holdfast**)

L. longipes Bory (mucilage ducts present in blade only, monomorphic gametophytes)

L. sinclairii (Harv.) Farlow, Anderson et Eaton (mucilage ducts present in stipe and blade, dimorphic gametophytes)

Subgenus **Laminaria** Ju. Petrov (**hapteral holdfast**)

Section **Fasciatae** (blade entire with longitudinal folds)

L. angustata Kjellman (blade with 1 fold)

L. japonica Areschoug (blade with 2 folds)

Section **Digitatae** (blade ontogenetically digitate)

L. digitata (Huds.) Lamouroux (stipe flexible)

L. setchellii Silva (stipe rigid)

Section **Simplexes** (blade undivided)

L. groenlandica (Rosenvinge) sensu Druehl (mucilage ducts present in stipe and blade)

L. saccharina (L.) Lamouroux (mucilage ducts present in blade only)

Cymathere J.G. Agardh (**hapteral or discoid holdfast, blade with 2 to 4 longitudinal folds**)

C. triplicata (P. & R.) J.G. Agardh (discoid holdfast, annual, blade with 3 longitudinal folds, mucilage ducts present in blade only)

Costaria Greville (**hapteral holdfast, blade with 5 percurrent midribs, annual**)

C. costata (C. Ag.) Saunders (mucilage ducts absent)

Taxa included in the Phylogenetic Analyses

Eleven species, belonging to three genera within the kelp family Laminariaceae, were included in the phylogenetic analyses. Nine of these species belong to Laminaria and include:

One species from the subgenus Solearia:

L. ephemera Setchell - delicate sporophytes have small, discoid holdfasts, flexible stipes and undivided blades. Mucilage ducts are absent from both stipes and blades. Ephemeral sporophytes inhabit the subtidal or lower intertidal surge channels. L. ephemera is a Northeast Pacific endemic species, distributed from Ucluelet, British Columbia to San Luis Obispo County with an isolated population at Volga Island, Alaska (Druehl 1969; Klinger 1984) (Fig. 2).

Two species from the subgenus Rhizomaria:

L. longipes Bory - displays a rhizomatous holdfast from which multiple stipes arise. Each stipe supports a simple, undivided blade. Both the holdfast and the stipes are perennial. Proximal remnants of the blades are retained while new blades are produced. Mucilage ducts occur only in the blades. L. longipes is unusual among Laminaria species in having indistinguishable (monomorphic) gametophytes. An inhabitant of lower intertidal and upper subtidal regions, this species is found growing on rocks in moderately exposed or moderately sheltered environments. L. longipes is distributed in the Kuriles and from Attu Island to Coronation Island, Alaska, with a single, isolated

subtidal population at San Juan Island, Washington, U.S.A. (Druehl 1969; Markham 1972) (Fig. 3a).

L. sinclairii (Harv.) Farlow, Anderson & Eaton - the sporophyte of this species is morphologically similar to L. longipes; a rhizomatous holdfast supports numerous stipes, each with a single, undivided blade. The holdfast and the stipes are perennial while the entire blade is lost prior to the production of a new blade. Mucilage ducts are found in both the stipes and blades. L. sinclairii is found attached to rocks in lower intertidal, wave-exposed environments. The sporophyte may tolerate seasonal sand burial. Endemic to the Northeast Pacific, L. sinclairii occurs from central British Columbia (Hope Island) to Ventura County, California (Markham 1972) (Fig. 3b).

A total of six species from the subgenus Laminaria with two representatives of the Fasciate Section:

Laminaria angustata Kjellman - the sporophyte of this alga has a hapteral holdfast, a flexible stipe, and a blade with a single longitudinal fold. Mucilage ducts occur in both the stipe and blade. The sporophyte is perennial (Hasegawa 1962) and inhabits lower intertidal to subtidal regions, along exposed coasts where strong, cold water currents are mixed with warmer currents (Miyabe 1902). L. angustata is a Northwest Pacific endemic species, found along the southern coast of Hokkaido in Japan and the Kuriles in Russia (Fig. 4a).

L. japonica Areshoug - has a hapteral holdfast, a flexible stipe and a blade with two parallel, longitudinal folds. Mucilage ducts are present in both the stipe and blade. Perennial sporophytes inhabit

moderately wave-exposed, lower intertidal and subtidal regions.

L. japonica is a Northwest Pacific endemic species, distributed along the coasts of northern Japan, the Kuriles (Tokida et al. 1980) and Korea (Kang 1966) (Fig. 4b).

Two species represented the Digitate Section:

L. digitata Lamouroux - sporophytes have hapteral holdfasts, flexible stipes and digitate blades, which are the result of ontogenetically-induced splitting. Mucilage ducts are absent in the stipe. Perennial sporophytes inhabit wave-exposed lower intertidal and upper subtidal zones and thrive especially in a strong reversing current (Kain 1979). L. digitata is distributed in the northeast and northwest Atlantic (Kain 1979) and in Canadian Arctic coastal waters (Lee 1980) (Fig. 5a).

L. setchellii Silva - has a hapteral holdfast, a rigid stipe, and an ontogenetically-induced digitate blade. Mucilage ducts are present in both the stipe and blade. Perennial sporophytes inhabit wave-exposed rocky, intertidal regions. L. setchellii is endemic to the Northeast Pacific coast, from Yukatat, Alaska to southern California (Druehl 1968) (Fig. 5b).

Two species represented the Simplicis section:

L. groenlandica (Rosenvinge) sensu Druehl - has a hapteral holdfast, a flexible stipe, and a blade that often displays environmentally-induced digitation. Bullae, when present, form two longitudinal rows parallel to the blade margins. Perennial sporophytes inhabit rocky intertidal and upper subtidal regions with moderate wave-exposure or the subtidal regions of estuaries.

L. groenlandica is distributed in the Canadian Arctic (Lee 1980) and along the east Pacific coast, from Alaska to Oregon (Druehl 1968) (Fig. 6a).

L. saccharina (L.) Lamouroux - each sporophyte has a hapteral holdfast, a flexible stipe, and a blade that is either undivided or displays environmentally-induced digitation. Mucilage ducts are found only in the blade. Bullae, when present, occur in two longitudinal rows parallel to the blade margins. Sporophytes of L. saccharina from the North Pacific are annual while north Atlantic sporophytes are perennial (Hruby 1976; Druehl & Hsiao 1977). The species grows subtidally or in wave-sheltered, intertidal habitats (Druehl 1967). L. saccharina has a cosmopolitan distribution in the northern hemisphere including the Arctic (Wilce 1989; Luning 1991) (Fig. 6b).

Two species, from the genera Costaria and Cymathere, are phenotypically similar to species within Laminaria and were therefore included in the phylogenetic analyses:

Costaria Greville is a monotypic genus and is distinguished from other genera of the Laminariaceae by possession of a blade with five, longitudinal ribs (Setchell & Gardner 1925).

Costaria costata (C. Agardh) Saunders - displays a hapteral holdfast, a stipe that may have numerous fine, parallel, longitudinal grooves, and a coarsely bullate blade with five prominent, longitudinal ribs. Mucilage ducts are absent. Annual sporophytes inhabit wave-sheltered or wave-exposed intertidal and subtidal

regions. Costaria costata is a Northeast Pacific endemic species, commonly found from the Bering Sea to San Pedro, California (Setchell & Gardner 1925; Druehl 1968) (Fig. 7).

Cymathere (J.G. Agardh) was originally established to accommodate a single species with a discoid holdfast and a blade with three parallel, longitudinal folds, previously known as Laminaria triplicata Postels & Ruprecht. Agardh moved this species to its own genus because of its supposedly unique discoid holdfast. Subsequently, three kelp species with discoid holdfasts were included in Laminaria. Meanwhile, Miyabe (1936) added two Japanese species to Cymathere (C. japonica and C. fibrosa); both have hapteral holdfasts and fasciate blades similar to the fasciate laminarians. There are no consistent, gross morphological features which separate species of Cymathere from those of Laminaria. However, anatomically, Cymathere species can be distinguished by the size and shape of the paraphyses (sterile filaments interspersed among the sporangia of the soral tissue) (Okamura 1936).

Cymathere triplicata - has a discoid holdfast, a flexible stipe and an undivided blade, with three parallel, longitudinal folds. Mucilage ducts are present in both the stipe and blade. Annual sporophytes inhabit rocky, moderately wave-sheltered intertidal areas or protected, deep tidepools in wave-exposed regions. C. triplicata is endemic to the Northeast Pacific from the Bering Sea to Puget Sound (Druehl 1968) (Fig. 8).

Fig. 2. Subgenus **Solearia** (Petrov 1974) - species representative:

- a). **Laminaria ephemera** Setchell from British Columbia
Canada



Fig. 3. Subgenus **Rhizomaria** (Petrov 1974) - representative species:

- a). **Laminaria longipes** Bory from Washington, U.S.A.
- b). **L. sinclairii** (Harv.) Farlow, Anderson & Eaton from British Columbia, Canada.

Fig. 3

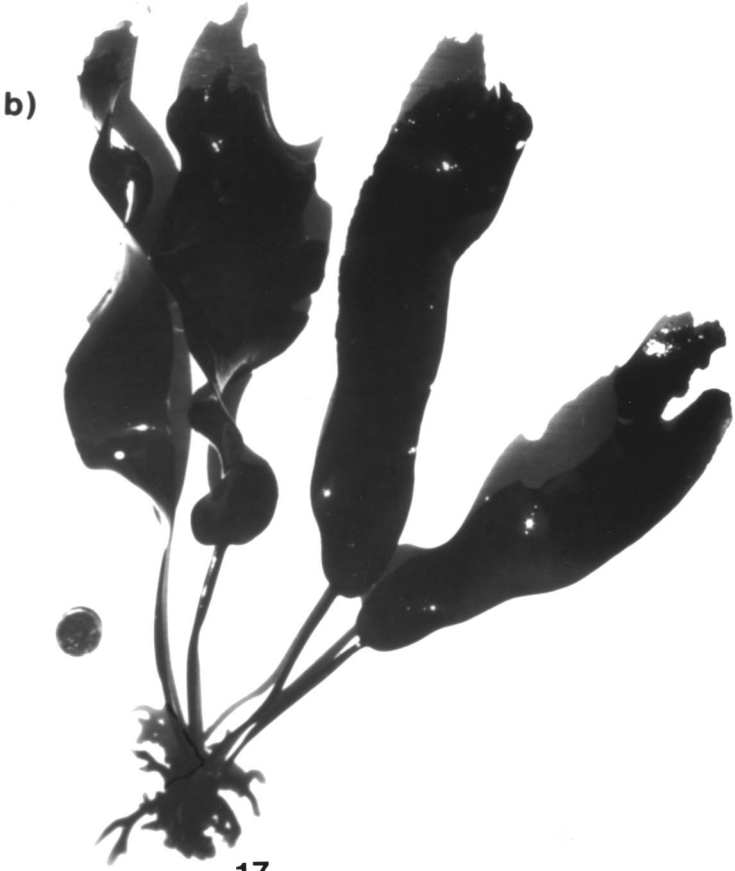
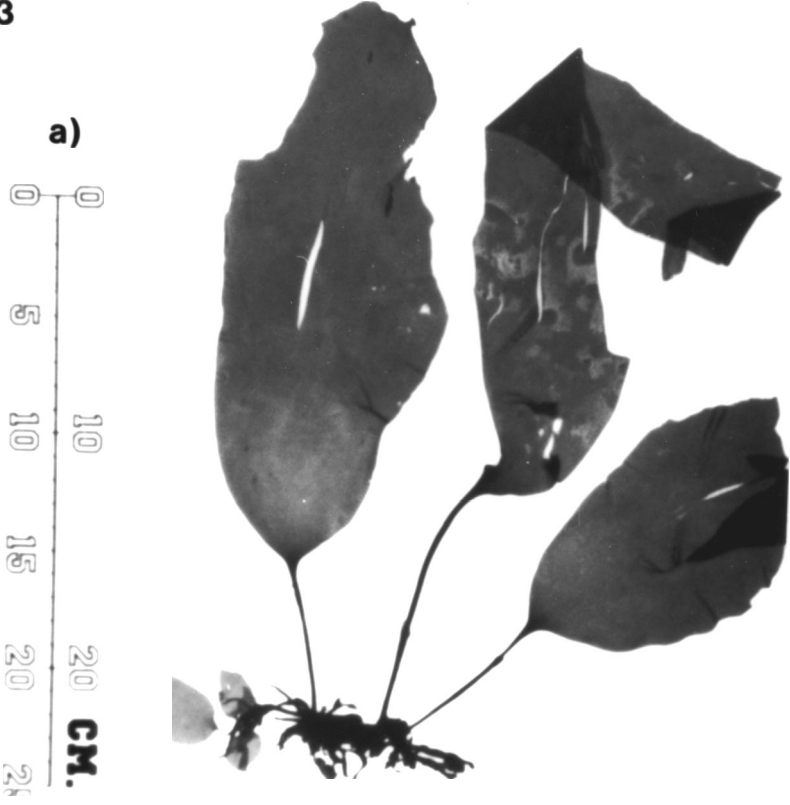
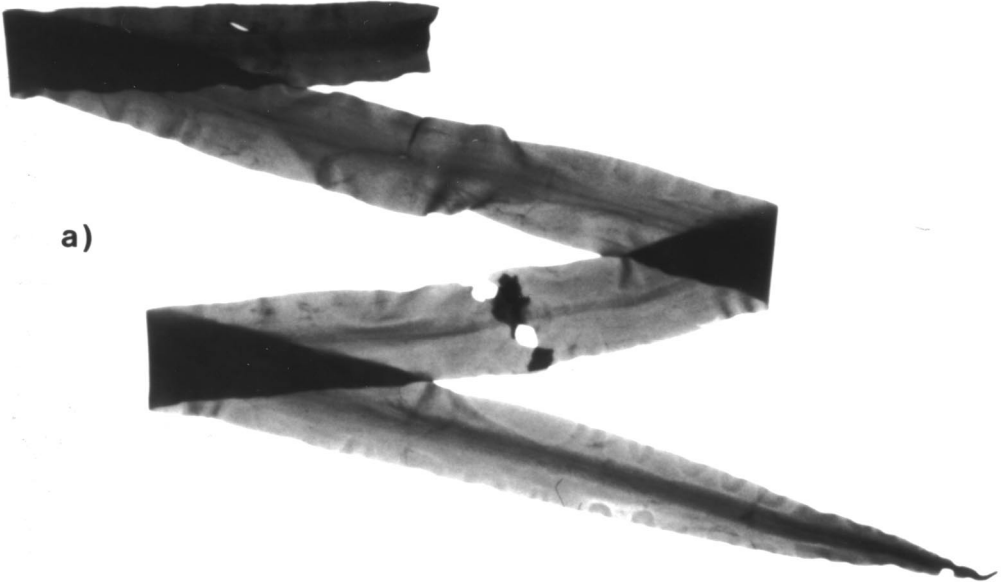


Fig. 4. Subgenus **Laminaria** (Petrov 1974) - Section Fasciatae:
representative species:

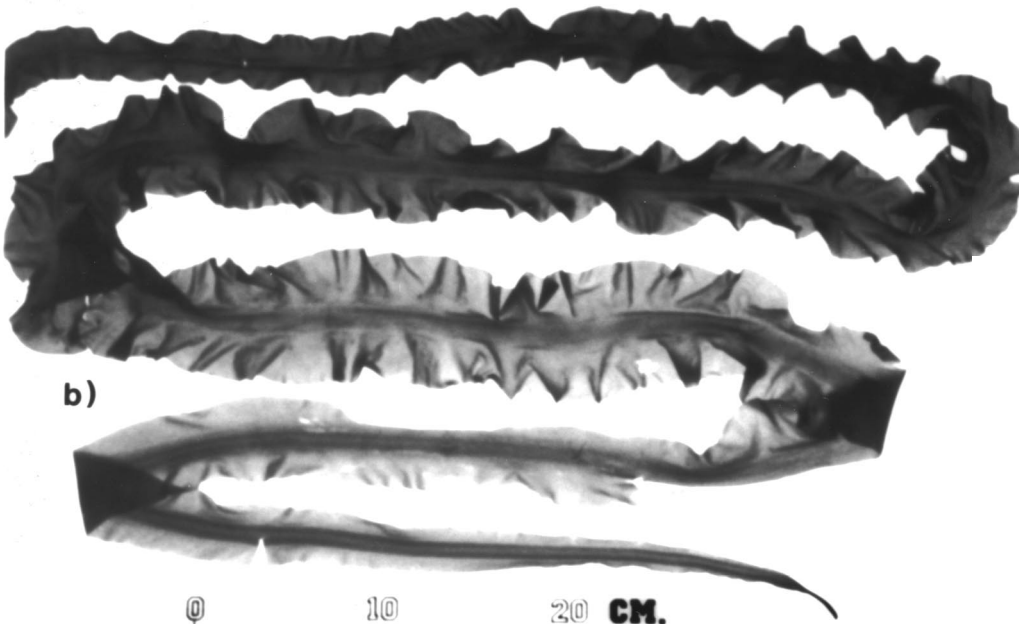
- a). **Laminaria angustata*** Kjellman from Japan,
- b). **L. japonica*** Areshoug from Japan.

*Hapteral holdfasts are missing from
these specimens.

Fig. 4



a)



b)

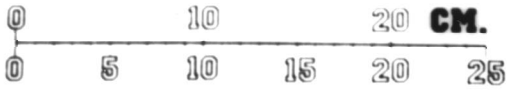


Fig. 5. Subgenus **Laminaria** (Petrov 1974) - Section Digitatae:
representative species:

- a). **Laminaria digitata** (Huds.) Lamouroux from
Hudson Bay, Canada.

- b). **L. setchellii** Silva from British Columbia, Canada.

Fig. 5



b)

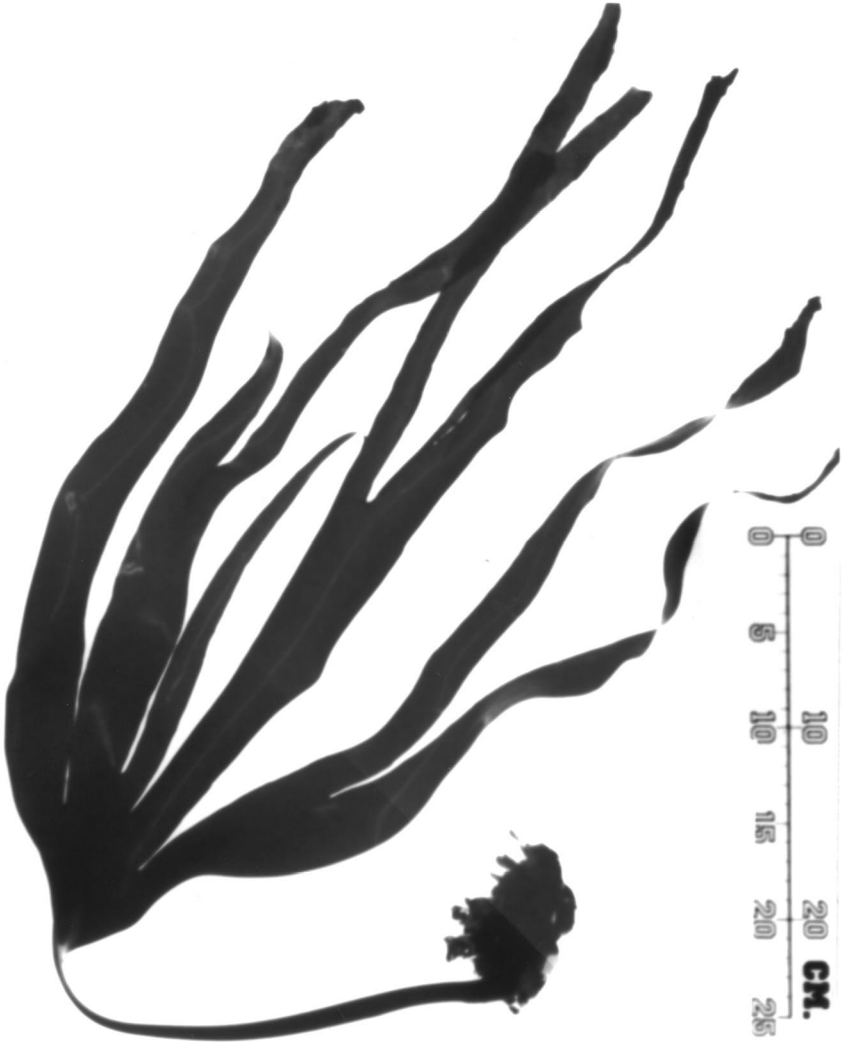


Fig. 6. Subgenus **Laminaria** (Petrov 1974) - Section Simplicis:
representative species:

- a). **Laminaria groenlandica** (Rosenvinge) sensu Druehl
from British Columbia, Canada.
- b). **L. saccharina** (L.) Lamouroux from Helgoland,
Germany.

Fig. 6

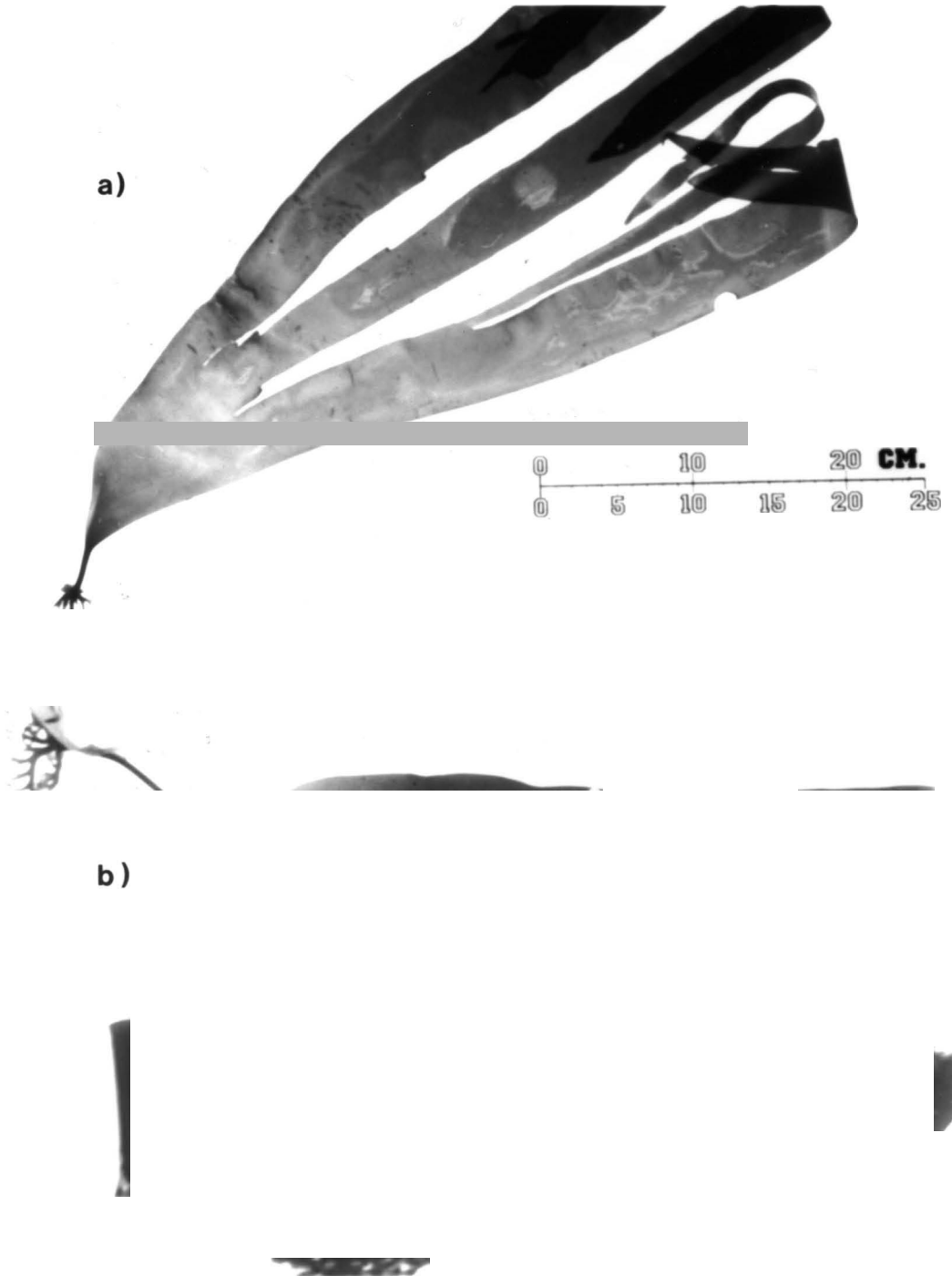


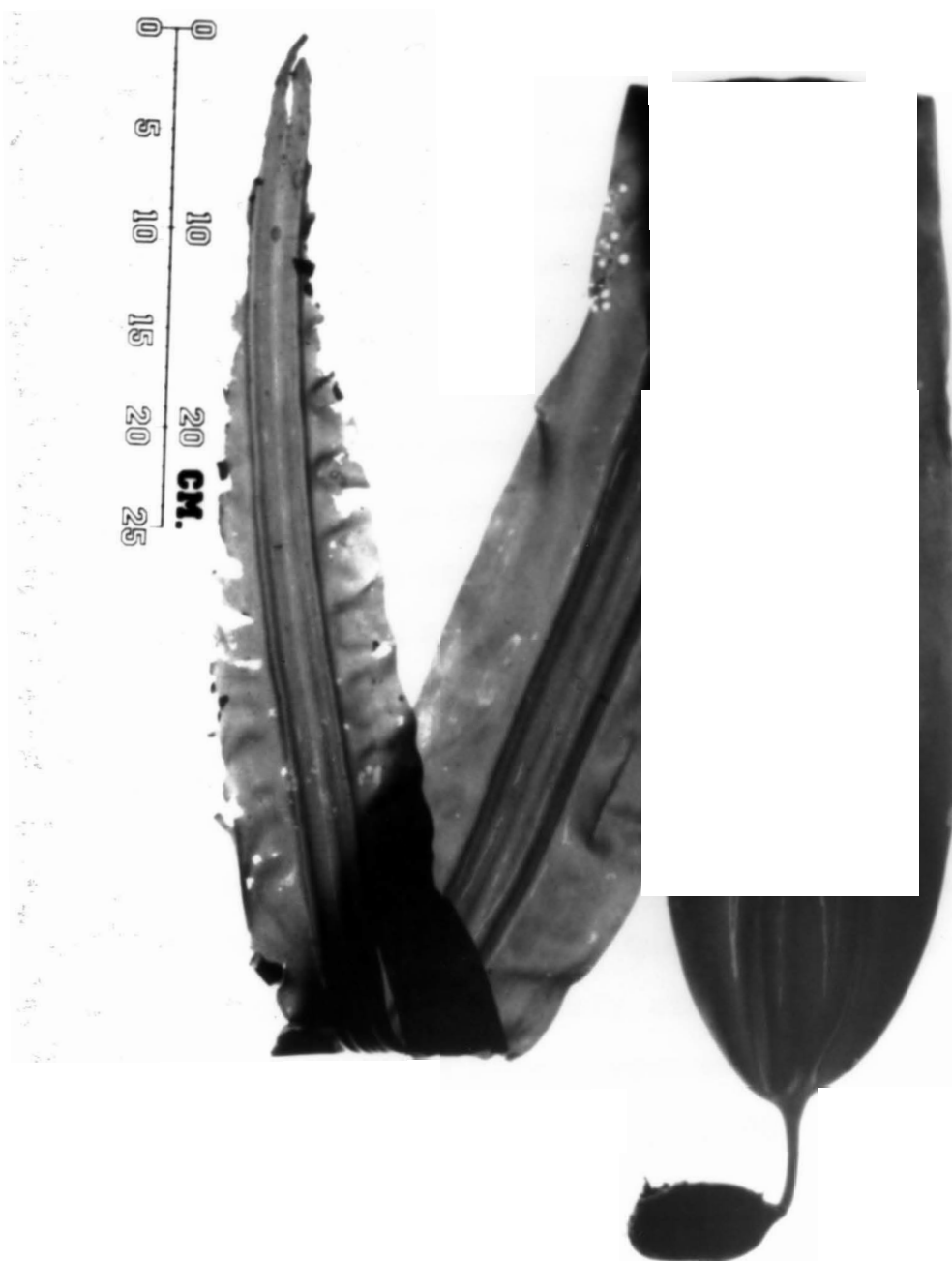
Fig. 7. Costaria costata (C. Ag.) Saunders from British Columbia, Canada.

Fig. 7



Fig. 8. **Cymathere triplicata** (Post. & Rupr.) J.G. Agardh from British Columbia, Canada.
The discoid holdfast is partially obscured by the rock to which it is attached.

Fig. 8



Molecular Phylogenetics

Molecular phylogenetics involves the comparison of molecular data for the purpose of inferring evolutionary relationships among organisms (Hillis 1987; Hillis & Moritz 1990; Li & Graur 1991). Molecular characters, in particular DNA sequences, can be advantageous alternatives to morphological characters in phylogenetic inference. DNA sequences constitute the heritable record of organismal genetic history. They not only comprise the largest possible set of characters (nucleotide substitutions, insertions, deletions, rearrangements) for phylogenetic analyses (Hillis 1987) but also range considerably in variation, from highly conserved, slowly evolving gene sequences to relatively unconserved, rapidly evolving, intron and spacer sequences (Nei, 1987; Li & Graur 1991). This range in sequence variation is particularly useful for phylogenetic comparisons across all taxonomic levels (Moritz & Hillis 1990). In addition, similarity (and dissimilarity) of molecular sequence characters is easily quantified (Fitch 1970) whereas morphological similarity is difficult to quantify (Patterson 1988). Non-transcribed or post-transcriptionally eliminated DNA sequences have the further advantage of being independent of morphological and environmental influences (Perry 1976; Gerbi 1986). Molecular characters must not only be homologous (share a common ancestry) but must also be orthologous (share a common ancestry with divergence based on speciation, rather than gene duplication)

(Hillis 1987; Olsen 1990).

However, like morphological data, molecular data are also subject to unequal rates of character evolution and misleading similarity (homoplasy) (Mishler et al. 1988). Although a positive correlation exists between accumulating nucleotide substitutions and time (Nei 1987), there are considerable differences in evolutionary rates between various groups of organisms and among different regions of the genome (Britten 1986; Hillis 1987). Therefore it is prudent to chose methods of phylogenetic analysis that do not assume constant or equal rates of change (Hillis 1987; Moritz & Hillis 1990; Swofford & Olsen 1990; Li & Graur 1991). Homoplasy occurs when a given nucleotide site has undergone multiple base changes (Sanderson & Doyle 1992). As sequence divergence between taxa increases, the expected number of homoplastic sites also increases. Given enough time, a sequence may become saturated for change, thus obscuring the historical record of change in a comparison between homologous sequences (Williams 1992). This may be especially problematic for transitions which are biochemically more likely to occur than transversions (Kimura 1980; Nei 1987; Li & Grauer 1991). Homoplasy will also result in underestimates of divergence between compared taxa. Therefore, it is vital to ascertain the regions of the genome that remain useful for inference of evolutionary relationships among variously diverged groups of organisms (Smith 1989; Swofford & Olsen 1990). For phylogenetic inference among highly diverged groups of organisms, slowly evolving, functionally conserved, gene sequence variation is commonly assessed. In these sequences the

variation observed is less likely to be obscured by homoplasy. Conversely, comparison of conserved gene sequences among recently diverged organisms may not provide enough variation for phylogenetic inference. Rapidly evolving sequences are more likely to contain the necessary variation for phylogenetic resolution at lower taxonomic levels. In addition, homoplasy is less likely to have reached saturation levels, in rapidly evolving sequences, among recently diverged organisms (Sanderson & Doyle 1992). Corrections for homoplasy, based on models of nucleotide substitution such as the Jukes & Cantor (1969) single parameter or the Kimura (1980) two parameter model are commonly applied to transform pairwise sequence dissimilarity values to estimates of evolutionary distance (Nei 1987; Hillis 1987; Swofford & Olsen 1990).

Orthologous sequences must be aligned so that the positional identity of the nucleotide sites is established; then the variation among and within sites can be assessed. Alignment of sequences by visual inspection is considered the most reliable method currently available (Swofford & Olsen 1990). Gaps are commonly inserted to assist sequence alignability in regions of high variability and to account for the occurrence of insertion or deletion events among the sequences. Although the most appropriate treatment of gaps in phylogenetic analyses is currently unresolved, they can be either treated as a fifth character state or as missing information (and eliminated from the analyses) (Swofford & Olsen 1990; Felsenstein 1993). Regions of ambiguous alignment (where site homologies are

difficult to infer without the inclusion of numerous gaps) may contribute erroneous information to subsequent phylogenetic analyses and should therefore, be removed (Swofford & Olsen 1990).

From a statistical point of view, multiple as opposed to single data sets are preferable for phylogenetic analyses. In practice however, obtaining a large number of data sets for comparison is infeasible. However, bootstrap analysis is a sampling method which enables the generation of multiple data sets from a single data set of aligned sequences (Felsenstein 1985; Swofford & Olsen 1990). The nucleotide sites are subjected to repeated random sampling (with replacement) in order to generate multiple data sets. The collection of data sets can in turn be analyzed by the phylogenetics algorithms. Bootstrapping is used to place confidence limits on phylogenies (Felsenstein 1985; Swofford & Olsen 1990).

Methods of molecular phylogenetic analyses generally fall into one of two categories; distance methods or maximum parsimony methods. Distance methods involve pairwise comparisons of aligned sequences and calculation of distance values (based on sequence dissimilarity) between all possible pairs of sequences. 'Distance' is a representation of the number of nucleotide differences between compared sequences (Swofford & Olsen 1990). The values are corrected for homoplasy (as described above) and a matrix of the transformed evolutionary distance estimates between sequence pairs is tabulated, followed by the application of a clustering algorithm from which phylogenetic trees are inferred (Nei 1987). Clustering methods group taxa based on highest similarity (lowest distance

estimates). The Neighbor-joining method of Saitou & Nei (1987) is considered a superior clustering method because it does not assume uniform rates of nucleotide change among organisms. This method chooses a pair of taxa to minimize distance of the rest of the tree (Saitou & Nei 1987). Neighbor-joining has also been found to be a superior method in inferring the correct distance tree (Saitou & Imanishi 1989).

Rather than distance values, maximum parsimony methods use character state data as the basis for phylogenetic inference (Nei 1987; Williams 1992). In these methods, only the character states of the nucleotides at informative sites are considered. A site among aligned, orthologous sequences is considered phylogenetically informative if at least two of the taxa compared share a character state different from that of the other taxa (Nei 1987). Informative sites group organisms into clades, thereby preferentially supporting certain phylogenies. This information is used to infer the character states at the same sites in the ancestral sequence (Maddison *et al.* 1984). Maximum parsimony methods then find the evolutionary tree(s) which explain the evolution of the characters observed in the extant taxa, through the minimum number of mutational changes (Felsenstein 1983; Nei 1987).

The results of phylogenetic analyses are summarized in tree diagrams. Information concerning branching order (genetic affinity or relative recency of common ancestry), and branch lengths (the amount of change each taxon has accumulated since divergence from their common ancestor) can be depicted by these trees. Phylogenetic trees

may be rooted or unrooted. 'Rooting' helps to put into context the observed distance or the direction of character state change between the taxa compared. When a fossil record is depauperate for a group of organisms, character state polarity, whether primitive or derived, of the characters being compared, can be hypothesized by the use of an outgroup organism (Maddison et al. 1984; Moritz & Hillis 1990). For characters that vary in the group of interest (ingroup), the state of these characters in the outgroup is assumed to be the primitive state (Theriot 1989). Use of multiple outgroups is recommended because the character state at a given informative site, in a particular outgroup organism, could be the derived rather than the ancestral state. Outgroup organisms with presumed close evolutionary relationships to the ingroup organisms should be chosen (Maddison et al. 1984; Li & Graur 1991). If an outgroup organism is too distantly related to the ingroup, it may not have enough sites in common to provide unequivocal information about character state polarity (Williams 1992). In distance analyses, a too distant outgroup may cause serious errors in tree topology (Li & Graur 1991).

In 'strict consensus' trees, defined branches are in accordance with all contributing data. 'Majority consensus' trees are similar but differ in that they include monophyletic groups found in the majority of trees generated by the particular phylogenetic analysis (Hillis 1987; Felsenstein 1993). Both distance and parsimony methods have weaknesses and strengths: distance analyses use all of the sequence variation among compared taxa but reduce the variation to a single

numerical value and so, information is lost during the process. Parsimony methods, on the other hand, only examine the variation at informative sites and ignore autoapomorphies (variation unique to a single sequence).

According to Theriot (1989), the robustness of a phylogenetic tree can be assessed by testing it with the predictions it infers. In other words, do other lines of evidence (eg. biogeographic, interfertility studies, chromosome number similarities, temperature tolerances, independent molecular data), corroborate the groupings hypothesized by the cladogram? "It seems unlikely that several lines of evidence could support the same hypothesis through chance alone" (Theriot 1989, pg. 409).

Ribosomal Cistron

The nuclear encoded ribosomal cistron is considered an ideal system for molecular phylogenetics. rRNA genes in all extant organisms share a common ancestry (Sogin & Gunderson 1987). Further, this multi-copy, multigene family is easily isolated and contains a spectrum of sequence variation that facilitates phylogenetic resolution at all levels of the taxonomic hierarchy (Hillis 1987; Hillis & Dixon 1991; Druehl & Saunders 1992). The spectrum of sequence divergence results from the different levels of functional constraint on regions within the cistron (Hillis 1987; Hillis & Dixon 1991). Nuclear rDNA cistrons are composed of tandemly arranged repeats or units containing ribosomal RNA genes and spacer regions (Long & Dawid 1980; Appels & Dvorak 1982; Gerbi 1986; Appels & Honeycutt 1986) (Fig. 9a). The rDNA genes are post-transcriptionally complexed with proteins to form the subunits of the cytoplasmic ribosomes involved in protein synthesis (Perry 1976). In eukaryotes, the cistron occurs in several hundred to thousands of copies per nucleus (Rogers & Bendich 1987). The process of concerted evolution (accomplished through gene conversion and unequal crossing over) leads to the almost complete homogenization of the cistron copies within the cells of individuals and species (Dover 1982; Worton 1988; Sanderson & Doyle 1992). This property is especially beneficial for phylogenetic studies because it implies that any rDNA cistron copy can be sampled and compared among taxa as though it

Fig. 9: Schematic of the kelp nuclear-encoded Ribosomal Cistron.

a). Organization of one tandem repeat unit (cistron) of ribosomal DNA.

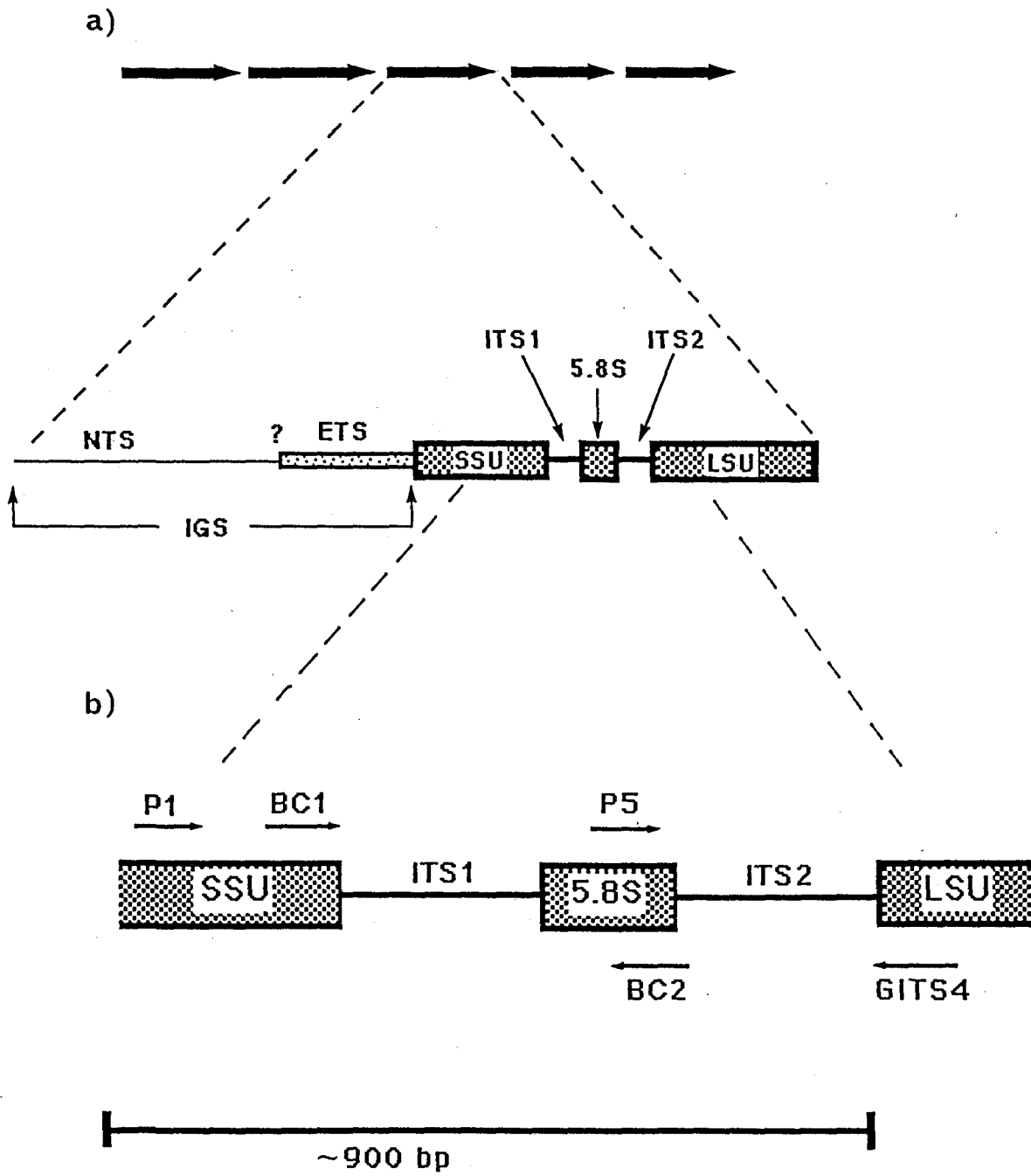
The Small Subunit gene (SSU), 5.8S and Large Subunit gene (LSU) alternate with spacer regions. The Internal Transcribed Spacers (ITS1 & ITS2) are moderately conserved, post-transcription eliminated sequences. The most variable sequence of the cistron, the Non-transcribed Spacer (NTS), together with the External Transcribed Spacer (ETS) make up the Intergenic Spacer (IGS). The exact location of the boundary between the NTS and the ETS is not known in the kelp and therefore marked with a "?".

b). Detail of the 3' SSU gene, ITS1, 5.8S gene, and ITS2 - 5' LSU gene regions of a ribosomal DNA repeat.

Approximately 900 bp of sequence were analyzed for variation and phylogenetic utility among 11 kelp species.

Universal rDNA primers, P1 and GITS4 were used to amplify the target region using the Polymerase Chain Reaction (PCR). Kelp specific, internal rDNA primers, BC1, BC2 and universal primer P5, were used to amplify and sequence subfragments of the target region.

Fig.9



were the only copy in existence (Sanderson & Doyle 1992).

Similar rDNA cistron organization (Fig. 9a) has been found among higher eukaryotes in general, (Gerbi 1986; Appels & Honeycutt 1986) in all higher plants studied (Rogers & Bendich 1987) and in algae (Berger & Schweiger 1982; Bhattacharya & Druehl 1989; Saunders & Druehl 1992). In kelp, the gene regions consist of the Small Subunit (SSU) or 18S-like gene, the 5.8S gene and the Large Subunit (LSU) or 26S-like gene. rDNA gene sequences are highly conserved (Gerbi 1986; Appels & Honeycutt 1986) and have been used to infer evolutionary relationships between highly diverged groups of organisms from the Eubacteria, Archebacteria and Eukaryota (Pace et al. 1986; Sogin 1989; Sogin et al. 1989, Hillis & Dixon 1991). Within the major divisions of the algae, rDNA gene sequences, particularly from the SSU gene, have been used for phylogenetic inference (Zechman et al. 1990 for chlorophytes; Bird et al. 1990 for rhodophytes; Bhattacharya & Druehl 1988, Tan & Druehl 1993 for phaeophytes). The 5.8S gene, by itself, has limited phylogenetic utility because of its small size (Walker 1985; Martin et al. 1990). At the other extreme, the intergenic spacer (IGS), composed of the non-transcribed spacer (NTS) and the externally transcribed spacer (ETS), is thought to be under little evolutionary constraint and accumulates mutations at a much higher rate than any other region of the cistron (Gerbi 1986; Bhattacharya et al. 1991). Molecular data from the IGS have been used to distinguish populations and species of a variety of eukaryotic organisms (Strauss et al. 1990; reviewed by Hillis & Dixon 1991), including kelp populations and species (Bhattacharya & Druehl

1989; Mroz 1989; Bhattacharya et al. 1991). In contrast to the gene regions and the IGS, the internally transcribed spacers (ITS1 and ITS2) which flank the 5.8S gene, are moderately conserved (Appels & Dvorak 1982; Verbeet et al. 1984; Gerbi 1986; Yokota et al. 1989) and as such, these spacers were deemed phylogenetically useful for resolution at the genus/species level (Hillis & Dixon 1991; Swofford & Olson 1990; Lee & Taylor 1991; Wesson et al. 1993).

The rDNA Internal Transcribed Spacers (ITS1 & ITS2)

The rDNA internal transcribed spacers, ITS1 and ITS2 are post-transcriptionally eliminated sequences, flanked by the gene sequences of the ribosomal cistron (Fig 9b). The ITS1 is thought to have originated as a true spacer sequence (Clark 1987) while the ITS2 is reported to have arisen from an intron-like structure and is similar to the variable Expansion Sequences (ES) found in the the LSU gene (Gerbi 1986; Torres et al. 1990) (Fig. 9b). The increased sequence variability of these spacers, relative to the rRNA genes, is the result of decreased functional conservation. Nonetheless, some sequence conservation is apparent, at least within various groups of organisms, and it is thought that these spacers must contain the signals for post-transcriptional processing (Torres et al. 1990; Wesson et al. 1992).

Partial or complete rDNA internally transcribed spacer sequences have been determined for a variety of organisms including

lower eukaryotes such as yeast (Veldman et al. 1981), fungi (Lee & Taylor 1991; Carbone & Kohn 1993), and slime mold (Ozaki et al. 1984), numerous higher eukaryotes, (Hindenach & Stafford 1984; Stewart et al. 1983; Goldman et al. 1983) including a number of primate species (Gonzalez et al. 1990) and several higher plant species (reviewed by Torres et al. 1990; Baldwin 1992). Many of the recently published ITS sequences have come from the algae. These include sequences for a variety of green algal species and isolates (Bakker et al. 1991; Kooistra et al. 1992; van Oppen et al. 1993), the brown algal species Desmarestia viridis/willii (van Oppen et al. 1993) and 12 kelp species representing the Alariaceae, Lessoniaceae and Laminariaceae (Saunders & Druehl 1993b).

Comparison of ITS rDNA sequence variation has been useful for delineating eukaryote populations and species (Gonzalez et al. 1990b; Lee & Taylor 1992; Bakker et al. 1992; Kooistra et al. 1992; van Oppen et al. 1993) but has also proved a valuable approach to phylogenetic inference among species and genera for numerous fungal groups (Bruns et al. 1991; Lee & Taylor 1992; Carbone & Kohn 1993), higher plants (Baldwin 1992) and kelp (Saunders & Druehl 1993b).

In addition to species delineation and phylogenetic inference, researchers have also investigated ITS sequence secondary structure in a search for post-transcription processing sites (Torres et al. 1990; Gonzalez et al. 1990a; Wesson et al. 1992).

Kelp Evolution and Phylogeny

The Laminariales are considered an evolutionarily advanced algal order based on life history, reproduction, tissue differentiation and ultrastructural features (Kain 1979, Clayton 1984, South & Whittick 1987). The advanced characteristics of kelp led to the assumption that the order was an ancient lineage (Tilden 1937; Clayton 1984); however, accumulating molecular evidence, in addition to biogeographical and hybridization data, support a recent divergence for this group (Fain 1986; Druehl & Saunders 1992; Estes & Steinberg 1988; Luning & tom Dieck 1990). Estes and Steinberg (1988) proposed a North Pacific origin and late Cenozoic radiation for the kelp based on the fossil records of kelp-associated organisms (saddle-shaped limpets and kelp-consuming marine mammals). Using chloroplast DNA restriction fragment analysis, Fain (1986) suggested the advanced families of kelp to have variously diverged between 8.5 to 34 mya. A more recent divergence of 10-7 mya is suggested for the order by the only known kelp fossil and putative ancestor, Julescrania (intermediate in morphology between two extant kelp species, Nereocystis and Pelagophycus) (Parker & Dawson 1965). Evidence from hybridizations between kelp species and genera (Sanbonsuga & Neushel, Luning & tom Dieck 1991, Mathieson et al. 1981, Cosson & Olivari 1982) also support a recent divergence of the kelp. In addition, Saunders & Druehl (1992) applied a molecular clock to kelp SSU rDNA sequence data and determined a 16-34 mya divergence for

kelp species of the Lessoniaceae, Alariaceae and Laminariaceae.

While a consensus among recent data has emerged regarding the time and place of origin of the Laminariales, accumulating molecular data contradicts traditional views of evolutionary relationships within the order. For example, Fain (1986) found species of the Lessoniaceae were polyphyletic using cpDNA restriction fragment length polymorphism (RFLP) analysis. In addition, Saunders & Druehl (1993b) concluded that genera within three kelp families (Lessoniaceae, Alariaceae and Laminariaceae) were polyphyletic based on phylogenetic analysis of rDNA sequences within the ITS1 spacer and 5.8S region of the nuclear ribosomal cistron.

Laminaria Evolution and Phylogeny

Of the 22 extant species of Laminaria found in the North Pacific, only one is also found in the North Atlantic (Laminaria saccharina) (Druehl 1968; Kain 1979). With this one exception, the Laminaria floras of each ocean are distinct. Access between the two oceans has been available from at least 3 mya, when the Bering Land Bridge was first breached (Gladenkov, 1986; Luning 1990). Sister species are hypothesized to exist in these two oceans due to the proposed dispersal of at least two North Pacific Laminaria ancestors through the Bering Strait into the Atlantic; one gave rise to the stiff-stiped digitate Laminaria species and the other to the flexible-stiped digitate species (Luning & tom Dieck 1990; tom Dieck 1992).

Based upon hybridization experiments and morphological similarities (tom Dieck 1992) proposed that Laminaria groenlandica (Northeast Pacific) and L. digitata (North Atlantic) shared the flexible-stiped Laminaria ancestor while L. setchellii (North Pacific) and L. hyperborea (North Atlantic) shared the stiff-stiped progenitor.

Within the genus Laminaria, only two previous studies have attempted an examination of phylogenetic relationships using a molecular approach. Stam et al. (1988) were unable to distinguish five species of Laminaria using DNA-DNA hybridization kinetics and therefore concluded the five species diverged simultaneously 15 - 19 mya. Bhattacharya et al. (1991) used a higher resolution technique of restriction mapping of the rDNA cistron and were able to distinguish 8 species of Laminaria. Although the data did not provide sufficient informative sites for a phylogenetic analysis, the genetic affinities elucidated by the restriction maps, enabled Bhattacharya et al. (1991) to reach a conclusion of taxonomic invalidity for the sections Simplices and Digitatae.

Initial Challenges: DNA Extraction from Kelp

An ongoing challenge in our laboratory has been the isolation of kelp DNA of sufficient quantity and purity for molecular analyses. Previous kelp DNA extraction techniques were developed primarily for harvesting chloroplast DNA (Barrett & Anderson 1980, Popovic et al. 1983, Fain et al. 1988). These protocols required 50-100 g of fresh blades that were subsequently quick frozen and subjected to a variety of manipulations, including a 36-48 hour ultracentrifugation step. Stam et al. (1988) developed a total cellular DNA extraction protocol for lyophilized kelp sporophyte tissue. Although DNA yields were excellent, the process requires in excess of three days. The DNA extraction methods reported in this thesis, exploited the varied life history stages of the heteromorphic life cycle of the kelp. DNA suitable for molecular applications was isolated and purified, from all three of these stages, in one day.

The procedure for DNA extraction from dried sporophyte blades incorporates a system of buffers used to stabilize organellar membranes and to help remove polysaccharides from an organelle preparation (Fain et al. 1988). Techniques for isolating gametophyte and meiospore DNA are similar to those applied to a variety of animal cells (Emmons et al. 1979, Sambrook et al. 1989). DNA samples were further purified on Sepharose columns (see Methods) to remove residual polysaccharides and RNA. This procedure eliminated the lengthy CsCl density gradient ultracentrifugation usually required for

DNA purification. These methods were used to isolate total cellular DNA from a variety of kelp species including high molecular weight DNA from herbarium specimens collected up to 22 years ago. DNA from all three sources (dried blades, fresh gametophytes and meiospores) was sufficiently pure for molecular applications, including restriction endonuclease digestion, Southern blot hybridizations and amplification by the polymerase chain reaction (PCR).

METHODS AND MATERIALS

Plant Collection

Sporophytes (including herbarium specimens), gametophytes and meiospores were obtained from local and international locations for a variety of kelp species (Tables 2 & 3). Species listed in Table 2 were used in the development of DNA isolation methods. The collection sites and life history stages of kelp species included in the phylogenetic analysis are shown in Table 3.

All processed blades were air-dried prior to DNA isolation, whereas gametophytes and meiospores were used fresh.

Kelp DNA Extraction

A. Sporophyte and Herbarium Specimen DNA Extraction

Cells walls were broken by freezing kelp blade tissue in liquid nitrogen and pulverizing in a cold mortar with a pestle (-70° C). Approximately 0.5 g of the blade powder was used per extraction. Organelles were suspended by gradually adding one half (0.25 g) of the kelp powder to 40 ml of buffer A [1.65 M sorbitol, 50 mM MES (2-[N-morpholino]-ethanesulfonic acid) pH 6.1, 10 mM EDTA (disodium ethylenediaminetetraacetic acid), 2% w/v PVP (polyvinylpyrrolidone Sigma P-6755), 0.1% bovine serum albumin (Sigma A-6793) and 5 mM

Table 2: Collection sites & life history stage(s) of the kelp species employed in the development of DNA extraction methods.

<u>Species</u>	<u>Site</u>	<u>Life Hx Stage</u>
<u>Thalassiophyllum clathrus</u>*	Attu Island ^a	Sporophyte
<u>Laminaria solidungula</u>*	Cape Farewell ^b	Sporophyte
<u>Saccorhiza polyschides</u>	Mulroy Bay ^c	Sporophyte
<u>Laminaria setchellii</u>	Cape Beale ^d	Sporophyte
<u>Laminaria yezoensis</u>	Port Hardy ^e	Gametophyte
<u>Lessoniopsis littoralis</u>	Bamfield ^d	Gametophyte
<u>Alaria nana</u>	Bamfield ^d	Gametophyte
<u>Cymathere triplicata</u>	Bamfield ^d	Gametophyte & Meiospore
<u>Laminaria saccharina</u>	Stanley Park ^f	Meiospore
<u>Laminaria ephemera</u>	Botanical Beach ^d	Meiospore
<u>Alaria tenuifolia</u>	Stanley Park ^f	Meiospore

*Herbarium specimens

^aAlaska, U.S.A., ^bGreenland, ^cDonegal, Ireland, ^dWest Coast, Vancouver Island, B.C., Canada, ^eNorth Coast, Vancouver Island, B.C., Canada, ^fVancouver, B.C., Canada.

Table 3: Species included in phylogenetic analysis:
collection sites and life history stage(s)

<u>Species</u>	<u>Site</u>	<u>Life Hx Stage</u>
<u>Laminaria</u>		
<u>angustata</u>	Muroran, Hokkaido ^a	Sporophyte
<u>L. digitata</u>	Herring Cove ^b & Cape Split ^b	Sporophyte
<u>L. ephemera</u>	Botanical Beach ^c	Meiospore
<u>L. groenlandica</u>	Bamfield ^c	Sporophyte
<u>L. japonica</u>	Muroran, Hokkaido ^a	Sporophyte
<u>L. longipes</u>	Salmon Bank, San Juan Island ^d	Sporophyte
<u>L. saccharina</u>	Stanley Parke ^e , Whyte Cliff Parke ^e & Garbary Bay ^b	Meiospore Sporophyte
<u>L. setchellii</u>	Cape Beale ^c	Sporophyte
<u>L. sinclairii</u>	Jordan River ^c	Sporophyte
<u>Cymathere</u>		
<u>triplicata</u>	Sooke ^c & Cape Beale ^c	Meiospore
<u>Costaria costata</u>	Stanley Parke ^e	Sporophyte

^aJapan; ^bNova Scotia, Canada; ^cVancouver Island, B.C., Canada,
^dWashington, U.S.A., ^eVancouver, B.C., Canada

beta-mecaptoethanol) with gentle stirring for 2-3 min (Fain et al. 1988). Cell wall debris was removed from the homogenate by filtration through Miracloth (Calbiochem) supported on each side by two layers of cheesecloth. All buffers, homogenates and filtrates were kept on ice during the organelle isolation process to minimize organellar membrane breakdown prior to the lysis step. The above steps were repeated for the second 0.25 g of powder. Organelle filtrates were combined and centrifuged for 5 min at 4,300 x g (-4° C) in an SS34 rotor (Sorval centrifuge). The supernatant was discarded and the organelle pellet was resuspended in 30 ml of buffer B (buffer A minus PVP) and centrifuged as above. Successive buffer B washes removed additional polysaccharides from the organelle suspension and, therefore this step was repeated one to several (3-6) times to reduce supernatant viscosity and improve pellet compactness. Pellets were then resuspended in 30 ml of buffer C (1.65 M sorbitol, 50 mM tris-HCl pH 7.5 and 25 mM EDTA) and centrifuged as before. The supernatant was discarded and the pellets were transferred to 1.5 ml microcentrifuge tubes and resuspended in 500 µl of protease buffer [50 mM EDTA, 100 mM tris hydroxymethyl aminomethane (tris-Base) pH 8.5 (pH'ed with HCl), 200 mM NaCl and 1% lauryl sulphate (SDS)] (Emmons et al. 1979). Proteinase K (Sigma P0390) was added to a concentration of 200 µg·ml⁻¹. Samples were incubated in a 65° C for 30-60 min and inverted frequently to ensure efficient organelle lysis. The appearance of a whitish flocculent material (presumably dissociated membranes and denatured proteins) indicated that lysis

had occurred. Proteins were removed from the lysate with a series of phenol, followed by chloroform-isoamyl alcohol, extractions (a modification of Maniatis et al. 1982). All subsequent centrifugations were 2 min each at 12,000 x g in a microcentrifuge. The upper aqueous phase resulting from the first phenol extraction was placed in a separate microcentrifuge tube. The interphase was added to a 0.5 vol of TE (10 mM Tris pH 8.0, 1 mM EDTA) and mixed by inversion for 7 min. The interphase-TE portion was extracted with phenol and centrifuged. At this point, aqueous portions were combined and extracted two additional times with phenol, as above, followed by two extractions with chloroform/isoamyl alcohol (24:1 v/v). DNA was recovered from the aqueous phase by precipitation with 2 vol. of cold 95% ethanol, 0.4 vol 5M ammonium acetate and storage at -20° C for 3-12 h, with subsequent centrifugation in a refrigerated microcentrifuge for 15 min at 12,000 x g. The DNA precipitation step was a convenient resting point in the procedure. However, lengthy precipitation was not necessary provided that ice cold (-20° C) 95% ethanol and a longer centrifugation (30-40 min) were used. Pellets were washed with 70% ethanol and recentrifuged as above. Vacuum desiccation removed remaining ethanol from the pellets which were then resuspended in 50 µl of TE containing DNAase-free ribonuclease A (10 µg·ml⁻¹) and incubated for 40 min at room temperature.

DNA Purification - Sepharose Columns

Following Ribonuclease A treatment, DNA samples were diluted with 150 μ l of TE and purified by gel filtration on Sepharose spin columns (Gabe Kalmar, Institute of Molecular Biology and Biochemistry, Simon Fraser University, pers. comm.). Columns were prepared by stoppering 1 cc syringes with porous polyethylene (Biolab) plugs cut to size with a 5.0 mm cork borer. Syringes were placed in 15 ml plastic test tubes and packed with Sepharose (Sigma CL-2B-300) that was allowed to settle by gravity. The columns were centrifuged 2 min at 650 x g (International HN-S centrifuge). The compact bed volume of Sepharose was approximately 0.75 ml. Columns were subsequently washed (200 μ l TE each) and recentrifuged as above, three times. The washings were discarded and a capless 1.5 ml microcentrifuge tube placed into the bottom of each test tube. Columns were replaced into the test tubes, with the tip of each column extending into a microcentrifuge tube. Each DNA sample (200 μ l or less) was applied to one or more column(s) and centrifuged as above. Columns were washed (50 μ l TE) and centrifuged as above, two times. Microfuge tubes ("A" tubes) containing column effluent were removed from the test tubes, capped and refrigerated at 4° C. New 1.5 mL microfuge tubes ("B") were placed beneath each column. Columns were washed (50 μ l TE) and centrifuged as above, two times. The DNA from column elutants "A" and "B" were precipitated separately because elutant "A" DNA is

usually of greater purity. DNA was ethanol precipitated as described above. Samples were centrifuged for 15-30 min at 12,000 x g (-4° C) in a Sorval centrifuge (SS34 rotor). Resulting pellets were washed with 70% ethanol to remove salts and recentrifuged as before. Pellets were vacuum dessicated and redissolved in 20-100 µl TE or double-distilled water.

B. Gametophyte DNA Extraction

Gametophytes, grown under unialgal conditions (Druehl 1980) were rinsed in millipore-filtered (0.45 µm) sea water and centrifuged 1 min at 3,000 x g in a microcentrifuge. Excess fluid was removed and approximately 25 µL of compacted filaments were placed in a mortar with 500 µl protease buffer and 5 µl Proteinase K (20 mg·mL⁻¹ stock). Tissue was vigorously ground into a homogenate, transferred to 1.5 ml microcentrifuge tubes (500 µl per tube) and incubated in a 65° C water bath for 30-60 min. The DNA extraction protocol outlined for dried blades, beginning with phenol extraction and including Sepharose column purification was followed from this point onward.

C. Meiospore DNA Extraction

Mature soral tissue was thoroughly wiped clean of surface contaminants with cheesecloth and sterilized 5 min in a 1% solution of bleach in millipore-filtered sea water (Druehl 1980). Tissue was blotted dry and allowed to partially desiccate (to facilitate spore release) at room temperature. After 2-3 h, tissue was transferred to clean, 2 L glass jars and emersed in millipore-filtered sea water. Jars were placed in natural light at room temperature. Spore release was determined by periodic sampling and microscopic examination. The increasing cloudiness of the the sea water-tissue solution was also a good indication of spore release. If few spores were released after 3-4 h, tissue was re-blotted, placed between damp paper towels, refrigerated at 4° C for 12 h and then re-emersed in sea water (Druehl 1980). The top two thirds of the spore solution was gently siphoned off and filtered through 4 layers of cheesecloth to remove mucilage released by the soral tissue. The bottom third was discarded to minimize possible contamination with diatoms or other settled organisms. The filtrate was transferred to 250 ml Sorval centrifuge bottles and centrifuged in a Sorval GSA rotor at 4,100 x g (-4° C) for 10 min to pellet the meiospores. Compact meiospore pellets were resuspended in filtered sea water and recentrifuged for 5 min to clean the pellets. Pellets were transferred to 1.5 ml microcentrifuge tubes. Approximately 50 µl of concentrated

meiospores were used per extraction. Protease buffer and Proteinase K were added to the meiospores as above and all subsequent steps were followed from the blade extraction and purification protocol.

DNA Yield Estimates

DNA yield estimates were obtained from ultraviolet spectrophotometric absorbance values at 260 nm. DNA yields from the second elutant ("B") samples were estimated following additional purification using new Sepharose spin columns.

Endonuclease Digestion and Gel Electrophoresis

One to two μg of total cellular DNA was digested with 10-20 units of one or two restriction endonucleases according to the manufacturer's recommendations [Pharmacia, Bethesda Research Laboratories (BRL)]. BglII, ClaI, DraI, PstI, SmaI and SacI were the restriction enzymes used in the analysis. Digested DNA was size fractionated by horizontal gel electrophoresis (0.7% agarose, 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide). Gels were run for 16-22 h at 1.1 V/cm in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 8 mM disodium EDTA pH 8.0) (Maniatis *et al.* 1982).

Southern Transfer and Hybridization

DNA was transferred bidirectionally, after brief acid hydrolysis, to BioTrace RP nylon membranes (Gelman Sciences) by an alkaline transfer method (Biorad recommendations). After a 6-8 h transfer, filters were rinsed in 2 x SSC (SSC = 15 mM NaCl, 1.5 mM sodium citrate) and then washed in three consecutive rinses of 0.1 x SSC and 0.1% SDS (sodium dodecyl sulfate) warmed to 42° C. Filters were blotted dry and stored in 5 x SSPE, 5 x BFP and 0.2% SDS (SSPE = 0.18 M NaCl, 10 mM sodium phosphate and 1 mM disodium EDTA, pH 7.0; BFP = 0.02% w/v Bovine Serum Albumin (Fraction V; Sigma A-6793), Ficol Type 400 (Sigma F-4375), PVP-40 (Polyvinylpyrrolidone, Sigma P-6755) at 4° C.

Filters were prehybridized at 65° C for 4-18 h. Hybridizations under conditions of moderately high stringency, using the probe pCc18 [random clone from the kelp, *Costaria costaria* homologous to 1595 bp of the ribosomal SSU gene and 204 bp of the upstream External Transcribed Spacer (ETS)] (Bhattacharya & Druehl 1989) were from 8-20 h at 65° C in 5 x SSPE, 0.2% SDS and 1 x BFP. The probe was radiolabelled by nick translation (Rigby et al. 1977). Unincorporated nucleotides were removed by application of the reaction volumes to 1.5 ml Sephadex G-50 spin columns (Maniatis et al. 1982). After hybridization, filters were washed for 15 min at 25° C in 1 x SSC and 0.1% SDS with gentle agitation, followed by three consecutive moderately high stringency washes (20 min each at 65° C) in 0.1 x SSC

and 0.1% SDS. Filters were blotted dry and heat-sealed in plastic bags to prevent desiccation. Ribosomal DNA fragments were visualized by exposing filters to pre-flashed Kodak (X-OMAT-K) x-ray film at -70°C for 24 h to 10 days. Band fragments were sized using the GEL program (Andrew Beckenbach, Institute of Molecular Biology and Biochemistry, Simon Fraser University) and the distance of migration of each band from the well.

PCR Amplification and Direct Sequencing

Universal and kelp-specific oligonucleotide primers for regions of the ribosomal cistron were used to amplify DNA via the polymerase chain reaction (PCR) (Mullis & Faloona 1987, Saiki *et al.* 1988) (Fig. 9b). Using the Gene-Amp Kit and its heat stable DNA polymerase (AmpliTaq), PCR amplifications of double stranded nuclear DNA templates (100-200 ng) were completed according to the manufacturer's recommendations (Perkin-Elmer-Cetus). Amplification reactions used the universal rDNA primers P1 (5' TAA TCT GTT GAA CGT GCA TCG 3') complementary to the coding strand of the 3' end of the 18S gene and GITS4 (5' CTT TTC CTC CGC TTA TTG ATA TG 3') complementary to the noncoding strand of the 5' end of the 26S gene of the fungus, *Saccaromyces* (Baroin *et al.* 1988) (Fig. 9b). Kelp-specific internal oligonucleotide rDNA primers BC1 (5' GAT TCC GGA CTG TGG CTC CGG TG 3') and BC2 (5' CGA GTG GTG TCA ACA GAC ACT CC 3') were complementary to conserved regions of the coding

strand of the 3' end of SSU gene and the non-coding strand of the 5' end of the 5.8S gene, respectively (Saunders & Druehl 1993a). They were used to amplify subfragments encompassing the ITS1 for each of the study species. Internal primer P5, (5' GCA TCG ATG AAG AAC GCA G 3'), complementary to the coding strand at the 3' end of the 5.8S gene, was modified from White et al. (1990) by Saunders & Druehl (1993a) (Fig. 9). P5 and GITS4, were used to amplify the ITS2 region of the study species. Primers were used at a working concentration of 1 μ M (Saunders & Druehl 1993a).

Amplification reactions were carried out in an automated thermocycler under the following regime: initial cycle (denature 4 min 95° C, anneal 30 sec 60° C, extension 1 min 72° C), 32-38 cycles of (denature 30 sec 95° C, anneal 30 sec 60° C, extension 1 min 72° C) with a final cycle (denature 30 sec 95° C, anneal 30 sec 60° C, extension 10 min 72° C). Positive and negative controls were run simultaneously with each series of reactions. Lambda DNA and complementary primers, supplied with the GeneAmp (Perkin-Elmer-Cetus) kit, were used in the positive control reactions. Amplified products were cleaned using either Spin columns (Pharmacia) or the Sephaglas DNA BandPrep kit (Pharmacia) following the manufacturer's protocols. Double stranded DNA cleaned by these methods was directly sequenced via the dideoxynucleotide chain-terminating method of Sanger et al. (1977) after an initial alkaline denaturation (1M NaOH) and subsequent neutralization with 1M HCl (Pharmacia, Analects 8, 1990) using the Sequenase (version 2.0) sequencing

system (manufacturer's directions, United States Biochemicals). Primers used in PCR amplification reactions were also used for sequencing.

Compression artifacts encountered in the amplified templates were often resolveable using PCR cycle-sequencing (American Biochemicals Incorporated [ABI]) with the Dye terminator Kit (ABI) and subsequent acrylamide gel electrophoresis using the ABI automated sequencer.

Preliminary Assessment of Intraspecific rDNA Sequence Variation

Intraspecific rDNA sequence variation was assessed for a subset of the study species: Laminaria digitata, L. saccharina, L. groenlandica, and Cymathere triplicata. Ribosomal DNA was isolated from three individuals representing geographically distinct stands of L. saccharina (Stanley Park, Vancouver, B.C., Whyte Cliff Park, West Vancouver, B.C. and Garbary Bay, Nova Scotia, Canada), from two sporophytes of L. digitata, (Cape Split and Herring Cove, Nova Scotia, Canada) and from two individuals of C. triplicata (Whiffen Spit, Sooke and Cape Beale, Vancouver Island, B.C., Canada). rDNA was amplified and sequenced as described previously. Two individuals of L. groenlandica from the same stand were also examined for rDNA intraspecific sequence variation.

Sequence Alignment and Phylogenetic Analysis

DNA sequences were aligned manually using a multisequence text editor [Eyeball Sequence Editor (ESEE)] (Cabot & Beckenbach 1989). Ambiguously aligned sequence was excluded from the phylogenetic analysis. Transition and tranversion frequencies were calculated for the aligned sequences using the COMPARE option of ANDYPROG (Andrew Beckenbach, Institute of Molecular Biology and Biochemistry, Simon Fraser University, B.C. Canada). Evolutionary distance estimates were obtained using the Phylogeny Inference Package (PHYLIP) (version 3.5c, Felsenstein 1993) by invoking the DNADIST algorithm. In addition, distance estimates were also computed for published ITS1 sequences of kelp species representing 8 genera (Saunders & Druehl 1993b). Restriction sites and short repeat motifs were located by visual inspection. Putative stem loop structures (adjacent to the 5' end of the 5.8S gene) were tentatively identified by comparison with previously reported kelp secondary structures (Saunders & Druehl 1993a). Percent G+C content was determined for each internal transcribed spacer.

In preparation for phylogenetic analyses, gaps were subjected to two treatments: a). they were considered as a fifth character state and included in the phylogenetic analyses and then b). they were considered as missing data and therefore, eliminated. Both distance and parsimony phylogeny inference algorithms of the computer package PHYLIP were employed. DNA distance analyses consisted of

invoking SEQBOOT to generate 500 replicates of the input data set (bootstrap analysis). DNADIST was next invoked to generate the 500 matrices for distance analyses. Jukes & Cantor (1969) single parameter and Kimura (1980) two parameter nucleotide substitution correction formulas were each employed with this algorithm. NEIGHBOR, the neighbor-joining (Saitou & Nei 1987) phylogeny inference program was used to infer phylogenies from the distance matrices. The input order of algal sequences was randomized in both distance and parsimony analyses. Parsimony analyses consisted of invoking SEQBOOT (200 replicates) followed by the DNAPARS algorithm. CONSENSE completed the bootstrap analyses via determination of a majority-rule consensus tree from the trees generated either by NEIGHBOR or DNAPARS (Felsenstein 1993).

RESULTS

Kelp DNA Extraction

Total cellular DNA of sufficient quantity and purity for nuclear molecular analyses was extracted in one day, from milligram amounts of dried sporophyte blades, and microliter volumes of fresh gametophyte filaments and meiospores. DNA from all three sources (including the herbarium specimens) was not extensively degraded and consisted of a size range of fragments useful for molecular biology techniques (Fig. 10a).

Estimated DNA yields from dried sporophyte tissue were 12.8 $\mu\text{g}\cdot\text{g}^{-1}$ (*Laminaria setchellii*) and 26.4 $\mu\text{g}\cdot\text{g}^{-1}$ for *Saccorhiza polyschides* (Lightf.) Batt. (Fig. 10a). DNA was isolated from herbarium specimens of *Laminaria solidungula* (22 years old) and *Thalassiophyllum clathrus* (Gmelin) Postels & Ruprecht (8 years old); however DNA yields were not estimated (Fig. 10a). DNA from the dried sporophyte and herbarium specimens was successfully amplified using PCR (Fig. 10b).

Total cellular DNA, obtained from 25 μl volumes of compact gametophytes of *Laminaria yezoensis* and *Cymathere triplicata* yielded 1.9 μg and 11 μg , respectively (Fig. 10a). Gametophyte DNA from *L. yezoensis*, *Alaria nana* Schrader and *Lessoniopsis littoralis* (Farl. & Setch.) Reinke was PCR amplified (Fig. 10b). The gametophyte

Fig. 10:

a). Total Cellular Kelp DNA:

Sporophyte DNA: Lane 1, Laminaria setchellii Silva; Lane 2, Sacchoriza polyschides (Lightf.) Batt.; Lane 3, Thalassiophyllum clathrus (Gmel.) Postels & Ruprecht, 8 year old herbarium specimen; and Lane 4, Laminaria solidungula J. Agardh, 22 year old herbarium specimen.

Gametophyte DNA: Lane "A", Cymathere triplicata (Post. & Rupr.) J. Agardh; Lane 5, Laminaria yezoensis Miyabe; and Lane 6, Laminaria ephemera Setchell. No corresponding PCR product shown in Fig. 10b for "A".

Meiospore DNA: Lane 7, Cymathere triplicata (Post. & Rupr.) J. Agardh; Lane 8, Laminaria saccharina (L.) Lamouroux and Lane 9, Alaria tenuifolia Setchell. M₁ and M₂ are Lambda-HindIII and 1Kb ladders respectively.

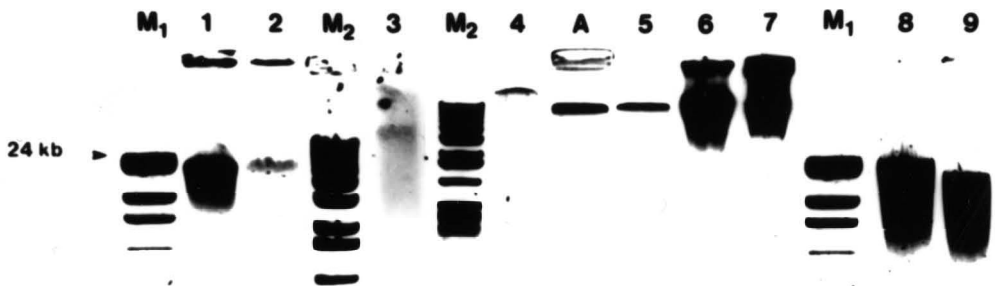
b). PCR Amplified Kelp DNA Fragments:

Kelp specific primers for regions of the ribosomal cistron were used.

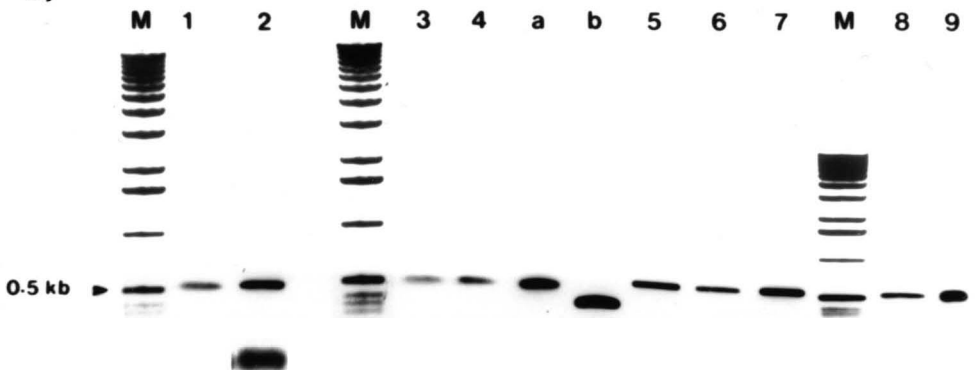
Lanes 1 - 9 correspond to the species (and life history stages) outlined above in Fig 10a. Lane "a", Alaria nana Schrader and Lane "b", Lessoniopsis littoralis (Farl. & Setch.) Reinke are amplified products from gametophyte DNA. Corresponding total cellular DNA not shown for "a" or "b" in Fig. 10a. M = 1 kb ladder.

Fig. 10

a)



b)



DNA of C. triplicata did not amplify.

Concentrated meiospores (50 μ l) from each of two individuals of C. triplicata yielded 43 μ g and 54.3 μ g of total cellular DNA respectively. DNA was isolated from the meiospores of three additional species: Alaria tenuifolia Setchell, Laminaria saccharina and Laminaria ephemera (Fig. 10a). Restriction fragments were identified following Southern blot hybridization of digested meiospore DNA from L. ephemera DNA (Fig. 11) whereas DNA from C. triplicata, Alaria tenuifolia, L. saccharina and L. ephemera was PCR amplified (Fig. 10b).

rDNA Sequence: Size, Composition and Alignment

rDNA sequences (approximately 900 bp each) spanning the 3' end of the SSU gene, through the adjacent ITS1 and 5.8S gene to the 3' terminus of the ITS2 were determined for nine species of Laminaria and Cymathere triplicata. In addition, complete ITS2 sequence was determined for Costaria costata. A highly conserved sequence of 204 bp comprised the 3' end of the SSU rRNA gene in all study species. The adjacent ITS1 region ranged in size from 236 to 249 bp among the Laminaria species (Table 4). The ITS1 was 251 bp in Costaria costata (as previously determined by Saunders & Druehl 1993b) and 266 bp in Cymathere triplicata. The 5.8S gene was absolutely conserved in size (160 bp) and sequence for all study species while the ITS2 varied in length from 239 to 267 bp among the nine Laminaria species (Table 4).

Fig. 11: Southern Blot Hybridization of Kelp Meiospore DNA.

Hybridization of the kelp specific rDNA probe pCc18 to restriction digested meiospore DNA isolated from Laminaria ephemera Setchell. Lanes 1-6: DNA was digested with PstI, SalI, BglII, SacI, ClaI and DraI, respectively.

Arrows indicate bands corresponding to estimated band size displayed along the left hand margin.

Fig. 11

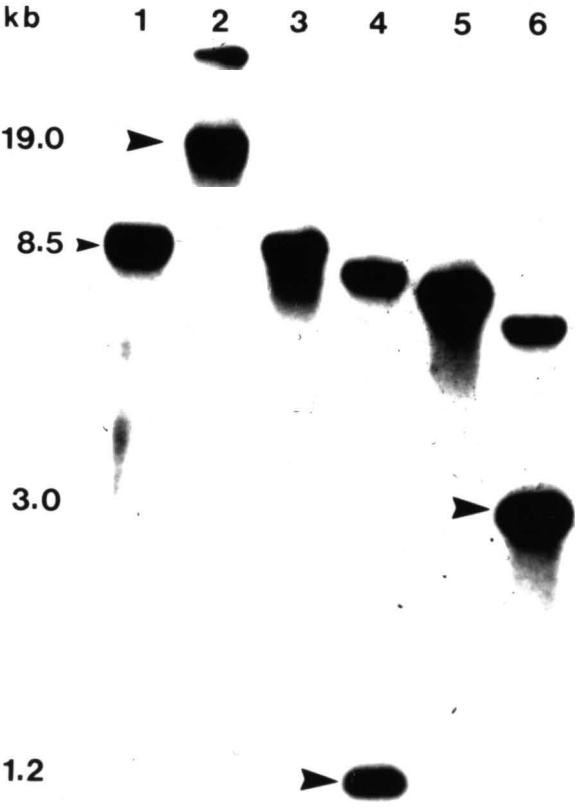


Table 4: Nucleotide length comparison of rDNA internal transcribed spacer (ITS1 & ITS2) and 5.8S gene regions among representative species of the Laminariaceae

SPECIES	ITS1	5.8S	ITS2
<u>Laminaria</u>			
<u>L. angustata</u>	238	160	263
<u>L. digitata</u>	245	160	263
<u>L. ephemera</u>	247	160	259
<u>L. groenlandica</u>	238	160	267
<u>L. japonica</u>	238	160	259
<u>L. longipes</u>	249	160	250
<u>L. saccharina</u>	243	160	263
<u>L. setchellii</u>	237	160	239
<u>L. sinclairii</u>	236	160	243
<u>Costaria</u>			
<u>C. costata</u>	251 ^a	160 ^a	269
<u>Cymathere</u>			
<u>C. triplicata</u>	266	160	279

^aSequences from Saunders & Druehl (1993b)

The ITS2 was 269 and 279 bp respectively in Costaria costata and Cymathere triplicata. The 5' and 3' termini of each rDNA region were assigned by comparison with other kelp sequences (Saunders & Druehl 1993a; 1993b) which in turn were assigned by alignment and/or comparison with other eukaryote sequences [Gunderson et al. 1987 (3' end 18S/5' end ITS1), Yokota et al. 1989 (5' and 3' ends 5.8S), Baroin et al. 1988 (3' end ITS2/5' end 26S)].

Figure 12 illustrates the alignment of the 3'SSU gene - ITS1 - 5.8S gene - ITS2 rDNA sequences of the study and outgroup taxa. A second alignment was made of the ITS1 and ITS2 sequences of the Laminaria species exclusively (Fig. 13). In most cases, the boundaries for the regions of ambiguous alignment were established by the nearest, flanking, conserved sites among all the sequences; the intervening nucleotide sites were removed and excluded from subsequent phylogenetic analyses (Figs. 12 & 13; non-underlined regions). Comparison of unambiguously aligned sequence regions of the study and outgroup taxa (785 sites) (Fig. 12) showed that 16.5% (131 sites) varied in at least one of the sequences. Of the variable sites, 53.4% (71) were informative: one site occurred in the 3' SSU, 20 in the ITS1 and 50 in the ITS2. The combined ITS1 and ITS2 data set had 464 unambiguously aligned nucleotide positions among Laminaria species only (Fig. 13) and 429 positions when the other taxa were included (Fig. 12).

Fig. 12: Sequence alignment of rDNA: 3'18S gene, Internal Transcribed Spacers (ITS1 & ITS2) and 5.8S gene of nine species of Laminaria; Costaria costata and Cymathere triplicata.

Underlined sequence regions were included in the phylogenetic analyses while regions of ambiguous alignment (non-underlined sequence) were excluded. V1 - V5 regions correspond to the variable sequence discussed in the text. The dashed lines denote the location of a putative stem loop near the 5' end of the 5.8S gene. The series of dots indicate two perfect, non-adjacent repeats in the 5.8S gene. The variable C-T tract, near the 5' end of ITS2, found in all the study taxa, is indicated by a double solid line. Restriction sites are bordered top and bottom with solid lines.

Species abbreviations are as follows:

L.ang = Laminaria angustata, L.jap = L. japonica,
L.sac = L. saccharina, L.groen = L. groenlandica,
L.sinc = L. sinclairii, L.set = L. setchellii, L.long
= L. longipes, L.eph = L. ephemera and L.digt =
L. digitata.

Fig. 12

3'SSU

L..JAP TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 L..ANG TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 L..SAC TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 L..GROEN TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 L..SET TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 L..SINC TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 L..DIGT TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 L..LONG TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 L..EPH TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 ALARIA TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 COSTARIA TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 CYMATHERE TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115
 POSTELSIA TCATCAGCTCGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCCACCTACCGATTGAATGTTTCGGTGAAGATTCCGGACTGTGGCTCGGGTGCCTCACGGCGGCTCT 115

L..JAP TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 L..ANG TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 L..SAC TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 L..GROEN TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 L..SET TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 L..SINC TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 L..DIGT TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 L..LONG TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 L..EPH TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 ALARIA TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 225
 COSTARIA TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 229
 CYMATHERE TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230
 POSTELSIA TGCCGTGGGAAGTTATCTAAACCTCAACATTTAGAGGAAGGTGAAGTTCGGTAGGTGAACCTGCGGAAGGATCATACCAGAAAGCGGGTTCGTTCAATCCCCC 230

ITS1

Fig. 12 cont'd.

ITS1 cont'd.

L..JAP CCGCTCTATAAATTGTCT-GTGAGGCCGCTTGG-----TGGGGCTC-TTTACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 318
 L..ANG CCGCTCTATAAATTGTCT-GTGAGGCCGCTTGG-----TGGGGCTC-TTTACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 318
 L..SAC CCGCTCTATAAATTGTCT-GTGAGGCCGCGGT-----TTCGGGGCTC-TGTACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 321
 L..GROEN CCGCTCTATAAATTGTCT-GTGAGGCCGCTT-----GGGGGCTCTCACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 316
 L..SET CCGCTCTATAAATTGTCT-GACACTCGCCCGC-----GGCGGTTGTTTACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 318
 L..SINC CCGCTCTATAAATTGTCT-GACACTCGCCCGC-----CGCGGTTGTTTACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 318
 L..DIGT CCGCTCTATAAATTGTCT-GACACTCGCCCGCT-----CITTGAGATTGGGGCGTTGTATACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 330
 L..LONG CCGCTCTATAAATTGTCT-GACACTCGCCCGT-----GAGATTGGGGCGTTGTTTACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 326
 L..EPH CCGCTCTATAAATTGTCT-GACACTCGCCCGA-----ATATGGGGCGTTG-TTACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 324
 ALARIA CTG-TCATAAAA---ACT-CATACTGCTGCCGT-----TGATACGGCGCTTGTAAACCC-C-GAGAAA-GAAAATCGTTATCGAAGTTGGCGAGGGGGCGCC 318
 COSTARIA CCGCTCTATAAATTGTCTCGGCAGCGCCATGA-----AAGAGGGCGAGTGGTCATACCC-C-GTGAATGAA-TTCGTTATCGAAGTTGGCGAGGGGGCGTC 326
 CYMATHERE CCGCTCTATAAATTGTCT-GAGACGTGGCGTAGTAACCTAACCCGAGGGGCGCGTATTACCC-C-GAGAAA-GAA-TTCGTTATCGAAGTTGGCGAGGGGGCGCC 341
 POSTELSIA CCGCTCTACAAAATTGTCT-GAGACT-CTGGCGC-----ATTTCAGAGAGGGCGCTTCTACCC-C-GAGAAA-GAA-GCCGTTATCGAAGTTGGCGAGGGGGCGCC 330



L..JAP TCGCCGAGGCT-GTG-----AAAAGGCCCTCGAATCAAAGGCCACCCACAT-TTCAA-CCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCTGGCT-----AGCC 416
 L..ANG TCGCCGAGGCT-GTA-----AAATGGCTCTCGAATCAAAGGCCACCCACAT-TTCAA-CCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCTGGCT-----AGCC 416
 L..SAC TCGCCGAGGTTTGTATA-----AAAAGGCTCTCGAATCAAAGGCCACCCACAT-TTCAA-CCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCTGGCT-----AGCC 421
 L..GROEN TCGCCGAGGCT-----AAGGCTCTCGAATCAAAGGCCACCCACAC-TTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGTGGCTGGGGCTC-TGCC 416
 L..SET TCGCCGAGGCT-----GAGGCTCTCGAATCAAAGGCCACCCACAC-TTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCTGGCT-----GGG 414
 L..SINC TC-CGGAGGCT-----GAAAGCTCTCGAATCAAAGGCCACCCACAC-TTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCTGGCT-----GGG 413
 L..DIGT TCGCCGGGAGG-----AAGTTTGGTCTCGAACCAAAGGCCACCCACAC-TTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCGAGGCT-----CGCC 423
 L..LONG TCGCCGAGAGG-----CTTTGGAAAGCTCTGAACCAAAGGCCACCCACAC-TTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCGAGGCT-----GAAAAC 425
 L..EPH TCGCCGAGGTTT-----CTTTGGAAAGCTCTGAACCAAAGGCCACCCACAT-TTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCGAGGCT-----TGTC 425
 ALARIA TC-CGGAGGTTTGTATT-----GAAA-CCCTCGACTCGAACCAAAGGCCACCCACAC---TTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCGAGGCTTTTACC 423
 COSTARIA TC-CGGAGGTT-----CTTCGCTAGACTCTGAACCAAAGGCCACCCACACAT-TTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCGAGGCTCGTCGCC 429
 CYMATHERE TCGGAGCGTCATCATGATGGTG-----CTCGAATCAAAGGCCACCCACAC-TTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCTGGCT-----TGTC 444
 POSTELSIA TC-CGGAGGCCAGCCAGCTCG-----CAACCAAAGGCCACCCACATTTCAAACCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGGCGAGGCT-----GCC 424

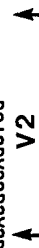


Fig. 12 cont'd.

ITS2 cont'd	738
L. JAP	GGCGAGTCCGGC-----GGCGGACTTTGAGTTCGGAG-TT-CCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
L. ANG	AGGGAGTCCGGC-----GGCGGACTTTGAGTTCGGAG-TT-CCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
L. SAC	GGGGGTCCGGC-----GGCGGACTTTGAGTTCGGAG-TT-CCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
L. GROEN	CGGGGTCCGGC-----GGCGGACTTTGAGTTCGGAG-TT-CCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
L. SET	TT-GGGAGGGCCCC-----GGCGGACTCTGAGTTCGGAG-TT-CCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
L. SINC	TTTGGAGGGCCCC-----GGCGGACTCTGAGTTCGGAG-TT-CCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
L. DIGT	TTTGGAGGGCCCC-----GGCGGACTCTGAGTTCGGAG-TT-CCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
L. LONG	GTCCGAGGGCC-----GGCGGACTCTGAGTTCGGAG-TT-CCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
L. EPH	GTTGGTAGGGCC-----GGCGGACTCTGAGTTCGGAG-TT-CCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
ALARIA	GTGTGGTGGGGCGTCG-----GGCGGACTCTGAGTTCGGAGCTT-----GCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
COSTARIA	AAGGTCTGGGCAAGAGAT-CGC GG--GGGACTCTGAGTTCGGAGCTTACCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
CYMATHERE	GGAGCCGGGGT-----CGCGGCGGACTCTGAGTTCGGAGCTTGA--GCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
POSTELSIA	CGGCGTCCGAGCGGGGTGGGGGGCGGACTTTGAGTTCGGAGCTTGA--GCTCCGAGTGCACCTAATCTCGTGAACGAAGCCTCTCGGCCCTGCCGCACAGAGT
	V4
L. JAP	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAA-CGTGCCAGGATGCCT-----GCCTCATTCGGGGCTCCGAGC-CGACCCCTTCT-----GGGTGAGCGTT--- 840
L. ANG	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAA-CGTGCCAGGATGCCT-----GCCTCATTCGGGGCTCCGAGC-CGACCCCTTAAAGAGGGTTCAGCGTT--- 844
L. SAC	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAA-CGTGCCAGGATGCCT-----GCCTCATTCGGGGCTCCGAGC-CGACCCCGTAAAAAGGTTCAGCGTT--- 849
L. GROEN	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAA-CGTGCCAGGATGCCT-----GCCTCATTCGGGGCTCCGAGC-CGACCCCGTAAAAAGGTTCAGCGTT--- 848
L. SET	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAAAGT-----AGGGTTC-----GGGGCTTC-TTCGGGGCTCC-CGATGAAG----- 822
L. SINC	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGA-TCCGAAAAAG-----CAGGCTGCTCGGGGGCTTC-TTCGGGGCTCC-CGA-GAAG----- 825
L. DIGT	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCGCCAAA-CGTGCCAGGCTGC-----GGGGCTTC-TTCGGGGCTCCCGAAGGGGTAAAAACCCCTCTCTTCGT--- 851
L. LONG	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCGGAAA-CGC-CGCAGGCTGC-----GGGGCTTC-TTCGGGGCTCCCGACCCAAAAGGG----- 840
L. EPH	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAA-CGTGCCAGGCTGC-----GGGGCTTC-TTCGGGGCTCCCGGAGACTTTTCAGAACTCTTT----- 847
ALARIA	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAA-CGTGCCAGGCTGCCT-----GCTTC-TTCGGGGCTCC-----TTCCCGCGGTCAAAAGGGGA--- 847
COSTARIA	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAA-CGTGCCAGGATGC-----GGGGCTCATTCGGGGCTCC-----AGAAGCTTAAAGGTGAT----- 862
CYMATHERE	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAA-CGTGCCAGGATGCCT-----GCTTC-TTCGGGGCTCCCGTGGGCTCAGCTTCTCGAAGCGGGGGCTTC 887
POSTELSIA	TGTTGACGGCGCTCGCTTCGGCGGCGACTCTCGACTCACCAAA-CGTGCCAGGCGCT-----GCTTC-TTCGGGGCTCCCGCTCGCTCGGCCCTCTCCAAACCGTTCACAGG 874
	V5

Fig. 12 cont'd.

	ITS2 cont'd		
	↑		
L. JAPGGAAACCGTAC--CACTTTCGTT	861	
L. ANGGGAAACCGTAC--CACTTTCGTT	865	
L. SACGGAAACCGTAC--CACTTTCGTT	870	
L. GROENGGAAACCGTAC--CACTTTCGTT	869	
L. SETGGAAA----AC-CCACTTTCGTT	840	
L. SINCGGAAA----AC-CCACTTTCGTT	843	
L. DIGTGGAAACCGTAC--CACTTTCGTT	872	
L. LONGGGAAACCGTACACCACCTTTCGTT	863	
L. EPHGGGAATCCGTAC--CACTTTCGTT	870	
ALARIAGGAAACCGTAC--CACTTTCGTT	869	
COSTARIAGGAAACCGTAC--CACTTTCGTT	883	
CYMATHERE	T-----GGAAACCGTAC--CACTTTCGTT	909	
POSTELSLIA	AAGGAAGAGGGGCGTTCGGCGCGGAATCCGTAC--CACTTTCGTT	919	
	↑		
	V5		

Fig. 13. Sequence alignment of rDNA: Internal Transcribed Spacers (ITS1 & ITS2) of nine Laminaria species.

Underlined sequence regions were included in the phylogenetic analyses. Regions of ambiguous alignment (non-underlined sequence) were eliminated from the analyses.

Species abbreviations as in Fig. 12.

ITS1

L. ANG CCGAAAGCGGGTTCGTTCAATCCCCCGGCTCTATAAATTTGTC-TGAGGCGCGTTCG-----TGGGGCTCTTT-ACCCC-GAGAAAGAAATTCGTTATGCGAAGTTG 101
L. JAP CCGAAAGCGGGTTCGTTCAATCCCCCGGCTCTATAAATTTGTC-TGAGGCGCGTTCG-----TGGGGCTCTTT-ACCCC-GAGAAAGAAATTCGTTATGCGAAGTTG 101
L. SAC CCGAAAGCGGGTTCGTTCAATCCCCCGGCTCTATAAATTTGTC-TGAGGCGCGGTT-----TTGGGGGCTCTGT-ACCCC-GAGAAAGAAATTCGTTATGCGAAGTTG 104
L. GROEN CCGAAAGCGGGTTCGTTCAATCCCCCGGCTCTATAAATTTGTC-TGAGGCGCGT-----GCGGGCTCTCACCCC-GAGAAAGAAATTCGTTATGCGAAGTTG 99
L. DIGT CCGAAAGCGGGTTCGTTCAACCCCGGCTCTATAAATTTGTC-TGACACTCGCCCTCTTTGAGATTGGGGGGTTCGTTATGCGAAGTTG 113
L. LONG CCGAAAGCGGGTTCGTTCAACCCCGGCTCTATAAATTTGTC-TGACACTCGCCCTCT-----GAGATTGGGGGGTTCGTTATGCGAAGTTG 109
L. EPH CCGAAAGCGGGTTCGTTCAACCCCGGCTCTATAAATTTGTC-TGACACTCGCCCTCT-----ATATGGGGGGTTCGTTATGCGAAGTTG 107
L. SET CCGAAAGCGGGTTCGTTCAACCCCGGCTCTATAAATTTGTC-TGACACTCGCCCTCT-----GCGGG-TTGTTCACCCC-GAGAAAGAAATTCGTTATGCGAAGTTG 101
L. SINC CCGAAAGCGGGTTCGTTCAACCCCGGCTCTATAAATTTGTC-TGACACTCGCCCTCT-----CGGG-TTGTTCACCCC-GAGAAAGAAATTCGTTATGCGAAGTTG 101

L. ANG GCGAGGGGCGCCTCGCCGAGAGTCT-GTA--AAATGGCTTCGAATCAAAGCGCACCCACATTTCAA-CCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGCGC-TGGCT- 208
L. JAP GCGAGGGGCGCCTCGCCGAGAGCT-GTG--AAAAGGCTTCGAATCAAAGCGCACCCACATTTCAA-CCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGCGC-TGGCT- 208
L. SAC GCGAGGGGCGCCTCGCCGAGAGTTTGTG-AAAAGAGCTTCGAATCAAAGCGCACCCACATTTCAA-CCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGCGC-TGGCT- 213
L. GROEN GCGAGGGGCGCCTCGCCGAGAGTGT-----AGAGCTTCGAATCAAAGCGCACCCACACTTCAACCCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGCTCGGGCT 205
L. DIGT GCGAGGGGCGCCTCGCCGGAGT-----GAAAGCTTCGAACCAAAGCGCACCCACACTTCAACCCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGCGC-AGCGCT- 215
L. LONG GCGAGGGGCGCCTCGCCGAGAGC-----AAGTTTGGTTCGAACCAAAGCGCACCCACACTTCAACCCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGCGC-AGCGCT- 215
L. EPH GCGAGGGGCGCCTCGCCGAGAGT-TTTCCTTGGAAAGCTTCGAACCAAAGCGCACCCACACTTCAACCCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGCGC-AGCGCT- 217
L. SET GCGAGGGGCGCCTCGCCGAGAGT-----GAAAGCTTCGAATCAAAGCGCACCCACACTTCAACCCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGCGC-TGCCAT- 206
L. SINC GCGAGGGGCGCCTCGGAGAGT-----GAAAGCTTCGAATCAAAGCGCACCCACACTTCAACCCCAT-TAAACTCT-GAATCTGAACCTCAAAGGGGCGC-TGCCG- 205

L. ANG ---AGCGCGGC-TCCCCCAACCTTT-AACGTTGTACACCACCTCGCCCTCT-----TCTCTCTGTCTCAGACAGGGAGTCCGGC--GGCGACTTTGAGTGTCCGGAG 311
L. JAP ---AGCGCGGC-TCCCCCAACCTTT-AACGTTGTACACCACCTCGCCCTCT-----TCTCTCTGTCTCAGACAGGGAGTCCGGC--GGCGACTTTGAGTGTCCGGAG 311
L. SAC ---AGCGCGGC-TCCCCCAACCTTT-AACGTTGTACACCACCTCGCCCTCT-----TCTCTCTGTCTCAGACAGGGGGTCCGGC--GGCGACTTTGAGTGTCCGGAG 316
L. GROEN CTCTGCCCGGC-TCCCCCAACCTTT-AACGTTGTACACCACCTCGCCCTCTC---TCTCTCTCGGCTTCGGGG-TGGCGGGTTCGGGC--GGCGACTTTGAGTGTCCGGAG 313
L. DIGT ---CGCGCGGC-TCCCCCAACCTTT-AACGTTGTACACCACCTCGCCCTCTC---TCTCTCTCGGCTGAATAA-GGGTGGAGGGCGG--GGCGACTCTGAGTGTCCGGAG 317
L. LONG -GAAAACCGGCTTCCCCCAACCTTTTAAAGTTGTACACCACCTCGCCCTCTC---CTCTCTCCCGTGAATAAAGGGTTCGGAGGGCGG--GGCGACTCTGAGTGTCCGGAG 324
L. EPH ---TGTCCGGC-TCCCCCAACCTTT-AACGTTGTACACCACCTCGCCCTCTC---CTCTCTCCCGTGAATAAAGGGTTCGGAGGGCGG--GGCGACTCTGAGTGTCCGGAG 320
L. SET ---CGCGCGGC-TCCCCCAACCTTTTAAAGTTGTACACCACCTCGCCCTCTC---GTCC-TCCCTCTCGTGGTT-GGAGGGCGGCGGCGGACTCTGAGTGTCCGGAG 313
L. SINC ---GGCGCGGC-TCCCCCAACCTTTTAAAGTTGTACACCACCTCGCCCTCTC---TCCCTCTCGTGGTTGGAGGGCGGCGGCGGACTCTGAGTGTCCGGAG 315

ITS2

Fig. 13 cont'd.

ITS2 cont'd

L. ANG ↑ TTCCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCCTCTCGGGCCCTGCCGCACAGAGTTGTTGACGGGGCTCGCTTCGGGGGGGACTCTCGACTCACCAAA-CGTGGCGCA 425

L. JAP TTCCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCCTCTCGGGCCCTGCCGCACAGAGTTGTTGACGGGGCTCGCTTCGGGGGGGACTCTCGACTCACCAAA-CGTGGCGCA 425

L. SAC TTCCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCCTCTCGGGCCCTGCCGCACAGAGTTGTTGACGGGGCTCGCTTCGGGGGGGACTCTCGACTCACCAAA-CGTGGCGCA 430

L. GROEN TTCCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCCTCTCGGGCCCTGCCGCACAGAGTTGTTGACGGGGCTCGCTTCGGGGGGGACTCTCGACTCACCAAA-CGTGGCGCA 427

L. DIGT TTCCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCCTCTCGGGCCCTGCCGCACAGAGTTGTTGACGGGGCTCGCTTCGGGGGGGACTCTCGACTCACCAAA-CGTGGCGCA 431

L. LONG TTCCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCCTCTCGGGCCCTGCCGCACAGAGTTGTTGACGGGGCTCGCTTCGGGGGGGACTCTCGACTCACCAAA-CGC-CGCA 437

L. EPH TTCCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCCTCTCGGGCCCTGCCGCACAGAGTTGTTGACGGGGCTCGCTTCGGGGGGGACTCTCGACTCACCAAA-CGTGGCGCA 434

L. SET TTCCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCCTCTCGGGCCCTGCCGCACAGAGTTGTTGACGGGGCTCGCTTCGGGGGGGACTCTCGACTCACCAAAAGT----A 424

L. SINC TTCCCATGCTCCGAGTGCACCTAATCTCGTGAACGAAGCCCTCTCGGGCCCTGCCGCACAGAGTTGTTGACGGGGCTCGCTTCGGGGGGGACTCTCGA-TCCCGAAAAG-----CA 425

L. ANG GGATGCCCT----GCCTCATTCGGGGCTCCGACG-CGACCCCTTTAAGAAGGTCAGCGTT-GGAAACCGTAC--CACTTTCGTT 501

L. JAP GGATGCCCT----GCCTCATTCGGGGCTCCGACG-CGACCCCTTCT----GGGTACCGTT-GGAAACCGTAC--CACTTTCGTT 497

L. SAC GGATGCCCT----GCCTCATTCGGGGCTCCGACG-CGACCCCGTAAAAAGGTCAGCGTT-GGAAACCGTAC--CACTTTCGTT 506

L. GROEN GGATGCCCT----GCCTCATTCGGGGCTCCGACGTCGACCCGGTAAAAAGGTCAGCGTT-GGAAACCGTAC--CACTTTCGTT 505

L. DIGT GGCTGC----GGGGTTC-TTCGGGGCTCCTCGAGAAGGT-GTAAAAACCCCTCTTCGTTGGAAACCGTAC--CACTTTCGTT 508

L. LONG GGCTGC----GGGGTTC-TTCGGGGCTCCCGACCAAAGGG-----GGAAACCGTACACCCTTCGTT 499

L. EPH GGCTGC----GGGGTTC-TTCGGGGCTCCCGG-AGACTTTTTCAGGAAGTCTTTGG-----GGAAACCGTAC--CACTTTCGTT 506

L. SET GGGTGC----GGGGTTC-TTCGGGGCTCCC-GATGAAG-----GGAAA-----AC-CCACTTTCGTT 476

L. SINC GGCTGCTCGGGGGTTC-TTCGGGGCTCCC-GA-GAAG-----GGAAA-----AC-CCACTTTCGTT 479

Sequence conservation and variation: ITS1 & ITS2

Different patterns of sequence conservation and variation characterized the two rDNA internal transcribed spacers of Laminaria. ITS1 regions of these species had an initial block of conserved sequence (93% similarity, nucleotide positions 1 - 44, Fig. 12) followed by a variable region, V1 (positions 45 - 82). The next region (positions 83-150) was 76% conserved among the species and was followed by a short variable region, V2 (positions 151 - 170). The conserved region (positions 171 - 244) following V2 was 80% similar. The adjacent variable region, V3 (positions 245 - 258) was the most challenging ITS1 region to sequence. Manual sequence autoradiographs showed that compression artefacts flanked V3. However, these compressions were usually resolved with cycle sequencing and automated sequencing technology (see Methods and Materials). The 3' region of ITS1 (positions 259 - 282) was 91% conserved.

In contrast, the ITS2 was dominated by a central block of highly conserved sequence (130 bp with 95% similarity; positions 70 - 200). A semi-conserved region next to the central block (positions 201 - 239) was 61% conserved. Variable regions (V4 and V5) occurred next to short, conserved sequences near the 5' and 3' termini. These were 100% (positions 1 - 16) and 83% (positions 297 - 318) conserved respectively. V4 (positions 17 - 49) included a variable dipolymer C-T tract and V5, near the 5' end of the LSU gene, (positions 240 - 296) encompassed the most divergent sequence found within these spacers. A sizeable gap was introduced into the V5 region in order to align the

outgroup sequence of Postelsia. Similar patterns of sequence variation and conservation were observed in the ITS1 and ITS2 of Costaria costata and Cymathere triplicata.

Percentage G + C content

The G+C content of the rDNA internally transcribed spacers surveyed across nine species of Laminaria ranged from 54.6 - 57.3% (ITS1) and between 61.4 - 65.8% (ITS2) (Table 5). G+C content of the ITS2 exceeded that of the ITS1 among the Laminaria species (Table 5); however, the percentage difference between the two spacers did not exceed 10% in any of the study taxa. G+C content of the ITS2 also exceeded that of ITS1 in both Costaria and Cymathere (Table 5).

ITS Sequence Landmarks: Homopolymer & Dipolymer Tracts, Stem Loops, Repeats and Restriction Sites

Both ITS spacers contained a number of sequence 'landmarks' which easily distinguished them on manual sequence autoradiographs and automated sequence chromatograms. The ITS1 contained a homopolymer cytosine tract located 21 bp downstream from the 3' terminus of the SSU gene. This tract consisted of 7 cytosines in all the Laminaria species except for L. ephemera which had 8 cytosines. L. sinclairii had an additional tract of 7 cytosines 52 bp downstream from the SSU gene 3' end. Cymathere triplicata shared

Table 5: Percent G+C Content of rDNA ITSs among representative species of the kelp family Laminariaceae

%G+C			
SPECIES	ITS1	ITS2	% DIFFERENCE
<u>Laminaria</u>			
<u>L. angustata</u>	54.6	61.6	7.0
<u>L. digitata</u>	56.3	63.0	7.3
<u>L. ephemera</u>	54.7	61.4	6.7
<u>L. groenlandica</u>	56.7	64.0	7.3
<u>L. japonica</u>	56.3	62.9	6.6
<u>L. longipes</u>	55.0	64.8	9.8
<u>L. saccharina</u>	54.7	62.4	7.4
<u>L. sinclairii</u>	57.2	65.8	8.6
<u>Costaria</u>			
<u>C. costata</u>	57.0	61.3	4.3
<u>Cymathere</u>			
<u>C. triplicata</u>	54.5	62.7	8.2

the first tract of 7 cytosines in common with the Laminaria species. ITS1 had several smaller homopolymer tracts of 4 - 5 nucleotides each. The ITS2 contained a long polyC-T tract, located approximately 11 nucleotides downstream from the 3' end of the 5.8S gene. The length and exact sequence composition of this tract varied among the species sequenced. Sequences of this tract could usually be obtained from only one strand and therefore, multiple reactions of the resolvable strand were performed for confirmation. Visual inspection indicated that both the ITS1 and ITS2 contained numerous, dispersed, repeat motifs (3-4 nucleotides in length; GAAT, GAGT, GCCG were a few of the examples). A visual inspection for restriction sites showed a homologous SacI site was located in the ITS1, near the 3' end of region V2, of L. groenlandica and L. saccharina (Fig. 12). Two SacI sites were identified in the ITS2 of Cymathere triplicata, in the 5' region of the centrally conserved sequence block, and are separated from each other by only 2 base pairs (Fig. 12).

Two perfect but non-adjacent repeats of 5' AAAACTTT 3' occurred in the 5.8S gene (Fig. 12) of each study species. Several small nonadjacent repeat motifs are also characteristic of the 5.8S gene sequences.

A putative stem loop structure may be present near the 5' end of the 5.8S gene in most, if not all, of the study taxa (Fig. 12) and was identified by comparison to similar structures reported in other kelp (Saunders & Druehl 1993a) and in higher plants (Torres et al. 1990).

ITS Length Mutations

A number of small insertion/deletions were present in several of the Laminaria species. The addition of Cymathere triplicata rDNA ITS sequence to the aligned Laminaria sequences indicated either a unique insertion of approximately 16 bp in the ITS1 spacer of this alga or that a deletion of this size had occurred in the other compared sequences. This sequence accounted for most of the size difference between Cymathere and the Laminaria species.

Preliminary Assessment of Intraspecific rDNA Sequence Variation

Very little intraspecific sequence variation was detected among the kelp individuals selected for investigation (Table 6). Sequence comparison of ITS1 and ITS2 rDNA, isolated from single sporophytes from each of three geographically distinct stands of L. saccharina, uncovered a single nucleotide difference. An additional nucleotide (thymine) was found in the ITS1 of the individual from the north Atlantic (Table 6). No nucleotide differences were found in the ITS1 sequences of the two C. triplicata individuals from distinct stands on Vancouver Island, B.C. ITS2 sequences were identical for the two L. digitata individuals collected from two locations in Nova Scotia, Canada. However, a single nucleotide difference was detected between two individuals of L. groenlandica collected from the same

Table 6: Intraspecific rDNA sequence variation of the internal transcribed spacers (ITS1 & ITS2) in selected study species

<u>Species</u>	<u>Location</u>	<u>Region Assessed</u>	<u>Intraspecific Variation</u>
<u>Laminaria</u>			
<u>saccharina</u>	Garbary Bay ^c & (46° 15' N 60° 10' E)	ITS1 & ITS2	Extra "T" (ITS1)
	Whyte Cliff Park ^b & (49° 22' N 123° 17' W)	"	None
	Stanley Park ^b (49° 18' N 123° 17' W)	"	None
<u>Cymathere</u>			
<u>triplicata</u>	Whiffen Spit ^a & (48° 22' N 123° 47' W)	ITS1	None
	Cape Beale ^a (48° 47' N 125° 12' W)	ITS1	None
<u>Laminaria</u>			
<u>digitata</u>	Herring Cove ^c & (45° 30' N 63° 30' E)	ITS2	None
	Cape Split ^c (43° 30' N 65° 40' E)	ITS2	None
<u>Laminaria</u>			
<u>groenlandica</u>	Whiffen Spit ^a (LG01) &	ITS2	Extra "C"
	Whiffen Spit ^a (LG03) (48° 22' N 123° 47' W)	ITS2	None

^aWest Coast, Vancouver Island, B.C., ^bVancouver, B.C., and ^cNova Scotia, Canada.

location. These sporophytes differed in sequence by an additional cytosine adjacent to the variable C-T tract at the 5' end of the ITS2.

Phylogenetic Analyses

a. Transition v.s. Transversion frequency

Transition and transversion frequencies were not necessarily equal to each other in all of the pairwise comparisons of the study and outgroup taxa (Table 7). In several instances, transversions appeared more frequently, especially between more distant taxa as indicated by pairwise distance estimates (see below).

b. Distance Estimates

Pairwise distance estimates among the study and outgroup taxa, based on unambiguously aligned rDNA sequences of the 3' SSU - ITS1 - 5.8S - ITS2 regions (Table 8), indicated that the least distant sequence relationships generally occurred among the Laminaria species. Distance estimates ranged from 0.26 to 4.5% among Laminaria species pairs (Table 8). With the exceptions of Postelsia / L. angustata and Postelsia / L. saccharina, distance estimates were lower between the species of Laminaria and Cymathere triplicata than between the laminarians and the other compared taxa (Table 8).

Table 7. Pairwise Transition/Transversion Determinations* Among the Study and Outgroup Taxa: values computed from unambiguously aligned rDNA sequences of the 3' SSU - ITS1 - 5.8S - ITS2 regions.

TAXON	LJ	LS	LG	LN	LT	LL	LD	LE	CY	CO	AL	PO
<u>L. angustata</u>	2/0	2/0	12/6	11/19	11/13	17/14	14/13	13/15	18/15	20/24	21/22	22/14
<u>L. japonica</u>		2/0	10/6	12/19	12/13	17/14	14/13	13/15	18/15	22/24	19/22	22/14
<u>L. saccharina</u>			10/6	10/19	10/13	15/14	12/13	11/15	16/15	20/24	19/22	22/14
<u>L. groenlandica</u>				14/19	14/13	14/14	11/12	12/15	13/17	18/24	20/22	23/18
<u>L. sinclairii</u>				0/4		9/12	8/12	9/18	14/22	19/32	18/24	25/23
<u>L. setchellii</u>						10/10	9/10	9/16	14/16	20/27	17/20	24/19
<u>L. longipes</u>							5/1	6/7	11/21	21/28	18/23	20/24
<u>L. digitata</u>								3/6	9/19	18/27	15/21	18/22
<u>L. ephemera</u>									10/21	14/25	18/28	16/23
<u>Cymathere</u>										20/18	17/21	26/13
<u>Costaria</u>											23/24	27/28
<u>Alaria</u>												31/20
<u>Postelsia</u>												

*Calculated using ANDYPROG (Compare Option).

Table 8. Pairwise Transformed Distance Estimates^a Among the Study and Outgroup Taxa: Values determined from unambiguously aligned rDNA sequences of the 3' SSU - ITS1 - 5.8S - ITS2 regions.

TAXON	LA	LJ	LS	LG	LN	LT	LL	LD	LE	CY	CO	AL	PO
<u>L. angustata</u>		0.26	0.26	2.4	4.1	3.3	4.2	3.7	3.8	4.5	6.1	6.0	4.9
<u>L. japonica</u>			0.26	2.1	4.3	3.4	4.2	3.7	3.8	4.5	6.4	5.8	5.0
<u>L. saccharina</u>				2.1	4.0	3.1	3.9	3.4	3.5	4.2	6.1	5.8	4.9
<u>L. groenlandica</u>					4.5	3.7	3.8	3.1	3.7	4.1	5.8	5.9	5.6
<u>L. sinclairii</u>						0.5	2.9	2.7	3.7	5.0	7.2	6.0	6.7
<u>L. setchellii</u>							2.7	2.6	3.4	4.1	6.6	5.3	6.0
<u>L. longipes</u>								0.8	1.7	4.4	6.8	5.8	6.1
<u>L. digitata</u>									1.2	3.8	6.2	5.0	5.5
<u>L. ephemera</u>										4.3	6.4	5.5	5.4
<u>Cymathere</u>											5.2	5.3	5.3
<u>Costaria</u>												6.7	7.7
<u>Alaria</u>													7.2
<u>Postelsia</u>													

^aKimura (1980) two parameter corrected distances expressed as percentages.

Distance estimates computed for published ITS1 sequences of several kelp species (Saunders & Druehl 1993b), representing an additional 8 genera (Table 9) were generally 1.5 - 4 times greater among these species than those determined from ITS1 sequences among the Laminaria species of this study (Table 10). However, there were notable exceptions; 5 of the generic representatives of the Saunders & Druehl (1993b) study displayed pairwise distances comparable to those estimated between species within Laminaria. I observed a similar result for Cymathere triplicata. Although from a traditionally separate genus, pairwise distances between C. triplicata and all of the Laminaria species were as low or lower in some cases, than estimated distances between species within Laminaria (Table 10). Although distance estimates were higher when calculated using the Jukes & Cantor (1969) correction compared to the Kimura two parameter (1980) corrected distances, the difference appeared to be only a matter of degree with the same hierarchical relationships seen among the pairwise comparisons.

c. DNA Distance Analyses

Jukes-Cantor one parameter corrected distances were also employed in the phylogenetic analyses; trees inferred from these distances, were essentially identical to those inferred from the Kimura two parameter corrected distances.

Table 9. Pairwise Transformed Distance Estimates^a Among Kelp Genera:
 Values determined from published rDNA ITS1 sequences^b.

GENUS	AL	PO	MA	NE	LP	PT	CO	EG	EI	DI	LO
<u>Alaria</u>	16.4	17.1	17.3	5.1*	5.1*	20.6	23.5	20.9	18.3	18.5	
<u>Postelsia</u>	4.8*	16.7	4.3*	15.0	18.3	15.1	13.1	17.0	8.3		
<u>Macrocystis</u>	3.7*	15.9	3.7*	15.5	20.1	12.4	12.3	16.8	10.1		
<u>Nereocystis</u>	16.1	17.1	18.4	13.7	10.1	16.3	10.2				
<u>Lessoniopsis</u>	7.4	22.4	24.7	21.2	19.5	17.2					
<u>Pterygophora</u>	22.3	24.7	22.7	18.3	17.0						
<u>Costaria</u>	22.9	20.9	7.2	20.5							
<u>Egregia</u>	16.2	21.2	16.5								
<u>Eisenia</u>	18.9	11.8									
<u>Dictyoneurum</u>	17.1										
<u>Lessonia</u>											

^aKimura (1980) two parameter corrected distances expressed as percentages.

^bSaunders & Druehl 1993.

*Distance values within the range of those observed among Laminaria species (Table 10).

Table 10. Pairwise Transformed Distance Estimates^a Among the Study and Outgroup Taxa:
 Values determined from unambiguously aligned rDNA ITS1 sequences.

TAXON	LA	LJ	LS	LG	LN	LT	LL	LD	LE	CY	CO	AL	PO
<u>L. angustata</u>		0.0	0.0	1.2	3.6	3.5	2.9	2.3	3.6	2.3	14.4	13.1	4.8
<u>L. japonica</u>			0.0	1.2	3.6	3.5	2.9	2.3	3.6	2.3	14.4	13.1	4.8
<u>L. saccharina</u>				1.2	3.6	3.5	2.9	2.3	3.6	2.3	14.4	13.1	4.8
<u>L. groenlandica</u>					2.9	2.9	2.3	1.7	4.1	1.7	13.6	13.0	5.3
<u>L. sinclairii</u>						0.0	2.9	2.3	5.4	3.5	15.8	14.5	6.6
<u>L. setchellii</u>							2.9	2.3	5.4	3.5	15.8	14.5	6.7
<u>L. longipes</u>								0.6	3.6	2.9	13.6	14.4	4.8
<u>L. digitata</u>									2.9	2.3	12.9	13.0	4.1
<u>L. ephemera</u>										4.7	15.8	14.6	4.8
<u>Cymathere</u>											14.3	12.3	5.9
<u>Costaria</u>												15.3	15.0
<u>Alaria</u>													13.6
<u>Postelsia</u>													

^aKimura (1980) two parameter corrected distances expressed as percentages.

The outgroup rooted, majority-rule consensus tree inferred from the neighbor-joining analysis of pairwise distance values among the study taxa, showed two distinct groups, both of which were supported by high bootstrap values (Fig. 14a). Group 1 consisted of L. angustata, L. japonica, L. saccharina and L. groenlandica; these four species grouped together in 92% of the trees generated. Bootstrap values of 90% or greater were considered to have resolved the branch points. Within Group 1, the branching order appeared well defined with the exception of L. angustata and L. japonica. L. angustata and L. japonica form an initial clade, with low support (64%) from which L. saccharina branched (100%) with strong support, followed by L. groenlandica (92%). Group 2 consisted of two clades: L. ephemera, L. longipes, L. digitata formed one clade with 90% support while L. setchellii and L. sinclairii formed the other (100%). Branching of Cymathere, Costaria, and the outgroup taxa, was unresolved with respect to the Laminaria species and to each other. The unrooted phylogeny inferred from ITS1 & ITS2 sequences, of Laminaria species only, supported the same groups with comparable bootstrap confidence limits (Fig. 15). A comparison of the holdfast type and blade morphology of the Laminaria species within each molecular inferred clade, contradicted the traditional taxonomic and phylogenetic relationships inferred from these morphological characters (Figs. 14b & 15).

Fig. 14a: Resulting majority rule consensus tree generated by the neighbor-joining method (Saitou & Nei 1987) with bootstrapping: Laminaria, Costaria, Cymathere and outgroup taxa.

Tree inferred from transformed distances (Kimura 1980) calculated between all pairwise combinations of the study and outgroup taxa. Distances based on rDNA sequence variation of the 3' SSU gene, ITS1, 5.8S gene and ITS2 regions. 500 bootstrap replicates were made of the original data set (Felsenstein 1993).

Numbers at each node are percentages that indicate the frequency of occurrence for that clade. See text for discussion of Groups 1 & 2.

Fig. 14 a

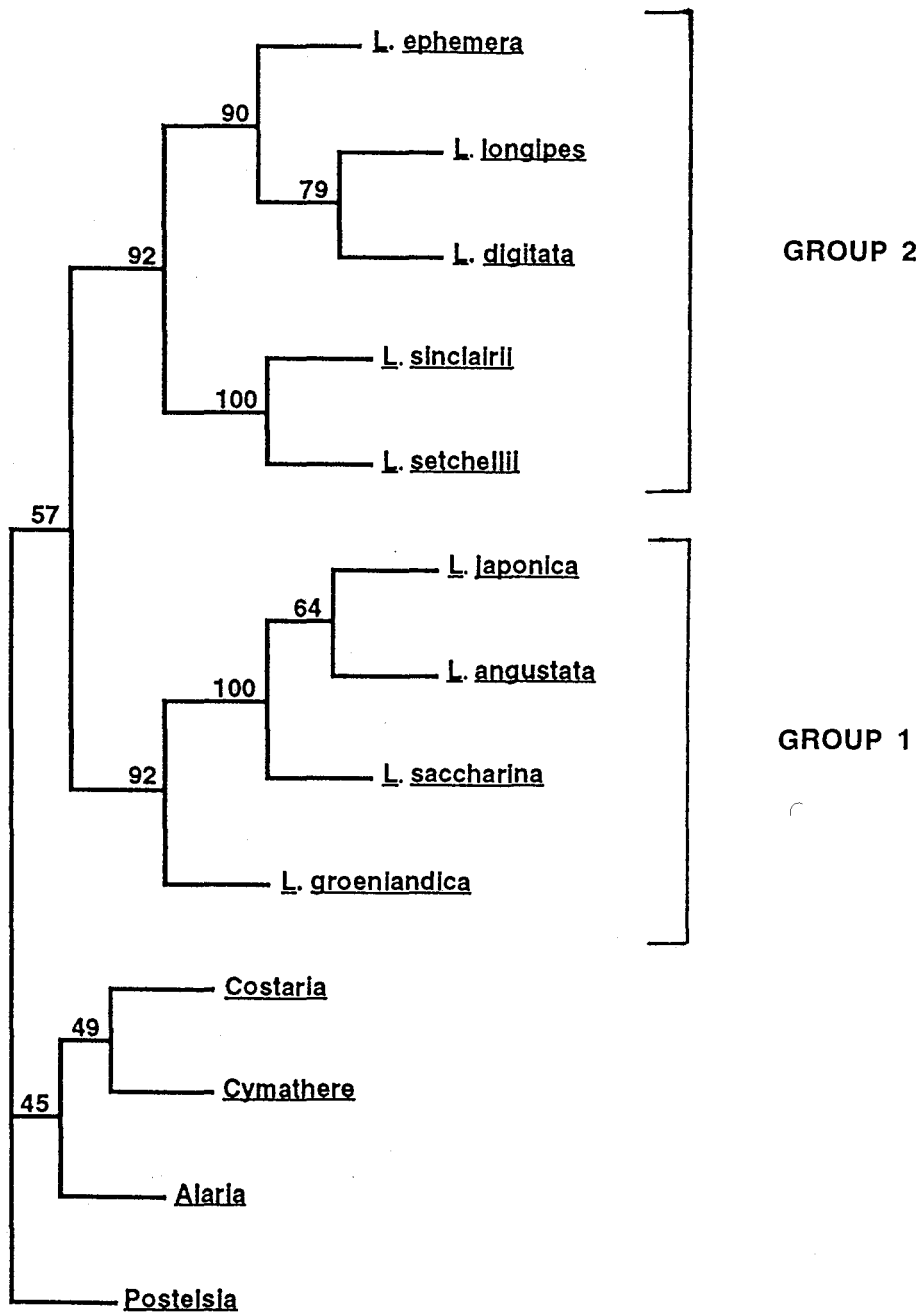


Fig. 14b: Comparison of morphological characters (holdfast and blade type) with inferred phylogenetic relationships based on molecular data:

Phylogenetic tree as in Fig. 14a. Morphological features (holdfast and blade types) of the study taxa displayed along the right hand figure margin. These features are currently used to delineate Laminaria species taxonomically and phylogenetically, however as can be seen, the species do not necessarily group according to these features. Cymathere triplicata and Costaria costata, two intrafamilial species with morphological similarity to Laminaria species, group with Postelsia palmaeformis and Alaria marginata, the designated outgroup taxa. *Costaria has a blade with ribs rather than folds or fascia. See text for further details.

Fig. 14 b

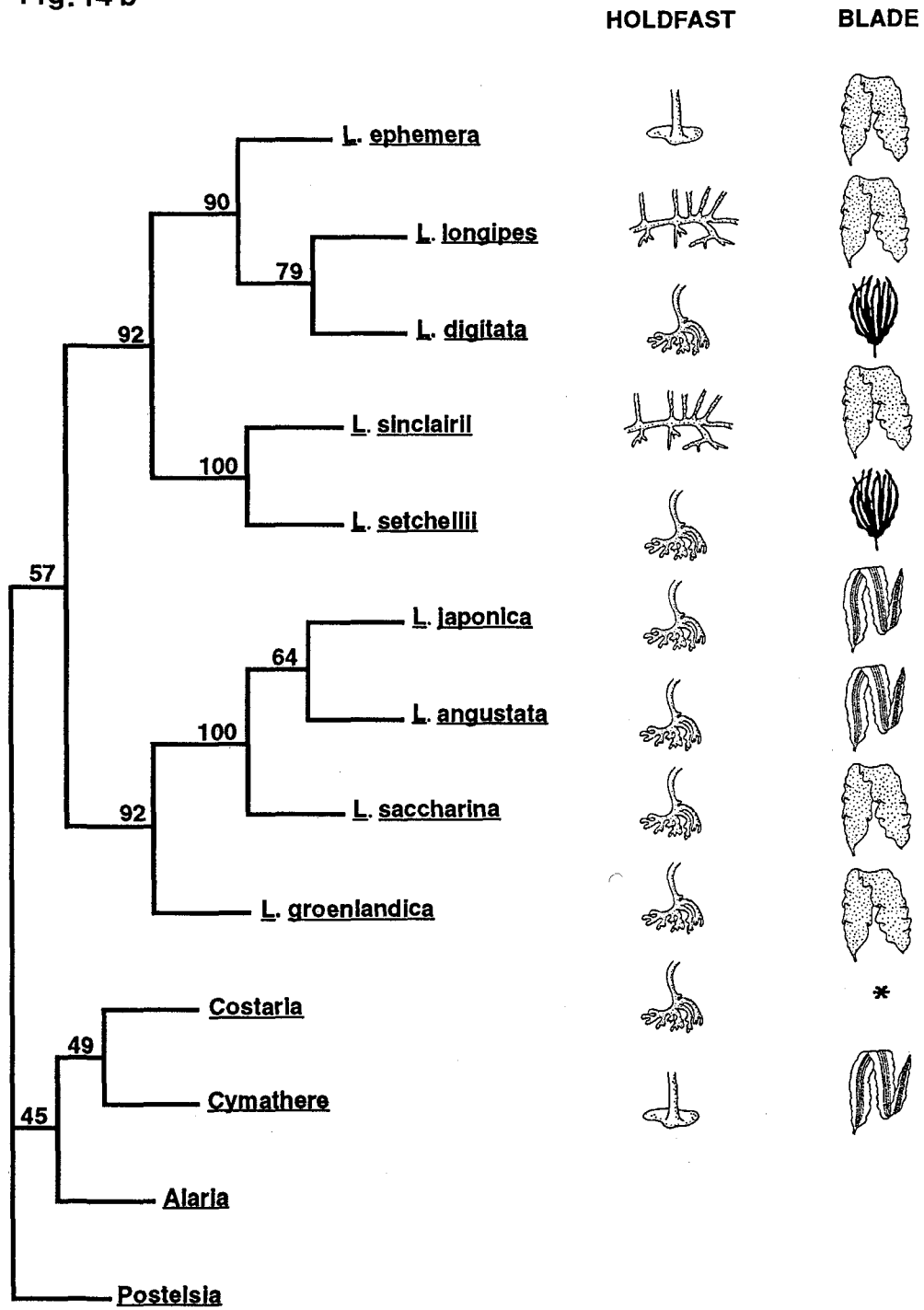
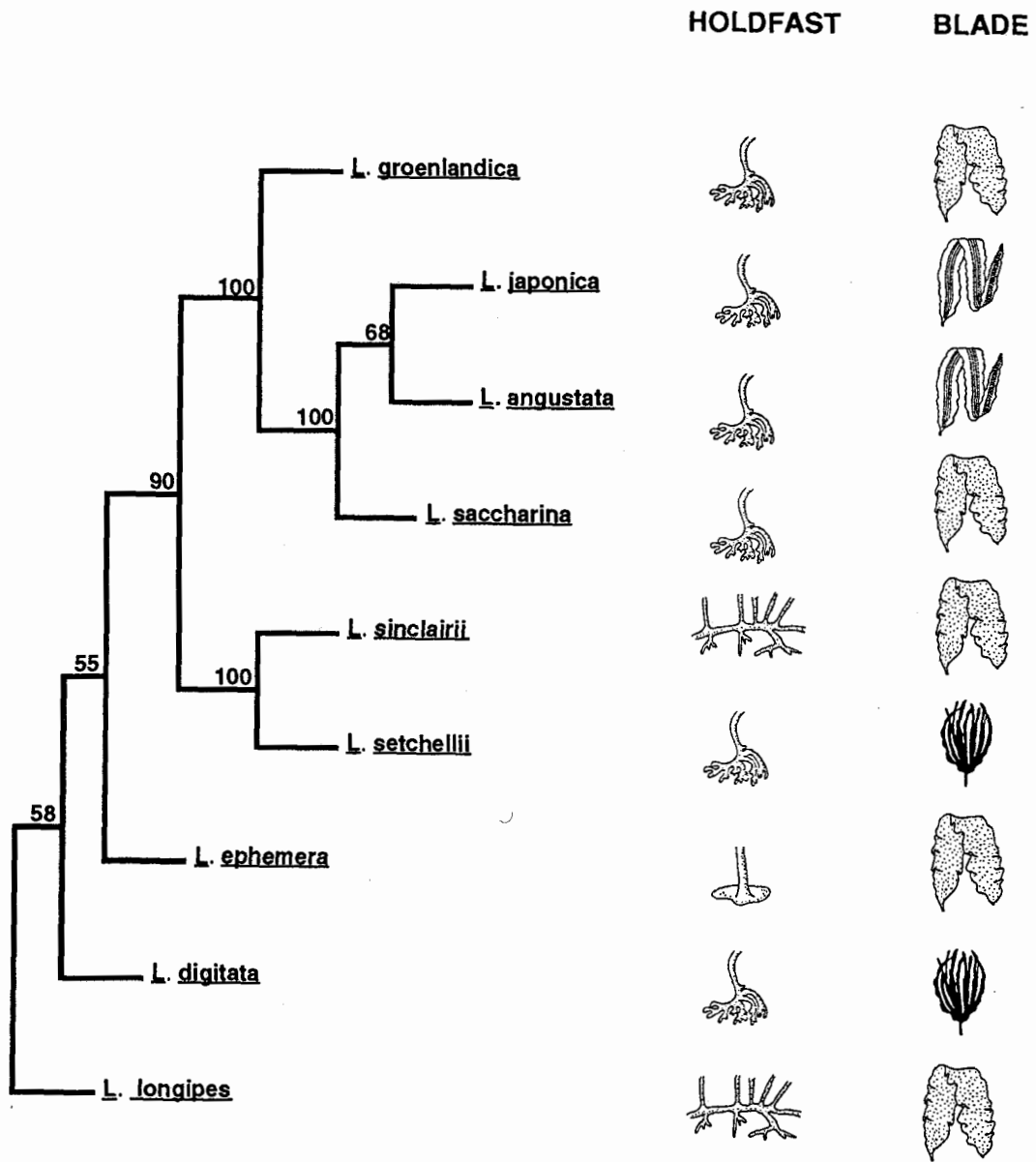


Fig. 15. Inferred Laminaria species relationships:
Unrooted majority rule consensus tree
generated by the neighbor-joining method:

Tree inferred from transformed distances
(Kimura 1980) calculated between all
pairwise combinations of the Laminaria
species only. Distances based on rDNA
sequence variation of the ITS1 and ITS2
only. 500 bootstrap replicates made of
the original data set (Felsenstein 1993).
Numbers at each node are percentages
that indicate the frequency of occurrence
for that clade.

Holdfast and blade type of each species is
displayed on the right hand figure margin.

Fig. 15



d. Parsimony Analyses

The outgroup rooted consensus tree, inferred from parsimony analysis of the study and outgroup taxa, was similar to the neighbor-joining tree inferred from distance data (Fig. 16). The same two groups were strongly supported by the bootstrap values. The tree differed in that analysis based on informative sites led to high bootstrap support for the clade of L. angustata and L. japonica (Group 1); the branch point of this pair was unresolved in the rooted neighbor-joining tree (64 v.s. 100). Although the clade of L. ephemera, L. digitata and L. longipes (Group 2) was highly supported (96%), the branching order among these species was unresolved in the parsimony analysis. The unrooted parsimony consensus tree (Fig. 17), based on ITS1 & ITS2 sequence data of the Laminaria species only, was almost identical to the unrooted neighbor-joining tree (Fig. 15).

Fig. 16. Inferred Laminaria, Cymathere, Costaria and outgroup taxa phylogeny: Outgroup rooted majority rule consensus tree generated from parsimony analysis. Comparison based on informative sites in rDNA sequences of the 3' SSU gene, ITS1, 5.8S gene and ITS2 of all study and outgroup taxa. 200 bootstrap replicates were made of the original data set (Felsenstein 1993). Numbers at each node are percentages that indicate the frequency of occurrence for that clade. See text for discussion of Groups 1 & 2.

Fig.16

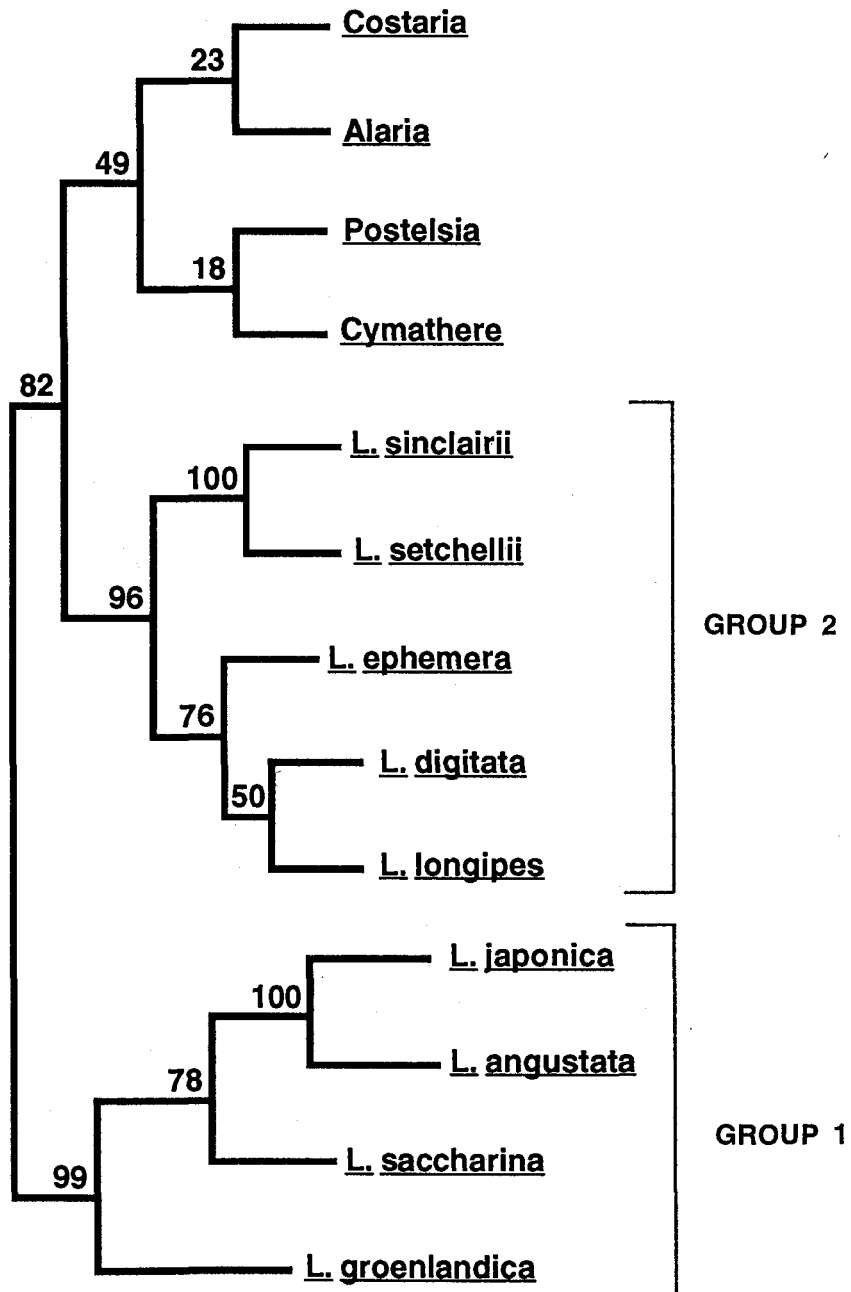
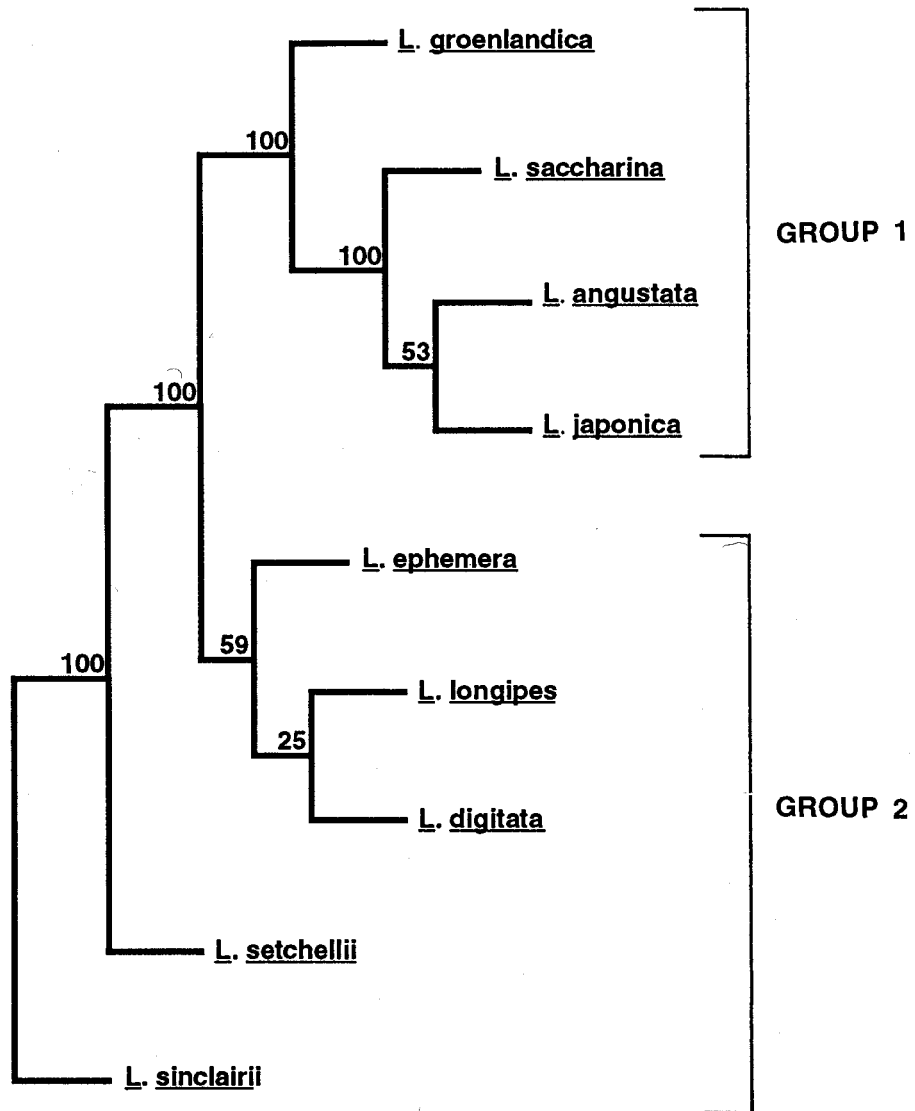


Fig. 17. Inferred Laminaria phylogeny:
Unrooted consensus tree generated
from parsimony analysis.

Analysis based on informative
sites in the rDNA ITS1 & ITS2
sequences of nine Laminaria
species. 200 bootstrap replicates
of the original data set were made
(Felsenstein 1993). Numbers at
each node are percentages that
indicate the frequency of
occurrence for that clade.
See text for discussion of Groups
1 & 2.

Fig. 17



DISCUSSION

Kelp DNA Extraction

Total cellular DNA of sufficient quantity and purity for nuclear molecular applications was quickly and easily obtained, in a single day, from milligram amounts of dried kelp sporophyte blades, fresh gametophytes and meiospores. Previous extraction techniques, developed primarily for kelp chloroplast DNA, were time consuming, labour intensive and required large quantities of fresh sporophyte tissue (Barrett & Anderson 1980, Popovic *et al.* 1983, Fain *et al.* 1988). In contrast, the new methods provide a number of substantial improvements. First, small quantities of tissue from the alternate life history stages of kelp can be exploited as DNA sources. Second, a considerable time savings is realized by elimination of lengthy ultracentrifugation steps to purify the DNA. Instead, DNA can be purified quickly and simply using gel filtration on Sepharose columns. In addition to the relative simplicity of these methods, the ability to extract DNA from gametophytes and dried sporophytes, both of which are easily transported from international locations, enhances the feasibility of including species from distant locations in molecular comparisons with local species. Finally, the possibility of extracting DNA from small quantities of dried sporophyte blades not only affords access to rare herbarium specimens for studies of molecular

evolution but also provides an alternate means of comparing field collected specimens with type material.

Sporophyte and Herbarium Specimen DNA

Fain *et al.* (1988) report total cellular DNA yields ranging from 0.3-1.7 $\mu\text{g}\cdot\text{g}^{-1}$ (wet wt) of kelp sporophyte tissue. These yields convert to 2.0-11.33 $\mu\text{g}\cdot\text{g}^{-1}$ (dry wt) assuming that dry sporophyte weight is approximately 15% of wet weight in *Laminaria* species (Stam *et al.* 1988). These yields border on the lower range of my DNA yields obtained from dried sporophyte tissue (12.8-26.4 $\mu\text{g}\cdot\text{g}^{-1}$) using the newly developed methods. Stam *et al.* (1988) report superior DNA yields from lyophilized sporophyte blades (117 $\mu\text{g}\cdot\text{g}^{-1}$), however, their protocol requires in excess of three days to obtain purified DNA for molecular applications.

Rogers and Bendich (1985) report successful DNA extraction from a variety of terrestrial plant herbarium specimens, 22 to 118 years old. To my knowledge, this is the first evidence of the ability to isolate DNA from herbarium specimens of kelp. Doyle and Dickson (1987) suggest drying as the most effective means of preserving higher plant material for DNA studies. Although DNA in dried samples degrades slowly over a several year period, such specimens still contain DNA fragments of sufficient size for molecular applications. The study results indicate this may also apply to kelp sporophyte and herbarium specimen DNA.

Gametophyte and Meiospore DNA

DNA yields from microscopic gametophytes and meiospores are greater compared to yields from macroscopic sporophytes because the former have greatly reduced polysaccharide content (Henry 1980). Greater yields from meiospores were expected; meiospore volume is dominated by nuclei, there is little evidence of storage products or secondary metabolites (polyphenolics) and most importantly, meiospores lack cell walls (Henry 1980). Direct lysis of gametophyte and meiospore organelles, along with the relatively low concentrations of polyphenolics found in these life history stages (Henry 1980), minimizes the problem of organelle loss, encountered with the sporophyte method, and nuclease degradation of DNA during extraction. By contrast, up to one third of the total dry weight of kelp sporophytes is polysaccharide (Rosell & Srivastava 1984). Further, the DNA extraction protocol developed here for kelp blades necessarily incorporated additional wash steps to remove polysaccharides and other contaminants; DNA is lost at each step, thus reducing the overall efficiency of the isolation. Another contributing factor to lower DNA yields from sporophyte tissue are the polyphenolic compounds contained in numerous phytol vesicles of sporophyte meristoderm cells (Ragan 1976). These compounds destroy organelle membranes (Fain 1986). However, in spite of the

greater polysaccharide contamination problems of sporophyte DNA, useful quantities were purified sufficiently with the Sepharose spin columns, to allow endonuclease digestion (data not shown).

The inability to amplify gametophyte DNA isolated from C. triplicata may be related to a residual PCR inhibiting contaminant or polysaccharide. I have also been unable to extract DNA from sporophytes of C. triplicata despite numerous attempts with fresh or dried young tissue. The lack of cell walls (source of structural polysaccharides) most likely accounts for the successful isolation of amplifiable DNA from C. triplicata meiospores. Indeed, meiospores are the preferred life history stage for kelp DNA isolation. The high nuclear to cytoplasm ratio and presumably minimal polysaccharide and polyphenolic content of these spores enhanced DNA yields. Of the life history stages, the greatest yields per volume were obtained from meiospores. Many kelp species produce meiospores only a few months of the year, however these can be germinated to provide an accessible source of gametophyte cultures for DNA extraction. Meiospores and gametophytes may be the only meaningful sources of DNA for some kelp species, whose sporophytes are extremely difficult to extract. In general, mature and/or thick bladed kelp are a greater challenge to DNA isolation and purification compared to young and/or thin bladed sporophytes. The increased polysaccharide-polyphenolic content of the former is the likely cause.

rDNA Sequence Features

A. Comparison of kelp ITS1, 5.8S and ITS2 rDNA regions with other eukaryotes

Although rDNA sequence analysis was undertaken for the purpose of obtaining morphology-independent characters for phylogenetic inference within the genus Laminaria, these sequences have interesting features in common with other eukaryotic ITS sequences. Short repeat motifs and homopolymer tracts, found in the study taxa, are also typical of rDNA internal transcribed spacer sequences in many eukaryote groups [for example, humans and other primates (Gonzalez 1990), fungi (Lee & Taylor 1992), chlorophytes, (Bakker et al. 1992), numerous higher plants (Torres et al. 1990; Baldwin 1992), and brown algae (van Oppen et al. 1993) including kelp species within the families Alariaceae, Lessoniaceae and Laminariaceae, (Saunders & Druehl 1993a; 1993b)]. Short repeat motifs and homopolymer tract regions are subject to higher rates of change than surrounding sequences because they tend to be targets for slipped-strand mispairing (Tautz et al. 1986; Levinson & Gutman 1987).

Although an analysis of secondary structure was beyond the scope of this study, a putative stem loop structure may exist near the 5' end of 5.8S gene in most of the study taxa. When non-canonical base pairing was used, stem loop structures similar in appearance to

those reported for other kelp (Saunders & Druehl 1993a and higher plants, could be drawn (Torres et al. 1990). The compression artefacts encountered during sequencing of the region of this putative secondary structure (Fig. 12) lend support to its existence. However, further investigations are needed (thermal stability and structural integrity) for its confirmation.

Laminaria, Costaria and Cymathere internal transcribed spacer sequences, were considerably shorter (2 - 3 times) than those reported for vertebrates (Michot et al. 1983; Gonzalez et al. 1990) but were most similar in length to the ITS sequences of other kelp species (Saunders & Druehl 1993a; 1993b) and of higher plants (Torres et al. 1990; Baldwin 1993).

ITS size, sequence composition and secondary structure are not only useful for comparative purposes across taxa but may also contribute to an understanding of rRNA processing systems among eukaryotes (Gerbi 1986; Torres et al. 1990).

B. Evolution of ITSs in Laminaria

Aligned sequence comparison of the ITS1 and ITS2 of nine Laminaria species showed these spacers differed in their pattern of evolutionary divergence. In the ITS1 a regular pattern of conserved sequence regions alternated with semi-conserved and variable blocks of sequence throughout the spacer. In the contrast, the ITS2 had a highly conserved central block of sequence (130 bp) flanked by

variable regions. This pattern was also observed in the two additional laminariaceans, Costaria costata and Cymathere triplicata and was also the pattern found in other advanced kelp representatives (Saunders & Druehl 1993a), the brown alga, Desmarestia viridis/willii (van Oppen et al. 1993), fungi (Carbone & Kohn 1992; ITS1 sequences), and several higher plant groups (Baldwin 1992; Torres et al. 1990).

C. GC Balance in the ITSs of Laminaria

'GC balance' refers to an equivalent or highly similar G+C content within rDNA internal transcribed spacers which Torres et al. (1990) suggest is a widespread phenomenon among eukaryotes. However, the ITSs of the slime mold, Dictyostelium discoideum do not exhibit GC balance (Torres et al. 1990) and recently, Saunders & Druehl (1993a) found that the advanced kelp species Alaria marginata only weakly displays GC balance. Kelp species examined in this study appeared to display GC balance (some more strongly than others (Table 5). At present, the significance of the GC balance phenomenon is unknown, however, it is hypothesized that it may be involved in some aspect of post-transcriptional processing (Torres et al. 1990).

Preliminary Assessment of Intraspecific rDNA Sequence Variation

Although the homogenization of nuclear ribosomal cistron repeats via concerted evolution is considered extreme (Sanderson & Doyle 1992), it is not necessarily complete in the faster evolving spacer sequences of individuals (Stewart et al. 1983). A range of intraspecific rDNA ITS variation has been reported in eukaryotes including fungal species (O'Donnell 1992), green algae (Bakker et al., 1992) and brown algae (van Oppen et al. 1993). The variation can be as high as 30% between individuals of a single fungal species (O'Donnell 1992) or as low as a single nucleotide difference between individuals of brown algal species (Desmarestia viridis/willis; van Oppen et al. 1993; Nereocystis lutkeana; G. Saunders, pers. comm.). My preliminary survey of intraspecific rDNA sequence variation was initiated to ascertain if substantial variation existed between individuals of a subset of the study species. The intraspecific variation detected was low and suggested the nature of the potential variation within the ITS1 & ITS2 of Laminaria species was primarily interspecific.

The single nucleotide difference found in the individual sporophyte of L. saccharina from the North Atlantic compared to the Pacific individuals may indicate that these populations have only recently been isolated from each other (Luning & tom Dieck 1990). Kain suggested (1979) that the Atlantic and Pacific versions of L. saccharina were not conspecific as the Pacific L. saccharina produces

an annual sporophyte while Atlantic sporophytes are perennial. However, tom Dieck (1992), crossed L. saccharina from the northeast Atlantic and northeast Pacific and obtained mature F1 sporophytes. The low level of variation in the ITS sequences of L. saccharina, along with the present northern hemisphere, cosmopolitan, distribution of this species, and conditions during the Pleistocene (access to the Arctic and Atlantic oceans through the Bering Strait and an eastward prevailing Arctic current) (Stam et al. 1988), could lead one to speculate on the possibility of genetic exchange between the North Pacific and North Atlantic L. saccharina. However, lack of a fossil record for Laminaria species keeps this in the realm of speculation.

Distance Estimates

Comparison of the distance estimates among the ITS1 sequences of Laminaria species, to that of other generic kelp representatives (Saunders & Druehl 1993b), suggested that the laminarian species probably arose and diverged more recently than most of the generic kelp representatives. However, there appeared to be a few exceptions (ie. Postelsia, Macrocystis, and Nereocystis) (Table 9). These generic representatives had pairwise distance estimates within the range of those calculated for the species of Laminaria. In addition, Cymathere triplicata (Table 8 & 10) had pairwise distance values with the Laminaria species on par or slightly higher than those calculated between these species. Druehl & Saunders (1992) found levels of

divergence among genera of kelp families equal to that of higher plant species. The results of this study's comparison of ITS1 sequence divergence further support their conclusion that Laminariales taxonomy is overinflated relative to other algal and plant orders.

Pairwise distance estimates were the lowest between L. angustata, L. japonica, and L. saccharina; the calculated distance between each pair was 0.26% (Table 8). The ITS1 & ITS2 sequences for these species were highly similar. L. angustata and L. japonica differed by only two transitions and a small insertion/deletion of 4 bp in the ITS2. The insertion/deletion occurred within an ambiguous alignment region that was not included in the phylogenetic analysis. The ITS sequence of L. saccharina differed from L. angustata and L. japonica by an additional 5 nucleotides.

Inferred Laminaria Phylogeny based on rDNA Sequence Data

Both distance and parsimony analyses inferred similar rooted and unrooted trees from the rDNA sequence data of Laminaria, Costaria costata, Cymathere triplicata and the outgroup taxa. In all of the inferred, consensus trees, two groups were supported with bootstrap confidence values of at least 90% (Figs. 14a - 17). The similarity of the phylogenies inferred from different analysis methods suggests the results were robust. The positions of Costaria, Cymathere and the outgroup taxa, were unresolved with respect to each other and to the Laminaria species. Additional sequence data and/or additional taxa may help to resolve the phylogenetic relationships between the Laminaria species and these non-laminarian taxa.

Within Group 1, the discrepancy in branch node support for L. angustata, and L. japonica, between the inferred distance trees (Figs. 14a, 14b, & 15), the unrooted parsimony tree (Fig. 17) (53 -68%) and the parsimony inferred phylogeny (Fig. 16)(100%) may be due to the high sequence similarity between L. angustata, L. japonica and L. saccharina.

In Group 2, the unresolved phylogenetic relationships among the strongly supported second clade (L. ephemera, L. longipes and L. digitata) may be resolvable with additional sequence data. The External Transcribed Spacer (ETS) of the ribosomal cistron may be a

possible candidate with sufficient variability for this purpose.

Molecular data did not group the species according to morphological similarity as predicted by the traditional taxonomy from which current evolutionary relationships among Laminaria species are inferred (Figs. 14b & 15). Instead, in Group 1, two fasciate species with hapteral holdfasts (L. angustata, and L. japonica), were grouped together with two simple-bladed species (L. saccharina and L. groenlandica) with hapteral holdfasts. In the Group 2 clades, L. setchellii (a digitate species with a hapteral holdfast) clustered with L. sinclairii (a species with simple, multiple blades and a rhizomatous holdfast), and in the second clade, L. longipes (with simple multiple blades and a rhizomatous holdfast) grouped with L. ephemera (simple blade and discoid holdfast) and L. digitata (digitate blade and a hapteral holdfast).

The reliance upon 'obvious' morphological similarity to delineate kelp species phylogenetically can be misleading due to phenotypic plasticity or convergent evolution. For example, all discoid holdfasts may not be equal among the kelp or even within Laminaria. There appears to be at least two routes by which this holdfast may arise: all juvenile kelp sporophytes develop an initial attachment disk from which the young stipe and blade arise. The haptera that form a hapteral holdfast develop later in a ring above this disk, eventually obscuring it (Kain 1979). In L. ephemera, the discoid holdfast could be a retained initial attachment disc. Laminaria ephemera is unusual and has perhaps evolved the strategy of a neotonous juvenile in having an ephemeral sporophyte, sporing early and disappearing much earlier in

the season than even the annual kelp species (Kain 1979; Klinger 1984). In contrast, the discoid holdfast of Cymathere triplicata sometimes has the appearance of fused haptera. In addition, Nagai (1940) found specimens of this kelp with rudimentary hapteres growing above the disc. Two other species of Laminaria, L. solidungula and L. yezoensis have discoid holdfasts. In these species the holdfast appears as a solid confluence with no suggestion of fused haptera (L. D. Druehl pers. comm). One can also speculate on the distinctiveness of the hapteral and rhizomatous holdfasts. The rhizomatous holdfast may be a variation of the hapteral type; (perhaps it is a combination of primary, decumbant stipes with clusters of hapteres while secondary stipes give rise to the blades). It appears to have arisen a number of times within the kelp (ie. Ecklonia stolonifera Okamura, Streptophyllopsis kurioshioensis (Segawa) Kajimura, and L. rodriguezii Bornet). Work has yet to be done to determine the extent of phenotypic plasticity of this morphological character. However, studies have determined that blade characteristics (width, thickness, texture, degree of bullation, and in some species, presence and abundance of mucilage ducts) are highly phenotypically plastic characteristics) (Burrows 1964; Bhattacharya & Druehl 1989).

Geographic distributions appear to group a number of the species in accordance with the groups inferred from the molecular data. For example, of Group 1 plants, L. japonica, L. angustata and L. saccharina all occur along the coasts of Japan and the Kuriles while

L. saccharina and L. groenlandica are both found along the northeast Pacific coast and in the Arctic. In addition, L. groenlandica and L. saccharina both have maximum upper limit temperature tolerances of 15°C (Luning & Freshwater 1988).

By contrast, Group 2 plants, L. sinclairii and L. setchellii have a relatively 'southern' distribution and are exclusively northeast Pacific species. L. setchellii extends further north to southern Alaska but shares the southern portion of its geographic range with L. sinclairii (both overlapping from central British Columbia to central California (Druehl 1970; Markham 1972). Within the geographic range, both occupy habitats with moderate to heavy wave exposure; however, L. sinclairii is further restricted to beaches where it colonizes rocks that are buried under sand on a seasonal basis (Markham 1972; Klinger & DeWreede 1988). Perhaps these species have evolved in sympatry. Further, both of these species display a mode of blade regeneration observed only in one other laminarian (L. farlowii Setchell). These kelp initiate a new blade by the elongation of the inner cortical cells within the transition zone. Elongation of these cells causes the outer cell layers to split horizontally with the consequent formation of collar-like structures on the stipe and blade base (Setchell 1905; Markham 1972; Klinger & DeWreede 1988).

This characteristic developmental pattern of blade regeneration may prove a useful phylogenetic character, especially if future investigations of molecular data from L. farlowii indicated a close

genetic affinity with L. sinclairii and L. setchellii.

The remaining Group 2 kelp span the morphological diversity found within Laminaria, and their close phylogenetic relationships based on rDNA sequence data are not easily explained by other lines of evidence. Geographically, L. digitata is separated from L. ephemera and L. longipes. Although the north Atlantic L. digitata extends into the Arctic, there are no Arctic distribution records of L. ephemera or L. longipes. Likewise, there appears to be little geographic overlap between L. longipes and L. ephemera: the former is distributed on both sides of the north Pacific from the Kuriles, through the Aleutians to Coronation Island, in southern Alaska, while the latter species has a relatively southern distribution (from southwest Vancouver Island to central California). However, an isolated population of each species occurs within the other's geographic range (Druehl 1970). Perhaps this indicates that L. longipes and L. ephemera once shared a continuous geographic range. Although highly speculative, one can propose that individuals of the laminarian ancestor for this clade may have dispersed through the Arctic giving rise to L. digitata in the north Atlantic while remaining populations of this ancestor gave rise to L. ephemera and L. longipes in the north Pacific.

North Pacific and North Atlantic Species Affinities of Laminaria

Discernment of laminarian sister species, along Pacific and Atlantic coasts, led previous authors to propose L. groenlandica

(Northeast Pacific) and L. digitata (North Atlantic) as sister species, whose recent common ancestor, dispersed from the Pacific to the Atlantic through the Bering Strait (Luning & tom Dieck 1990; tom Dieck 1992). Their proposal is based on morphological similarity, (both species have complanate, flexible stipes and divided blades (although the origin of blade splitting differs between them) and similar sporophyte longevities. In addition, non-viable sporophytes were produced from crosses of these species suggesting reproductive barriers had developed due to geographic separation (Luning & tom Dieck 1990; tom Dieck 1989). Results from the present study support the suggestion that sister species affinities of Pacific and Atlantic Laminaria are still discernable. However, the molecular data suggested a closer phylogenetic relationship between L. groenlandica and North Pacific/North Atlantic L. saccharina than with L. digitata. In addition, L. groenlandica and L. saccharina had a lower pairwise distance estimate (2.4 % versus 3.7% for L. groenlandica/L. digitata) (Table 8), and also shared a unique restriction site (SacI in the ITS1) (Fig. 12). Further, the molecular based phylogenies indicated that North Atlantic L. digitata had a closer affinity to other North Pacific Laminaria species (L. ephemera and L. longipes) than to L. groenlandica.

CONCLUSIONS

Total cellular DNA of sufficient quantity and purity for molecular applications can be quickly and easily obtained from dried kelp laminae, fresh gametophytes and meiospores. Previous kelp DNA extraction techniques were time consuming and labour intensive and some required large quantities of fresh sporophyte tissue. In contrast, the new methods use small quantities of tissue, exploit varied life history stages of kelp and save considerable time by elimination of lengthy ultracentrifugation steps.

The internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal cistron provide sufficient sequence variation for the delineation of Laminaria species and for phylogenetic inference within this kelp genus. The inferred phylogenetic relationships based on molecular data conflict with relationships inferred from morphology-based taxonomic characteristics.

Additional sequence and/or the inclusion of additional taxa may help to resolve the phylogenetic relationships among L. ephemera, L. longipes and L. digitata (Clade 2 of Group 2) and aid the resolution of the branching order of Costaria, Cymathere and the outgroup taxa included in this analysis. The ribosomal External Transcribed Spacer may prove a potentially useful sequence region with sufficient variation for this purpose.

Although speculative, the connections between present day geographic distributions of Laminaria species and their molecular inferred relationships may give us clues to past speciation events and a starting point for future investigations.

As superior alignment algorithms become available and/or when the details of ITS secondary structures are available to aid alignments among Laminaria species, superior hypotheses of evolutionary relationships among these species will likely emerge. Finally, corroboration of phylogenetic inference from as many data sets as possible (incorporating other molecular, physiological, developmental characters and so forth) would strengthen our understanding of evolutionary relatedness among kelp species.

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