

MOLECULAR BIOLOGICAL ANALYSIS OF MORPHOLOGICAL
VARIATION, POPULATIONS AND PHYLOGENY OF THE
KELP COSTARIA COSTATA (PHAEOPHYTA)

by

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B.Sc. (Hons.), Dalhousie University, 1981

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the department
of
Biological Sciences

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SIMON FRASER UNIVERSITY

June 1988

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Degree: Doctor of Philosophy

Title of Thesis:

MOLECULAR BIOLOGICAL ANALYSIS OF MORPHOLOGICAL VARIATION, POPULATIONS
AND PHYLOGENY OF THE KELP COSTARIA COSTATA (PHAEOPHYTA)

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Molecular Biological Analysis of Morphological Variation, Populations

and Phylogeny of the Kelp Costaria costata (Phaeophyta).

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ABSTRACT

Molecular biological analyses of the kelp, *Costaria costata* (C.A. Agardh) Saunders, were undertaken to study phenotypic variation, genetic subdivision of populations and phylogeny.

To study the basis of phenotypic variation in *Costaria*, individuals were collected from two morphologically distinct stands. Preliminary analysis of morphological variation using multivariate statistical techniques, indicated substantial differentiation between plants from the two sites. Phenotypic integration of the morphology of *Costaria*, on the basis of correlation analysis, was similar for wave-exposed and wave-sheltered plants. Restriction fragment length polymorphism (RFLP) analysis with 42 anonymous probes demonstrated only one polymorphism. Most of the probes (80.9%) encoded highly repeated, dispersed sequences. Primary sequence analysis of 1595 base pairs of small-subunit ribosomal DNA (rDNA, probe pCc18) and 204 base pairs of upstream sequence failed to show any divergence between plants from the two sites. It is suggested that differentiation of developmental regulatory systems and not general sequence divergence may account for the observed high level of morphological differentiation in *Costaria*.

Genetic subdivision of *Costaria* populations was inferred from usage of a polymorphic rDNA marker (pCc18) which appeared to uncover restriction site variation in the intergenic spacer of the repeat. Twenty sites were sampled and eight distinct

populations resolved. One variant (Variant A) identified a dominant, southern breeding group with plants from thirteen clustered sites sharing this banding pattern. The other seven variants, found in the northern portion of the study area, were unique and population-specific. It is postulated that the variant distribution was established following the last glaciation (Wisconsin). Surface water currents may have allowed the northward spread of *Costaria* as well as the genetic homogenization of stands sharing the predominant banding pattern. Transportation of tissues on boat hulls may also have led to the observed distribution of Variant A.

The small-subunit rDNA sequence (pCc18) of *Costaria* was aligned with those of other chlorophyll a+b- and chlorophyll a+c- containing vascular and nonvascular plants. Phylogenetic comparison of all sequences indicated a common ancestor for phaeophytes, chrysophytes and oomycetes. Phylogenies based on rDNA sequence data and those based on plastid characteristics were discussed and compared.

ACKNOWLEDGEMENTS

I would like to first thank the members of the examining committee for their helpful advice concerning this thesis. As well, I acknowledge the friendship and intellectual encouragement offered by Louis D. Druehl and David L. Baillie. Thanks also go to the many work study students and assistants for their support in the lab. And finally, I am grateful to my parents for their ongoing love and support and Claude for her companionship and criticism.

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CHAPTER I

GENERAL INTRODUCTION

Species of kelp comprise the conspicuous brown seaweeds (Phaeophyta) which inhabit much of the lower intertidal and subtidal regions of rocky shores in temperate climates. Though the object of many studies, there has been a dearth of data regarding molecular biological analyses of intraspecific variation, dispersal and taxonomy in kelp species. This absence of quantitative data is significant since kelps are characterized by high levels of phenotypic variation within species (Sundene 1958, Druehl 1978, Mathieson et al. 1981, Norton et al. 1981). Such variation is particularly evident in taxa which have the ability to exist in varying exposure (wave, current) settings. It is unclear whether general morphological differentiation among stands of kelps can be attributed to DNA sequence variation, phenotypic plasticity or both. The delimitation of populations (ie. interbreeding groups) has been hampered both by a lack of understanding of dispersability (van den Hoek 1987) and by a lack of unambiguous markers with which to tag populations. And finally, apart from the work of Lim et al. (1986), using 5S RNA sequence data, no molecular analyses of the evolutionary position of kelps relative to other chlorophyll-bearing plants (both nonvascular and vascular) exist.

This thesis shall describe several studies which have been carried out on the annual kelp, *Costaria costata* (C.A. Agardh) Saunders, using both morphological and molecular biological tools of analysis to begin to understand the nature and basis of phenotypic and population differentiation and the evolutionary position of this species. These studies have been divided into three chapters (Chapters II, III and IV) with each organized as a generally independent entity. In this chapter each study (Morphological and DNA Sequence Variation in *Costaria*, Population Analysis of *Costaria*, Evolutionary Position of *Costaria*) will be introduced. Experimental methods, analysis and discussion of results will follow separately for each chapter. The final chapter (Chapter V) will comprise a general discussion and integration of the findings.

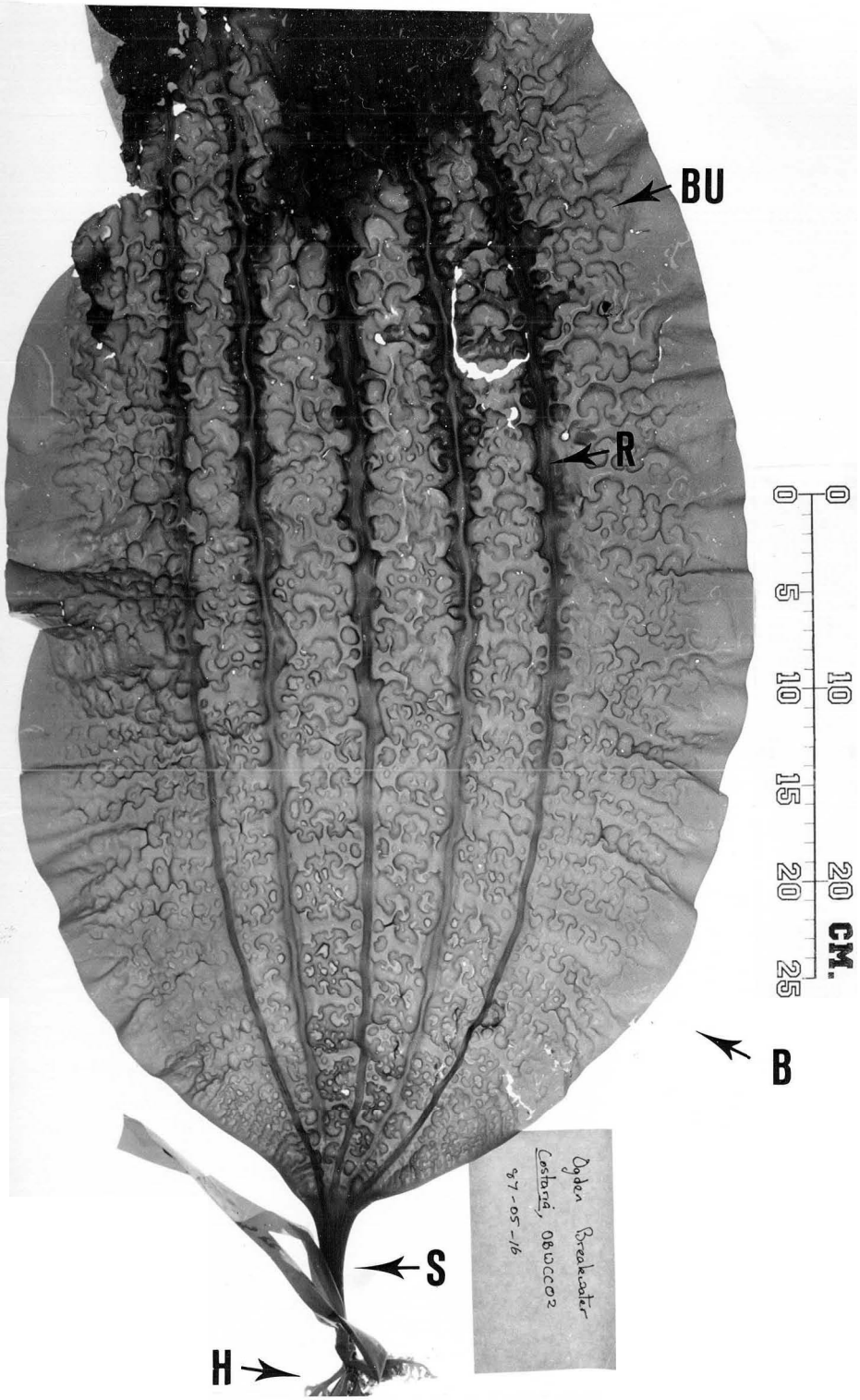
Costaria, a monotypic genus, is an ideal candidate for the study of variation and phylogeny since it has a simple morphology, its taxonomy is clear, and stands (or beds) of this species are accessible, being found intertidally in most regions of the northeast Pacific. The distribution of *Costaria* in the northeast Pacific ranges from central California to the eastern Aleutian Islands (Obrien 1972, see Fig. 1, p. 2b). Like many kelps, *Costaria* displays marked phenotypic differentiation in relation to its environment (Obrien 1972). *Costaria* exists on shores ranging from the most heavily exposed to those least exposed to waves. Plants display phenotypes according to the degree of exposure at sites.

Morphological and DNA Sequence Variation in *Costaria*

Characterizing morphological variation has been a central theme in phycology. Regarding intraspecific variation, the contribution of environmental and genetic components has been discussed (Norton et al. 1982, Innes 1984) and experimentally investigated (Sundene 1958, Sundene 1962, Chapman 1973, Nakahara and Yamada 1974, Espinoza and Chapman 1983). Statistical analysis of morphological variation in phenotypically distinct forms has also been carried out (Widdowson 1971, Druehl and Kemp 1982). As mentioned earlier, there is, however, little data concerning molecular biological analyses of intraspecific variation in kelps though many such studies have been carried out on higher plants and animals (Mitton 1978, Moran and Marshall 1978, Giles 1984, Nevo et al. 1986, Polans et al. 1986, Rieseberg et al. 1988, St. Louis and Barlow 1988).

This section will describe the techniques used in the analysis of both morphology and molecules (DNA) of plants arising from two phenotypically differentiated stands of *Costaria*. The simple morphology of *Costaria* consists of a holdfast, a corrugated stipe and a bullate, five-ribbed blade (Fig. 1). As is found in many kelps which have the ability to exist in varying exposure settings (Sundene 1958, Norton 1969, Nakahara and Yamada 1974), *Costaria* displays clinal phenotypic variance (Chapman 1974), plants originating from exposed sites are relatively narrower, thicker and more straplike than plants

Figure 1: Photo of a *Costaria* sporophyte showing its major anatomical features. The letters on the figure label the blade (B), stipe (S), holdfast (H), bullation (BU) and rib (R) where the meiosporangia are generally located in this species. In kelps, meiosporangia are grouped into sori which appear as dark areas on the blade. The five prominent ribs on the blade distinguish *Costaria* from other kelps. This plant was collected from a moderately exposed site (Ogden Breakwater, see Figure 7 for location).

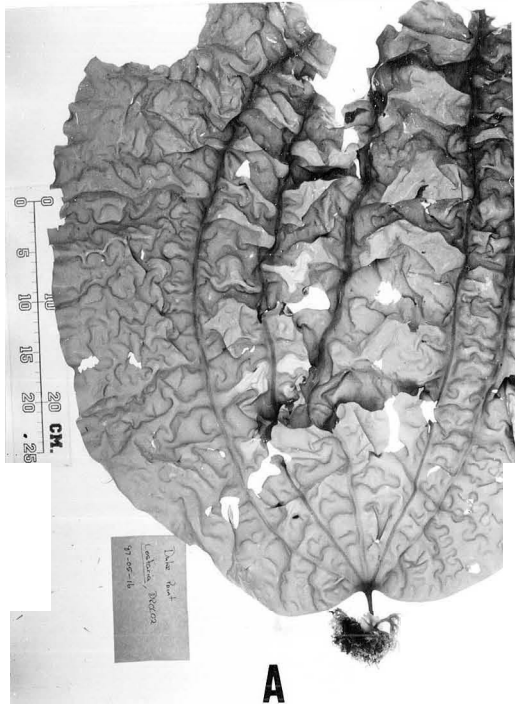


drawn from sheltered sites (Fig. 2). Intermediate forms are associated with sites having moderate exposure (O'Brien 1972).

Multivariate methods are valuable in morphometric studies because they can reveal biologically meaningful patterns of covariation among interrelated variables (phenotypic traits) which may not be discernible in the raw data (Rice and Chapman 1985, Rice et al. 1985, Shea 1985). In this thesis, cluster analysis (single linkage), principal components analysis (PCA) and discriminant function analysis (DFA) were used. The general theories underlying multivariate analysis of continuous morphometric data derived from seaweed genera have been reviewed (see Widdowson 1971, Marsden et al. 1983).

Single linkage cluster analysis involves the creation of a tree diagram by a stepwise amalgamation of variables based on the absolute values of their correlations (Hartigan 1975). Clusters are joined using the minimum distance rule. This technique allows the elucidation of linkages between variables. Statistical linkage leads to the generation of hypotheses regarding common developmental pathways influenced by both the genotype and the environment (Riska 1985). Atchley et al. (1981) postulated that morphological characters in an organism are strongly interwoven with changes in the structure of one trait leading to changes in the structure of others. Such assumptions have formed the basis for many correlative studies of organismal evolution (Berg 1960, Arnold 1981, Cheverud 1982). In some instances, when both statistical and quantitative genetic

Figure 2: Photos showing *Costaria* from wave-sheltered (Duke Point, A) and wave-exposed (Bordelais Islets, B) sites. In comparison note the elongation of the stipe, narrowing and thickening of the blade, reduced depth of bullations and the existence of perforations in the blade of the wave-exposed plant. The origin and function of the perforations is unknown (Obrien 1972). See Figure 7 for locations of sites described here.



correlations have been studied, phenotypic traits have been found to be both phenotypically and genetically integrated (Cheverud 1982).

In principal components analysis a correlation or covariance matrix is usually used as input. This technique involves the geometric rotation of axes describing original variables. Albrecht (1978) gives a clear example of axis rotation in a bivariate case of multi-group PCA. A new set of axes are created for which the maximum variation is expressed in the first principal component (a series of correlation coefficients). Subsequent components are orthogonal to the first and account for less and less of the variation (Shea 1985). In essence, PCA summarizes important trends in variation by reducing the data set to a smaller set of independent variables which reflect the original information (Albrecht 1978). The value of PCA lies in its power to contrast known groups within the data set on the basis of size and shape differences.

Stepwise discriminant function analysis is a method of extracting the linear combinations of variables which most clearly discriminate between groups in a data set when all morphometric variables are considered together. Results include F-values of each variable entered into the function. This statistic is computed from a one-way analysis of covariance where the covariates are the previously entered variables (Dixon 1985). As well, a Bartlett's Chi-square statistic may be computed to test for independence of groups in the data set

(Maxwell 1977). In this study DFA will be utilized to illustrate the basis of population discrimination and to compute a probability value reflecting the degree of independence between populations.

Population differentiation at the molecular level may be assessed with hybridization analysis using randomly cloned DNA fragments as probes against genomic DNA fixed on a solid support (Southern blot, Southern 1975). Probes of unknown sequence may be valuable for uncovering restriction fragment length polymorphisms (RFLPs) since they may correspond to both coding and noncoding DNA sequences (Gusella 1986). Noncoding sequences, such as intervening sequences (introns) and spacers (nontranscribed or transcribed but not translated) accumulate mutations more readily than conserved sequences, such as those coding for genes or regulatory regions (Long and Dawid 1980). RFLP analysis with anonymous probes has been used successfully to detect intra- and interspecific sequence divergence (Rose et al. 1982, Sapienza and Doolittle 1982, Natvig et al. 1987). Rose et al. (1982) were, for example, able to estimate sequence divergence between two interbreeding, closely related strains of the nematode *Caenorhabditis elegans* using this technique. The screening procedure also provides valuable information regarding the proportion of dispersed repeat sequences and, therefore, the genomic organization in study organisms.

The greatest resolution of the genetic divergence between organisms is derived from the sequencing of DNA or RNA (see Lim

et al. 1986, Bhattacharya and Druehl 1987, Gunderson et al. 1987). Homologous fragments, derived from either coding or noncoding regions, can be compared at the sequence level, often providing the resolution necessary for detecting intraspecific variation.

Population Analysis of Costaria

Though described as essentially continuous in latitudinal distribution within their range (Druehl 1981), kelp species may be demarcated into distinct stands or beds. Past researchers have been unable to distinguish between beds and *bona fide* populations (interbreeding groups) of kelps due to a dearth of quantitative data describing their dispersability (van den Hoek 1987) and due to the paucity of studies utilizing contemporary tools of population analysis (ie. molecular biology). The present literature indicates a very limited ability for dispersal, within 10 m of origin, for species studied (Sundene 1962, Anderson and North 1966, Dayton 1973). Druehl (1981), however, has pointed out the colonization of Surtsey, a newly-formed volcanic island (1963), by kelp species within 3 years of its origin. Surtsey is located approximately 5 km from the nearest Icelandic shore. As well, van den Hoek (1987) has reviewed the considerable evidence supporting the long-range dispersal of seaweeds. Crucial to this issue are the numerous observations of stable and rich seaweed floras on volcanic islands on or near the Mid Atlantic Ridge (ie. Azores, Tristan de Cunha) even though the nearest donor floras are more than 1000 km distant. These data suggest that kelps are able to disperse both into the immediate environment and distantly. Dispersal within 10 m of a kelp plant by its meiospores has been experimentally proven; the basis of longer-range dispersal is not clearly understood. Drifting plants may allow for such

spread. In this section of the thesis, the methods by which the analysis of kelp populations using a polymorphic ribosomal DNA (rDNA) molecular marker was carried out, will be introduced. This marker proved useful in uncovering the extent and identity of local breeding groups and led to the generation of hypotheses regarding their current distribution.

The life cycle of *Costaria* consists of an obligate alternation of heteromorphic generations (Angst 1927). The visible sporophyte, which may reach 3 m in length, undergoes meiosis in sporangia located on its blade (Fig. 1). Flagellated, heterokont meiospores are released from the sporangia and, after spending an unknown period of time in the water column, settle and ultimately give rise to equal numbers of microscopic, filamentous male and female gametophytes. The male gametophytes release motile sperm which fertilize the eggs retained on the female gametophytes. Following fertilization, the diploid sporophytes overgrow the female gametophytes eventually becoming the visible kelp plants. Though completed in the laboratory, the life cycle of *Costaria* is not apparent in the field due to the microscopic size of the gametophytes (O'Brien 1972). The annual sporophytes of *Costaria* generally appear in January-February and all are generally removed by August-September. Sporangia develop on sporophytes by May-June and meiospores are liberated from June onwards (O'Brien 1972). Either or both gametophytes and microscopic sporophytes may be the overwintering stage of this plant (O'Brien 1972). Dispersal in kelps, such as *Costaria*, is

presently impractical to study if meiospore dispersal and distribution of gametophytes are chosen for analysis due to their cryptic nature. This analysis has, therefore, been limited to visible sporophytes. In this section of the thesis, nuclear DNA samples prepared from individual sporophytes were screened with a ribosomal DNA (rDNA) probe (pCc18) which appeared to differentiate *Costaria* populations.

Ribosomal DNA is arranged in repeating units with small and large subunit genes separated by intergenic spacers (IGS). Ribosomal DNA variation has been described in many plant taxa primarily in the copy number of genes and in the length of and distribution of restriction sites within the IGS (see Rogers and Bendich 1987 b for review, Schaal et al. 1987). Small and large subunit gene sequences appear to be evolutionarily conserved (Pace et al. 1986). Copy number variation cannot necessarily be used to describe populations since individuals within a population may exhibit heterogeneity for this trait (Rogers and Bendich 1987 b). Spacer length variants have, however, been shown to be stable across generations and inherited in a simple Mendelian fashion (Polans et al. 1986), and have been used to infer genetic subdivision in wild plant populations (Saghai-Marooof et al. 1984, Systma and Schaal 1985, Flavell et al. 1986, Schaal et al. 1987). Learn and Schaal (1987) felt that population level forces such as gene flow and genetic drift would influence the fate and distribution of rDNA variants. And, since the rDNAs (including the IGS) are members of a multigene

family, the repeats within individuals of a population are likely to come under the homogenizing influence of molecular drive through such processes as unequal-crossing over and gene conversion (Dover 1982, Worton et al. 1988). The above data suggest that rDNA polymorphisms may act as stable markers of populations.

Evolutionary Position of *Costaria*

With the exception of the Cyanophyta and the Rhodophyta, which have as their sole chlorophyll, chlorophyll a, all other photosynthetic taxa fall into two major groups: chlorophyll a+b and chlorophyll a+c plants. Within these two groups the taxa are further distinguished on the basis of other plastid features and characteristics such as accessory pigments, flagellar and nuclear envelope structure and storage products (Dodge 1974, Taylor 1976).

The photosynthetic plastids of eucaryotes are believed to have arisen from symbiotic photosynthetic procaryotes (Gray and Doolittle 1982, Cozens and Walker 1987). Further, there is evidence that eucaryotic plastids evolved in this manner more than once (Ludwig and Gibbs 1985). Thus there are in photosynthetic eucaryotes at least two systems (the plastid and the host eucaryote) having different origins, each potentially evolving independently at different rates. Our understanding of evolutionary relationships between photosynthetic groups of organisms is primarily based upon the nature of the plastid (Taylor 1976).

Cavalier-Smith (1981) reasons that eucaryote classification should be based on fundamental cell structure rather than mode of nutrition (autotroph, heterotroph). In a recent study, employing cytoplasmic small-subunit rRNA sequence data, Gunderson et al. (1987) established a closer phylogenetic

relationship between a chrysophyte and an oomycete than between the chrysophyte and a chlorophyte. These observations suggest the necessity of understanding "host cell" phylogenies, independent of plastid features, as a prelude to appreciating evolutionary relationships of extant photosynthetic organisms.

Use of rRNA sequence divergence data as the basis for constructing a molecular chronometer is supported by the literature. Sogin et al. (1986) pointed out the universal distribution and functional equivalency of rRNA as well as their superiority to 5S RNA sequence data since the rRNAs are relatively larger and offer more information with which to evaluate near and distant phylogenetic relationships. Woese (1987) noted that the functional equivalency of the rRNA sequences assured relatively good clocklike behaviour. The phylogenetic usefulness of rRNA sequence data has led to their widespread usage and availability relative to many other genic sequences.

In this section of the thesis, 1595 base pairs (bp) of DNA sequence were determined for the small-subunit rDNA gene in *Costaria* and compared to existing RNA sequences for this gene. The addition of the *Costaria* rDNA sequence enlarged upon the phylogeny of Gunderson et al. (1987) and allowed the resolution of two questions. Firstly, is the clustering of diverse chlorophyll a+c plants similar to that observed for chlorophyll a+b plants? And secondly, what is the phylogenetic position of *Costaria* relative to the chlorophyll a+c chrysophyte *Ochromonas*

danica and the closely related aquatic oomycete *Achlya bisexualis* (Gunderson et al. 1987) ?

Small-subunit rRNA sequences were drawn for species from three Divisions of chlorophyll a+b plants and three Divisions of chlorophyll a+c plants and the oomycete *Achlya bisexualis*. The chlorophyll a+b plants are represented by two unicellular flagellates, *Chlamydomonas reinhardtii* (Chlorophyta) and *Euglena gracilis* (Euglenophyta) and two vascular plants, *Oryza sativa* and *Glycine max* (Spermatophyta). The chlorophyll a+c plants are represented by two unicellular flagellates, *Ochromonas danica* (Chrysophyta) and *Prorocentrum micans* (Pyrrophyta) and the relatively morphologically complex *Costaria*.

General Summary

It was my intention, in this thesis, to explore through molecular biological analyses, the relationship between phenotypic and genotypic variation, the genetic subdivision of populations and the phylogenetic relationships of *Costaria*.

CHAPTER II

MORPHOLOGICAL AND DNA SEQUENCE VARIATION IN *COSTARIA*

In this chapter the relationship between phenotypic variation, as inferred from *Costaria* sporophyte morphology, and genetic variation, as inferred from RFLP analysis, is examined. To facilitate this study, two morphologically distinct and geographically isolated stands of *Costaria* are analysed.

Materials and Methods

Sampling

Costaria sporophytes were sampled once from a strongly wave-exposed site (Cape Beale, west coast of Vancouver Island, 48° 47' 15" N, 125° 12' 45" W) and once from a sheltered site (Coal Harbour, Vancouver, 49° 18' N, 123° 07' 30" W, Canada). The approximate distance between Cape Beale and Coal Harbour is 278 km. Forty-five plants were collected in May, 1985 from a rocky outcrop at Cape Beale and from scattered boulders in a muddy bottom at Coal Harbour. Sampled individuals were separated by a minimum distance of 20 cm and when clumps of plants were found only one was taken from each clump to avoid sampling the same genotype twice. Plants were discontinuously distributed at both sites and reproductively mature, as indicated by the presence of sori. They were collected within a two week period. Plants were transported to the laboratory on ice while wrapped in seawater-soaked paper towels or inside sealed polyethylene

bags. *Costaria* is an annual genus, therefore, all individuals collected were approximately the same age.

Morphometrics

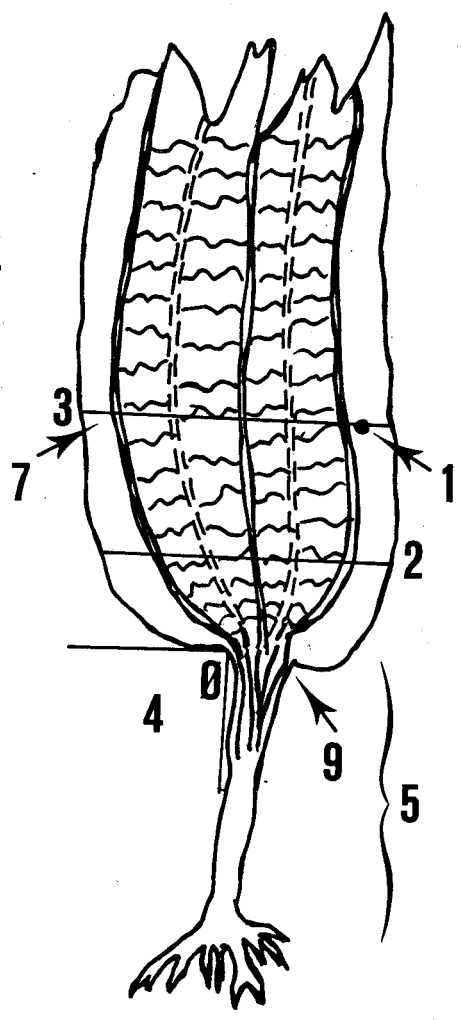
Values were obtained for each plant for eleven continuous variables presented in Figure 3 and Table I. To facilitate comparison of data, measurements of basal blade angle were transformed to linear values using the sine rule for triangles (Ellis 1982),

$$\frac{\sin b}{\text{side B}} = \frac{\sin a}{\text{side A}}$$

where "b" is the basal blade angle and "a" denotes equal and opposite angles in an isosceles triangle. Sides A were set as 1 whereas side B was computed. Data for nine variables were log (base 10) transformed. Stipe length and basal blade thickness were not transformed since the raw data displayed normal distributions. Departure from normality was tested by measures of skewness and kurtosis. None of the variables displayed correlations greater than 0.90. Levene's (1960) and Bartlett's (Dixon and Massey 1969) tests for homogeneity of variance were also performed. All data sets fulfilled the former test while some failed the more sensitive Bartlett's test by a small amount. All preliminary analysis of data were carried out with the Biomedical Computer Programs 1985 Version (BMDP: Dixon 1985) BMDP2D, BMDP3D and BMDP9D. Subsequent calculations (cluster analysis, PCA and DFA) were carried out with BMDP1M, BMDP4M and BMDP7M respectively.

Figure 3: The location of variables measured on *Costaria* sporophytes. Numbers correspond to those in Table I. The lower figure represents a cross-sectional view of the blade.

BLADE



STIPE

HOLDFAST

CENTRAL RIB

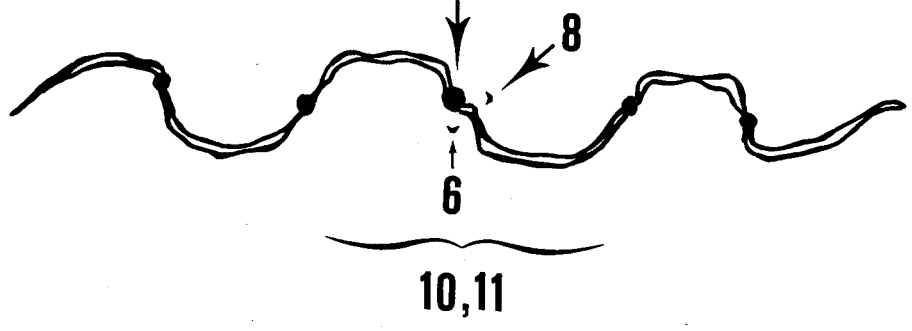


Table I: List of variables analysed on *Costaria*
sporophytes.

NUMBER	DESCRIPTION	ABBREVIATION
1	DRY WEIGHT AT 20 CM [*] OF TISSUE REMOVED BY HOLE PUNCHER OF 1 CM DIAMETER.	DW
2	BLADE WIDTH, 5 CM [*]	W5
3	BLADE WIDTH, 20 CM [*]	W20
4	BASAL BLADE ANGLE	BA
5	STIPE LENGTH	SL
6	CENTRAL RIB WIDTH, 20 CM [*]	RW20
7	BLADE THICKNESS, 20 CM [*]	T20
8	BLADE THICKNESS OF CENTRAL RIB, 20 CM [*]	TR20
9	BASAL BLADE THICKNESS	BT
10	BLADE WIDTH BETWEEN MIDDLE RIBS, 20 CM [*]	WR20
11	BLADE WIDTH BETWEEN MIDDLE RIBS, 5 CM [*]	WR5

*CM DISTAL TO BLADE BASE

Molecular Analysis

In the laboratory, healthy blades were removed from plants and individually processed. Blades were first carefully cleaned of surface contaminants by scrubbing with cheesecloth and then cut into 2 cm by 2 cm pieces and soaked in distilled water for 5 minutes. Following this, blade tissue was blotted dry with paper towels and ground manually in a mortar and pestle in the presence of liquid nitrogen and stored as powder in plastic bags at -70°C (Fain et al. submitted). Nuclear DNA was extracted from ten plants from Cape Beale and from ten plants from Coal Harbour using the method of Fain et al. (submitted). Essentially, following an organelle extraction, the nucleii and chloroplasts were lysed and the DNAs purified with two rounds of ultracentrifugation in cesium chloride. The first spin was carried out in the presence of Hoechst dye (H33258) to separate nuclear and chloroplast fractions. The collected nuclear fraction was then further purified with a second round of ultracentrifugation in an ethidium bromide gradient. One microgram of nuclear DNA prepared by this method generally required 10 units of restriction enzyme with an incubation time of 3 hours at 37°C to allow complete digestion.

Randomly cloned probes were derived from an Eco RI restriction digest of a Coal Harbour plant under conditions recommended by the manufacturer (Pharmacia). Digest products were ligated (T4 DNA ligase) into the plasmid vector pUC19 (Vieira and Messing 1982) which was also digested with Eco RI

under conditions recommended by the manufacturer (Bethesda Research). Ligation products were introduced into *Escherichia coli* strain JM83 by the method of Morrison (1978). Chimeric plasmids were selected and plasmid DNA prepared by alkaline lysis (Davis et al. 1980). Plasmids were radiolabelled with ^{32}P by nick translation (Rigby et al. 1977) and unincorporated nucleotides removed by passing the reaction volume through a 1.5 mL Sephadex G-50 spin column (Maniatis et al. 1982) and then used as probes against genomic digests (Southern blots) of Cape Beale and Coal Harbour plants. All anonymous probes were digested with Eco RI to release the cloned fragments and sized relative to a marker (lambda phage DNA digested with Eco RI and Hind III) on 0.7% agarose gels containing ethidium bromide.

Restriction enzymes used in genomic digests of Cape Beale and Coal Harbour plants were Eco RI, Hind III and Xba I. Digested genomic DNAs were size-fractionated on 0.7% agarose gels containing ethidium bromide and bidirectionally transferred to nitrocellulose filters (Schleicher and Schuell) by the method of Smith and Summers (1980). Filters were pretreated at hybridization temperature (68°C) in 5XSSPE, 5XBFP and 0.2% SDS (1XSSPE= 0.18M sodium chloride, 10 mM sodium phosphate, 1mM disodium EDTA pH 7.0; 1XBFP= 0.02% w/v bovine serum albumin, Ficoll (m.w.= 400,000), polyvinyl pyrrolidone). Hybridizations were carried out in 5XSSPE, 1XBFP and 0.2% SDS. Filters were washed at 60°C in 2.5XSSPE and 0.1% SDS. Initially, one plant from Coal Harbour was screened with each of 42 anonymous probes.

Those inserts which produced a resolvable number of bands after autoradiography (Eco RI= 6 probes, Hind III= 10 probes, Xba I= 8 probes) were used to probe several plants from Cape Beale and Coal Harbour for RFLPs.

The random clone library generated in this study was screened by the colony hybridization method of Grunstein and Hogness (1975) using the 5.8S-18S-26S rDNA cistron of the nematode *Caenorhabditis elegans* as a probe (pCes370, kindly provided by David L. Baillie). Though *Costaria* and *Caenorhabditis* are evolutionary distant organisms, the conserved nature of rDNA sequences allowed the successful usage of a heterologous (nematode) probe in this experiment. Recombinant plasmids carrying fragments of *Costaria* rDNA were identified and analysed. One fragment (pCc18) of size 1799 base pairs (bp) was found to encode 1595 bp of small-subunit rDNA and 204 bp of upstream sequence (see Chapter IV for details of procedure). One strand of the entire fragment was then sequenced for both a Cape Beale and a Coal Harbour individual using the dideoxynucleotide chain termination method of Sanger et al. (1977). The sequences were subsequently aligned and compared.

Culture

In the study of intraspecific variation, it is important to confirm the conspecificity of plants involved in study groups (Espinoza and Chapman 1983). Several healthy sporophytes were chosen from Cape Beale and Coal Harbour and meiospores released

in culture. These were allowed to develop into gametophytes over a two to three week period and healthy males and females isolated (Druehl and Boal 1981) and placed in PES medium (Provasoli 1968). Gametogenesis was induced in these plants according to Luning and Dring (1972) and reciprocal crosses were initiated between males and females from pairs of Cape Beale and Coal Harbour plants. Sporophytes were noted and crosses were termed successful when products reached a size exceeding 1 cm.

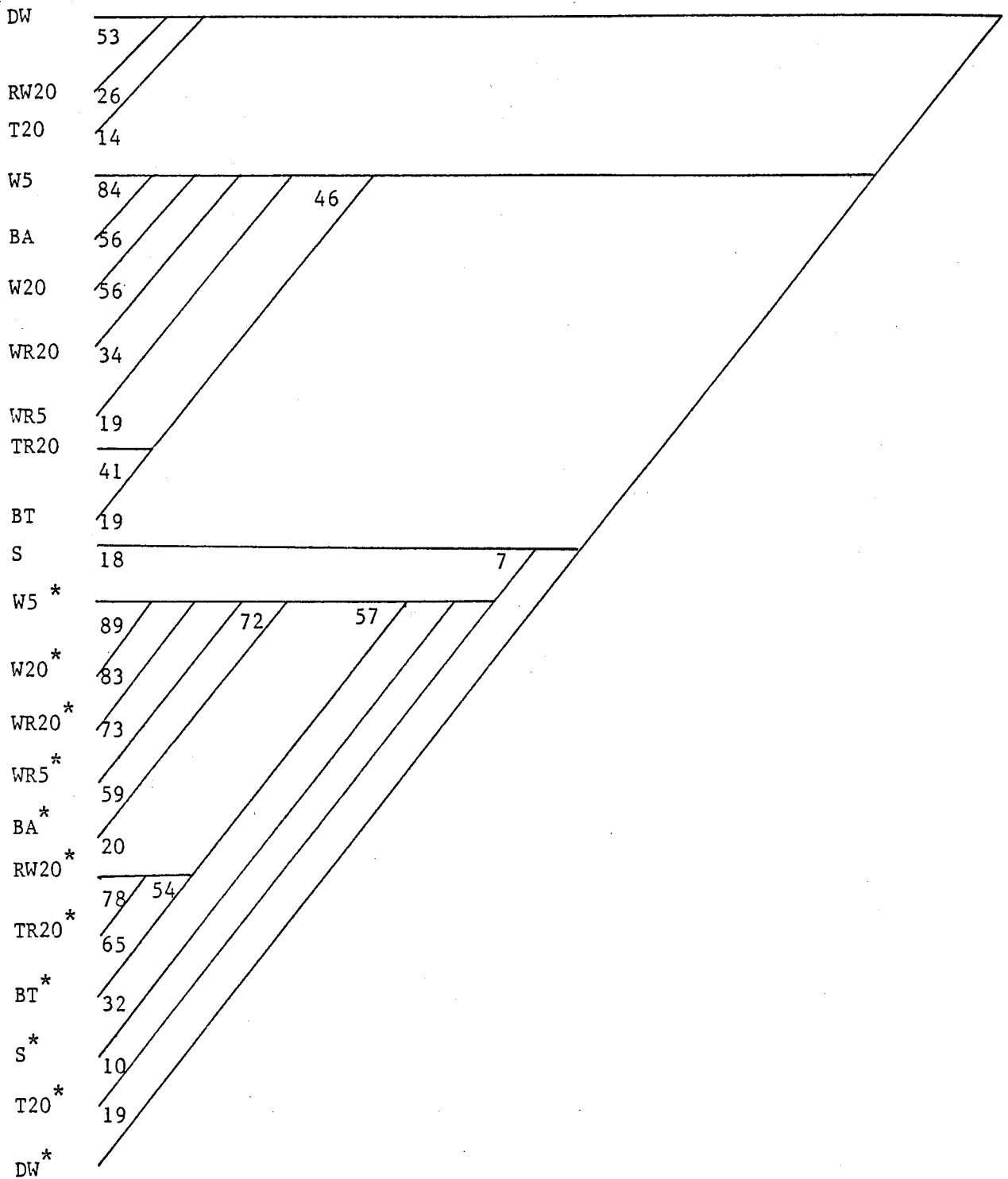
Results

Morphometrics

Multivariate statistical analyses performed on the chosen characters indicate that significant phenotypic differences exist between Cape Beale and Coal Harbour plants. Cluster analysis revealed a clear delineation of the data set into two groups (Fig. 4). Only stipe length of Coal Harbour plants was found to overlap into the Cape Beale group. The cluster diagram, which was produced from the absolute values of correlations, showed that correlations of similar magnitude existed between similar variables in the Cape Beale and Coal Harbour plants. Width measurements and basal blade angle assorted together, as did blade thickness measurements, in each population.

Results of the grouped PCA run on Cape Beale and Coal Harbour plants are presented in Table II. The first principal component separated the populations according to shape since the

Figure 4: Result of cluster analysis on *Costaria* sporophytes using the absolute correlation matrix of variables from Cape Beale and Coal Harbour plants. Numbers within and between clusters represent correlations which have been recoded to a measure of similarity between 0 and 100. A correlation of 0 is recoded to zero indicating minimum similarity.



* variables derived from Cape Beale plants

Table II: Result of grouped principal components analysis run on Cape Beale and Coal Harbour sporophytes of *Costaria*.

VARIABLE	FACTOR 1
DW	-0.907
W5	0.935
W20	0.977
BA	0.917
S	-0.554
RW20	0.074
T20	-0.884
TR20	-0.864
BT	-0.860
WR20	0.937
WR5	0.844
VARIANCE EXPLAINED	69.7%

loadings were bipolar (Shea 1985). Only this component is presented because it accounted for a majority of the total variation in the data set (69.7%). By examining the loadings on the first component it is possible to identify those variables primarily responsible for the separation. The stronger the positive or negative loading the greater its effect on explaining the components share of total variation. Positive and negative loadings increase in opposite directions relative to each other. The variables dry weight, blade thickness at 20 cm, blade thickness of the central rib at 20 cm and basal blade thickness all have strong negative loadings, whereas blade width at 5 cm, blade width at 20 cm, basal blade angle and blade width of the middle rib at 20 cm show strong positive loadings. Based on the general morphology of individuals studied, this component suggests that Cape Beale plants contribute the negative loadings and Coal Harbour plants contribute the positive loadings.

Discriminant function analysis results are summarized in Table III. The most important variable in discriminating the two populations is blade width at 20 cm followed by basal blade thickness, which has an F-value one order less in magnitude. The Bartlett's Chi-square statistic which tests for independence of groups in the data set (Maxwell 1977) and is derived from the eigenvalues of each case is $X^2 = 128.30$ which places the probability of association between the two populations at $p < 0.0005$. Cape Beale and Coal Harbour plants are, therefore, significantly different from each other with respect

Table III: Results of discriminant function analysis showing the six variables most important in discrimination of Cape Beale and Coal Harbour stands of *Costaria* sporophytes.

STEP NUMBER	VARIABLE	F- VALUE
1	W20	1071.9
2	BT	67.4
3	WR20	8.1
4	DW	8.8
5	RW20	6.4
6	TR20	5.2

to the variables used in this analysis.

Molecular Analysis

The random cloning procedure produced insert fragments of varying sizes (mean= 2086 bp; range= 203 bp to 6313 bp). The total number of base pairs of nuclear DNA sampled in this experiment was 87620 bp. Some recombinant plasmids contained more than one insert (pCc24, pCc29) but the majority had accepted only one. Hybridization analysis with the 42 anonymous probes resolved only one RFLP in plants from Cape Beale and Coal Harbour (pCc18). The vast majority of the probes were useless for this type of analysis since they hybridized to an unresolvable number of bands creating a smear on the autoradiograph. This result was found even when hybridizations and washes were carried out at higher stringencies. Such data may indicate that these probes contain dispersed sequences which are highly repetitive (Young 1979). Of the probes which were moderately dispersed or which existed in only one form, only pCc18 encoded a polymorphic sequence. Table IV summarizes the hybridization results, indicating the size of the Eco RI probe used and the number of homologous fragments resolved when hybridized to genomic DNAs digested with Eco RI, Hind III and Xba I. Examples of hybridizations with three anonymous probes (pCc6, pCc5B, pCc26) which gave results typical of probe sequences existing in many forms (smears), a few forms and as one form are shown in Figure 5.

Table IV: Results of hybridization analysis with anonymous probes showing the number of bands resolved with each hybridization. X represents total base pairs of cloned nuclear DNA from *Costaria* sporophytes and \bar{x} the average size of fragments.

PROBE	SIZE (BP)	ECO RI	HIND III	XBA I
pCc2	1222	smear	smear	smear
pCc4	847	smear	smear	smear
pCc5	308	smear	smear	smear
pCc6	6313	smear	smear	smear
pCc8	2545	smear	smear	smear
pCc9	1674	smear	smear	smear
pCc10	3485	smear	smear	smear
pCc11	1859	smear	smear	smear
pCc12	4951	smear	smear	smear
pCc13	502	smear	smear	smear
pCc14	284	smear	2	3
pCc16	1160	smear	smear	smear
pCc17	3870	smear	smear	smear
pCc18	1799	1 major, 10 minor	2	1 major, 2 minor
pCc19	2141	smear	2	smear
pCc20	1039	smear	smear	smear
pCc21	987	smear	smear	smear
pCc23	3588	smear	smear	smear
pCc24	1570, 653	smear	smear	smear
pCc26	531	1 major, 1 minor	1	1 major, 1 minor
pCc27	4412	smear	smear	smear
pCc28	3778	smear	smear	smear
pCc29	1242, 947, 203	smear	11	8
pCc30	937	smear	12	1 major, 10 minor
pCc31	2374	smear	smear	smear
pCc32	4313	smear	13	11
pCc34	850	smear	smear	smear
pCc35	1176	smear	smear	smear
pCc38	4085	smear	smear	smear
pCc1B	2800	smear	smear	smear
pCc5B	1542	2 major, 6 minor	1 major, 14 minor	smear
pCcI	805	smear	smear	smear
pCcN	4873	smear	smear	smear
pCcM	2252	9	smear	smear
pCcJ	2134	smear	smear	2 major, 4 minor
pCcK	3666	1 major, 1 minor	smear	smear
pCcD	4085	smear	7	5
pCc4A	2021	smear	smear	smear
pCc5A	947	smear	smear	smear
pCc7A	840	smear	smear	smear
pCc9A	897	1	1 major, 9 minor	smear
pCc10A	551	smear	smear	smear

N= 42 x= 87620 RANGE= 203 - 6313 \bar{x} = 2086

Figure 5: Autoradiographs of Southern blots probed with anonymous DNA fragments, pCc6, pCc5B and pCc26. Genomic digestions of a *Costaria* plant from both Coal Harbour and Cape Beale were carried out with Eco RI in the pCc6 hybridization and with Hind III in the pCc5B and pCc26 hybridizations. Probe pCc6 represents a sequence which exists in many forms, probe pCc5B a sequence which exists in a few forms and probe pCc26 a sequence which probably exists as a single copy sequence.

pCc6
SP CB



pCc5B
SP CB



pCc26
CB SP



Additional hybridizations, involving restriction digests of genomic DNAs with enzymes other than Eco RI, Hind III and Xba I, were carried out using probes which produced discrete bands. For example, probe pCc5B was hybridized to genomic DNA of plants from Cape Beale and Coal Harbour digested with the following six base recognition sequence enzymes Bam HI, Pst I, Pvu II, Sal I and Xho I and the following four base recognition sequence enzymes Hae III and Hpa II. None of these enzymes resolved an RFLP in the sequence encoded by pCc5B.

The polymorphic pattern produced by probe pCc18 is shown in Fig. 6. Ten plants from each site produced an invariant pattern which was site-specific. As stated earlier, probe pCc18 encodes the majority (1595 bp) of the small-subunit rDNA gene in *Costaria*. The rDNA cistron, though highly conserved over genic sequences, is known to be polymorphic in plants with regards to restriction site variation and the number of subrepeats in its intergenic spacer (Yakura and Tanifuji 1981, Saghai-Marroof et al. 1984, Polans et al. 1986). Restriction site variation immediately upstream of the start of the coding region of the small-subunit rDNA gene in *Costaria* is hypothesized to be responsible for the variable banding pattern uncovered by pCc18 (see Chapter III for details).

Estimations of the relative proportion of dispersed repeated sequences and sequences existing as one form in *Costaria*, based on the results of the hybridization analysis, are shown in Table V. Assuming that DNA fragments were randomly cloned, the

Figure 6: Autoradiograph of Southern blot probed with DNA fragment pCc18 which encodes 1595 base pairs of small-subunit ribosomal DNA. Genomic DNAs of two *Costaria* plants from Coal Harbour (Lanes 1,3) and Cape Beale (Lanes 2,4) were digested with Eco RI. Numbers on right margin indicate fragment sizes in base pairs. The 1799 base pair fragment is the major Eco RI fragment containing the small-subunit rDNA gene in *Costaria* and was sequenced in this thesis. The 4500 base pair fragment shared by all plants is an internal control consisting of uncut plasmid pCc18.

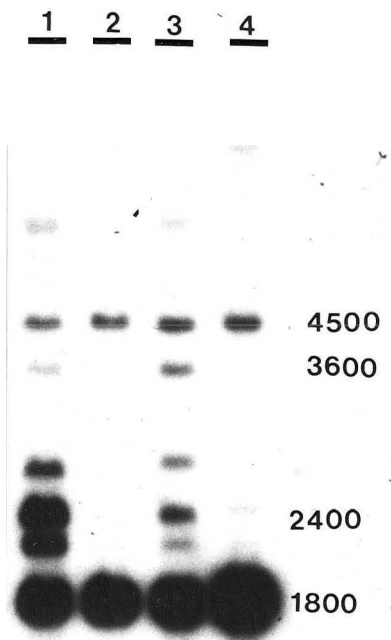


Table V: Estimations of percentage dispersed DNA
in *Costaria* sporophytes
on the basis of hybridization analysis
with 42 anonymous probes.

HYBRIDIZATION RESULT	Eco RI	HIND III	XBA I	MEAN
SMEAR	85.7	76.2	80.9	80.9
+ 10 BANDS	2.4	9.5	4.8	5.6
5 - 10 BANDS	4.8	4.8	7.1	5.6
2 - 4 BANDS	4.8	4.8	7.1	5.6
1 BAND	2.4	4.8	0	2.4

presence of repetitive sequences within most of these cloned fragments analysed (particularly those forming smears) suggests that repeats are interspersed over short intervals in the nuclear genome of *Costaria* (Emmons et al. 1979). The percentage of high copy number dispersed DNA, when averaged over the results of genomic digestions with Eco RI, Hind III and Xba I is 80.9%. This value may be an underestimate since the copy numbers of sequences existing in one or several forms cannot be measured with this method. As well, the value may be an overestimate if there are a small number of dispersed, highly repeated sequence elements in the genome of *Costaria*. It is possible that, in the latter case, if such elements were positioned within fragments containing single copy sequences that these clones would behave as highly repeated sequences in hybridization analysis producing smears on autoradiographs. Single copy DNA within such clones would not be resolved using random probe hybridization analysis. Regardless, the value of 80.9% is similar to that of many plant species which have been analysed by reannealing kinetics to measure proportions of repeat sequences in nuclear DNA. Flavell et al. (1974) found an average proportion of $80 \pm 2.0\%$ repeated sequences in a reannealing kinetics study of fifteen higher plant genomes.

Sequence analysis of the 1799 bp fragment encoding upstream and small-subunit rDNA failed to show any divergence between Cape Beale and Coal Harbour plants. This result is not surprising considering the conserved nature of this gene. Sogin

et al. (1986) found complete sequence conservation between several species of *Tetrahymena* and between strains of *T. pigmentosa* in an analysis of small-subunit rRNA. The sequence of the small-subunit rDNA and the region immediately upstream of it are not, therefore, sensitive enough to uncover intraspecific genetic divergence in *Costaria* over the geographic distance represented by Cape Beale and Coal Harbour.

Culture

Successful reciprocal crosses were established for Cape Beale and Coal Harbour plants. Sporophytes, derived from the male and female gametophytic stages, were produced in culture and exceeded 1 cm in length. It is concluded that, although plants from Cape Beale and Coal Harbour have undergone great morphological differentiation, they are conspecific.

Discussion

An important aspect of intraspecific differentiation is the relationship between genetic and morphological variation. A broad spectrum of genotype/ morphology relationships are possible. Some species of higher plants, such as *Hordeum spontaneum* exhibit substantial isozyme variation among local, ecologically-subdivided populations, with protein variation correlated with particular environments (Nevo et al. 1986). In

the case of *H. spontaneum*, Nevo et al. (1986) cited water availability as a possible cause of both genotypic and phenotypic differentiation. Giles (1984) reported contradictory results in an analysis of biochemical and quantitative variation in *Hordeum murinum*. Flowering time and aspects of seed and plant anatomy displayed differences at the population level for both character means and heritability, while the isozyme data revealed genetic uniformity. Jain et al. (1980) also found isozyme mobilities to be relatively uniform for grain amaranths even though the populations sampled were geographically diverse (derived from all over India) and morphologically variable. Emmons et al. (1979) reported yet another type of genotype/morphology relationship. These authors interpreted their anonymous probe hybridization data as indicating a large amount of nucleotide sequence divergence (at least 20%) between the nuclear genomes of the nematode species *Caenorhabditis elegans* and *C. briggsae*, even though the two species are morphologically very similar. What then, is the position of *Costaria* in this continuum of genotype/ morphology responses ?

The results of this study suggest phenotypic integration of the relatively simple morphology of *Costaria*. Plants from both sites appear to follow a common developmental pathway with respect to blade morphology since phenotypic correlations are similar for plants from Cape Beale and Coal Harbour. There is, however, strong geographic patterning of overall morphology, with blade width at 20 cm being the major discriminating

variable. Norton et al. (1982) reviewed both morphological and hydrodynamic data relating to seaweed phenotypic differentiation. These authors postulated that the sturdier, straplike nature of plants growing in wave-exposed sites relative to those of the same species growing in calmer waters may be a response to greater hydrodynamic drag in the exposed habitats. Gerard (1987) was able to induce phenotypic differentiation in *Laminaria saccharina* in culture by imposing mechanical stress on plants. Mechanical stress was applied by hanging weights from the distal ends of blades thereby eliciting a morphology similar to that resulting from increased wave exposure. Stressed plants produced longer, narrower blades though total tissue production and cell size in thalli were equal in stressed and non-stressed individuals. Gerard (1987) postulated that differences in the orientation of cell division, caused by mechanical stress, led to the phenotypic differentiation. Narrower, longer blades resulted from a higher frequency of longitudinal cell divisions relative to divisions in the transverse plane. If the mechanical stress applied by Gerard has a similar effect as that caused by wave action then it is likely that a modification in development manifested by control of the plane of cell division may allow kelps such as *Costaria* to produce an adaptive configuration in widely variable environmental conditions.

In both Cape Beale and Coal Harbour populations, the basal blade angle may act as a primary point of blade width

determination. As the basal blade angle increases so does the width of the blade and the distance between middle ribs at both 5 cm and 20 cm distal to the transition zone. Basal blade thickness and blade thickness of the central rib at 20 cm are also integrated in plants from both sites (see Fig. 4). Not surprisingly, the meristematic transition zone, which is the point of basal blade angle and basal blade thickness measurements, determines widths and thicknesses distal to it.

The genetic uniformity of Cape Beale and Coal Harbour plants, with respect to phenotypic integration revealed by the statistical data, is suggested by the results of the interfertility and DNA sequence analyses. Plants of *Costaria* from the wave-exposed and sheltered sites are phenotypically distinct and constitute separate populations (see Chapter III for further data). Individuals from Cape Beale and Coal Harbour have, however, not undergone sufficient genetic differentiation of traits, particularly those related to sexual compatibility and fusion, to deter interfertility. This genus may be termed phenotypically "plastic". Scheiner and Goodnight (1984) described phenotypic plasticity as being characterized by an organism which could grow and reproduce in a range of environments by varying its phenotype. Phenotypic plasticity can itself be under genetic control and therefore be under selective pressure (Bradshaw 1965). Some authors have hypothesized that selection for phenotypic plasticity and genetic variation would be antagonistic (Bradshaw 1965, Marshall and Jain 1968). In this

view, populations of species would be selected to be either phenotypically variable or genetically variable.

Costaria appears to respond to environmental heterogeneity (ie. varying exposure) in a fashion similar to species such as *H. murinum* and the grain amaranths, which display phenotypic plasticity in the absence of resolvable genetic variation. Jain et al. (1980) postulated that genetic uniformity of diverse populations of a species may result from: 1. a high rate of gene flow between populations leading to homogeneity, 2. selection for a common successful genotype in all areas, and 3. founder effects. These explanations cannot account for morphological variability between populations.

Intraspecific genetic differentiation between morphologically distinct members of algal taxa has been reported. Chapman (1974) found significant genetic differentiation between populations of *Laminaria* for stipe morphology. Gerard (1988) was able to differentiate ecotypes of *Laminaria saccharina* on the basis of light-related traits (ie. photosynthetic capacity). Sideman and Mathieson (1985) found that plants of *Fucus distichus*, derived from phenotypically variable natural populations, retained their distinctive forms when grown in a common environment. Espinoza and Chapman (1983) found genetic differentiation of growth rate and maximum uptake rate of nitrate between geographically separated populations of *Laminaria longicruris*. These data suggest that genetic differentiation is likely to account for some level of

phenotypic distinction in plants from Cape Beale and Coal Harbour. Yet, this level of sequence divergence has not been sufficient to block interfertility and not widespread enough over the nuclear genome to allow easy detection by random probe RFLP analysis. It is possible, however, that those sequences randomly cloned in this study, which were amenable to RFLP analysis, did not happen to encode polymorphic regions of DNA by chance alone. Such an explanation may be tested by expanding the library of probes to accommodate chance events or to find rare polymorphic sequences. Presently, phenotypic plasticity, for those traits analysed in this study, may be suggested as a possible explanation for the observed morphological variability in *Costaria*. Such plasticity may not be dependent on general nucleotide sequence divergence but rather on subtle modifications of genetic developmental regulatory systems. Sequence divergence of this kind in *Costaria* would likely not be resolvable with a technique such as RFLP analysis with anonymous probes.

CHAPTER III
POPULATION ANALYSIS OF *COSTARIA*

In this chapter, genetic subdivision of stands of *Costaria*, concentrated in a limited geographic region of the northeast Pacific, is explored.

Materials and Methods

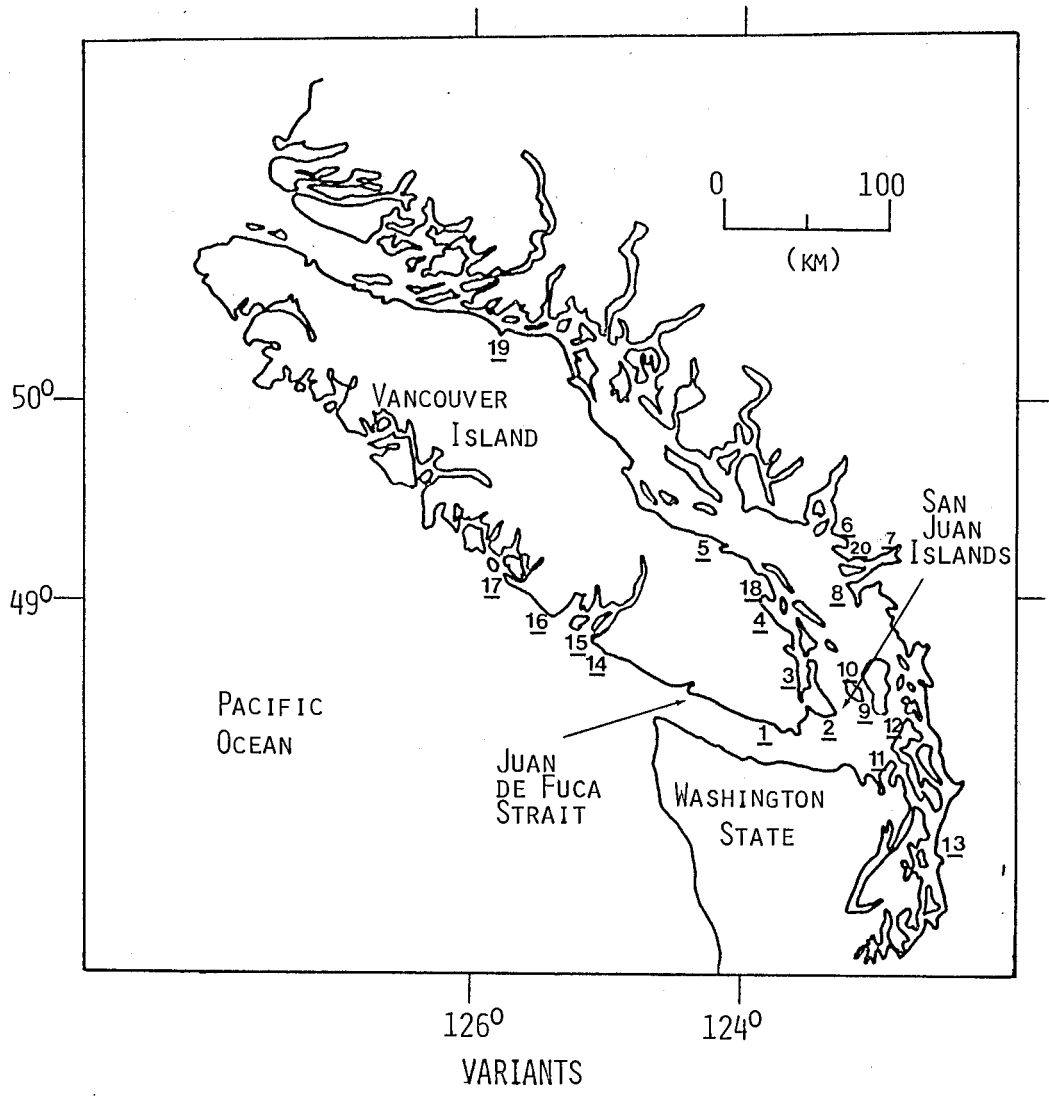
Sampling

Plants were collected once from twenty intertidal sites in southern and central Vancouver Island, British Columbia, Canada and northern Washington, U.S.A. (Fig. 7). Generally, two to three plants separated by at least 20 cm of shoreline were taken from each location. The sites varied in wave and current exposure from highly exposed (Bordelais Islets, Cape Beale) to sheltered (Lantzville, Coal Harbour).

Detection of rDNA Variation

Nuclear DNA was prepared as described in Chapter II. Restriction digest products were size-fractionated by electrophoresis on 0.7% horizontal agarose gels containing ethidium bromide at 10 mA for 20 hours in 1XTBE (89 mM Tris-borate, 89 mM boric acid, 8 mM disodium EDTA). Restricted DNA was acid-hydrolysed, denatured and transferred bidirectionally to nitrocellulose filters (Schleicher and

Figure 7: Geographic distribution of sites in the northeast Pacific from which *Costaria* was sampled. Numbers on map indicate site locations. These locations have been grouped according to which variant is found there.



- | | | | | |
|----------------------|--------------------|-----------------------|-------------------|--|
| A | | B | | |
| 1 (WHIFFEN SPIT) | 8 (TSWASSEN) | 14 (CAPE BEALE) | | |
| 2 (OGDEN BREAKWATER) | 9 (FRIDAY HARBOR) | C | | |
| 3 (CANOE COVE) | 10 (TURNER ISLAND) | 15 (BORDELAIS ISLETS) | | |
| 4 (MAPLE BAY) | 11 (PORT TOWNSEND) | D | | |
| 5 (LANTZVILLE) | 12 (ROSARIO BEACH) | 16 (UCLUELET) | | |
| 6 (HORSESHOE BAY) | 13 (BREMERTON) | | | |
| 7 (BROCKTON POINT) | | | | |
| E | | F | | |
| 17 (TOFINO) | 18 (DUKE POINT) | G | | |
| | | 19 (KELSEY BAY) | H | |
| | | | 20 (COAL HARBOUR) | |

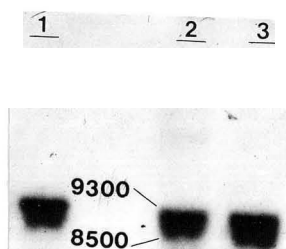
Schuell) by the method of Smith and Summers (1980). Restriction fragments were bound to the filters by baking at 80°C for 2 hours.

Prior to hybridization, filters were pretreated at the hybridization temperature (65°C) in 5XSSPE, 5XBFP and 0.2% SDS (1XSSPE= 0.18 M sodium chloride, 10 mM sodium phosphate, 1 mM disodium EDTA pH 7.0; 1XBFP= 0.02% w/v of bovine serum albumin, Ficoll (m.w.= 400,000), polyvinyl pyrrolidone). Hybridizations were carried out at 65°C in 5XSSPE, 1XBFP and 0.2% SDS. Filters were washed at 60°C in 2.5XSSPE and 0.1% SDS. Probes utilized in hybridizations were pCc18 and pCes370 (see Chapter II). Probes were radiolabelled with ³²P by nick translation (Rigby et al. 1977) and unincorporated nucleotides removed by passing the reaction volume through a 1.5 mL Sephadex G-50 spin column (Maniatis et al. 1982). Ribosomal DNA fragments were visualized by autoradiography at -70°C for 1-7 days using Kodak XAR-5 film.

A restriction map of the rDNA in *Costaria* was inferred from single and double digests using the following enzymes which all have six base recognition sites: Dra I, Eco RI, Hind III, Pst I and Xba I. Subcloned fragments of the nematode rDNA encoding only the 18S, 5.8S or 26S gene sequences were used in hybridizations to identify homologous fragments in *Costaria* in order to create the map. The rDNA in *Costaria* was found to exist in two size-classes, one of about size 8.5 kilo bases (kb) and the other of about size 9.3 kb, after hybridization analysis of Hind III or Pst I digested nuclear DNAs with pCc18 and pCes370

(Fig. 8). Both these enzymes cut once in the repeats. As well, the percentage rDNA in *Costaria* from Cape Beale, Duke Point and Friday Harbor was determined to detect possible copy number variation. In this dot-blot procedure (Rogers and Bendich 1987a), radiolabelled small-subunit rDNA of *Costaria* (pCc18) was used as a probe against 100-1000 ng of nuclear DNA bound to a nitrocellulose filter. Dots were cut out, dried and assayed by liquid scintillation counting. A standard curve of cpm/pg of pCc18 (insert only) was first constructed and then used to estimate pg of small-subunit rDNA in the population samples. These estimates were then divided by the total amount of DNA in each sample dot to get several estimates of percentage small-subunit rDNA in plants. These percentage values were halved and then multiplied by the ratio, in kilobases, of the size of the insert in pCc18 relative to the two size-classes of rDNA. These ratios were 4.7 and 5.2 respectively for the 8.5 kb and 9.3 kb size-classes. The percentage values of small-subunit rDNA were halved since it was estimated, from autoradiographs, that about equal numbers of rDNA genes were represented in the two size-classes (Fig. 8). The final values represented percentage rDNA in *Costaria*, from the three sites studied, totalled over the two size-classes of the repeat.

Figure 8: Autoradiograph of Southern blot showing the two size-classes of rDNA repeats in *Costaria*. The size of fragments is indicated in base pairs. DNA samples were digested with Hind III and probed with pCc18. Lanes 1,2 and 3 contain DNAs from plants originating in Duke Point, Bordelais Islets and Cape Beale respectively.

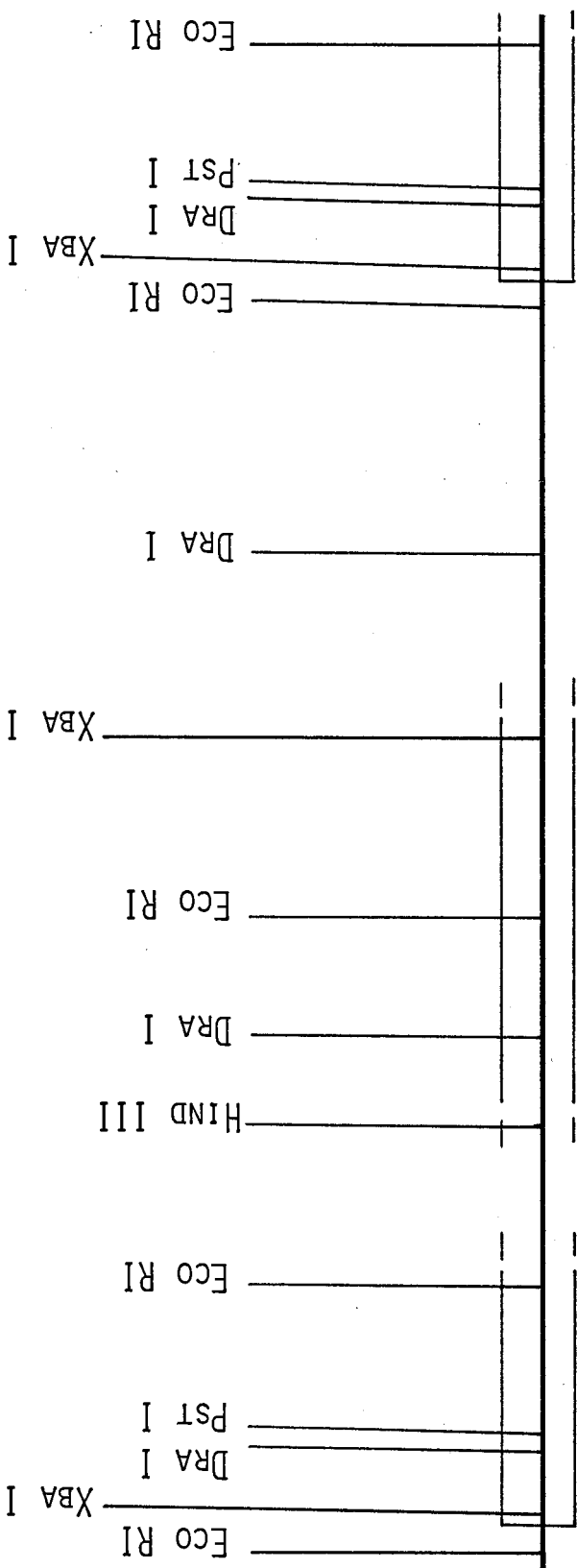


Results

A restriction map of *Costaria* rDNA is shown in Figure 9. Hybridization analysis revealed both length and restriction site heterogeneity in the rDNA of plants sampled. The length difference between the two size-classes found in this study is likely due to subrepeat variation in the IGS (Oono and Sugiura 1980, Learn and Schaal 1987, Schaal et al. 1987). The subrepeats appear to be about 200 bp in size (see below). The insertion of four such subrepeats would differentiate the 8.5 kb and 9.3 kb size-classes.

Though useful in understanding length variability in *Costaria* rDNA, hybridization analysis with pCc18 and pCes370 using Hind III digested DNAs did not resolve populations. All plants contained the two size-classes of repeats. Analysis of plants with pCes370 after digestion of DNAs with Pst I and Xba I also failed to show population-specific banding patterns. These results may be explained by the above enzymes having restriction sites only within the conserved coding region of rDNA (see Fig. 9). Resolution of populations was gained from Eco RI or Dra I digests of plants followed by hybridization analysis with pCc18 or pCes370. Both Eco RI and Dra I have restriction sites outside the rDNA coding region. Since the banding patterns produced by Eco RI digested DNAs were easier to resolve, two plants from each sampled site were digested with this enzyme and analysed with pCc18. Of the twenty sites studied eight distinctive

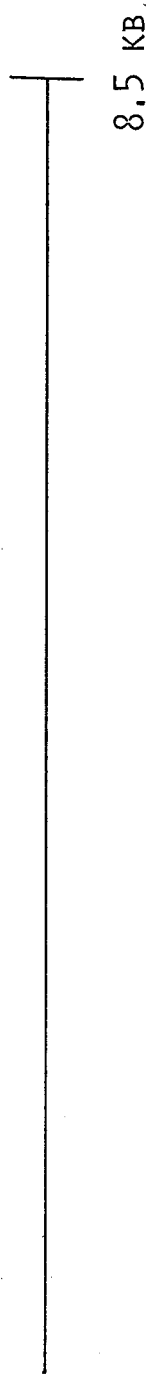
Figure 9: Restriction map of *Costaria* rDNA. The 8.5 kb length size-class of rDNA is shown.



SMALL-SUBUNIT

LARGE-SUBUNIT

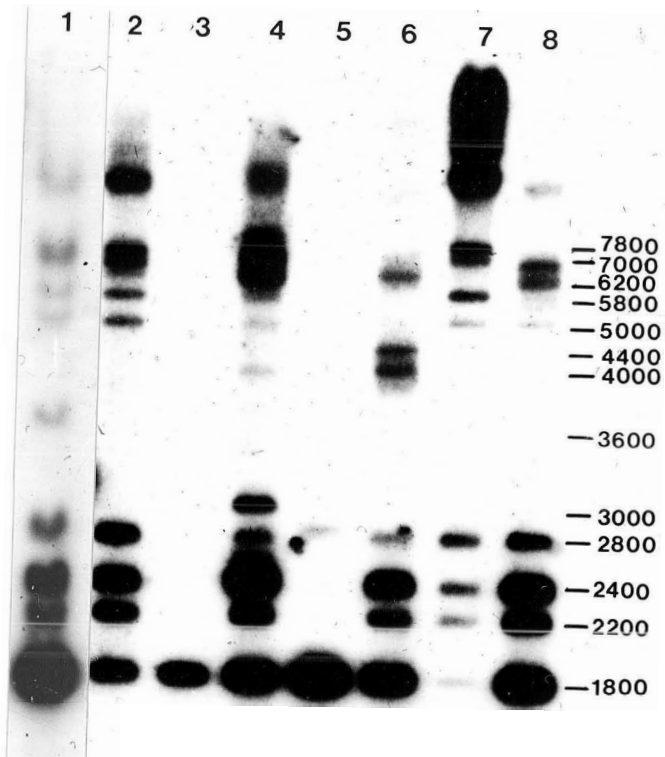
SMALL-SUBUNIT



restriction patterns were identified (Fig. 10). A predominant restriction pattern (Variant A) was shared by plants from thirteen, clustered sites (Whiffen Spit, Ogden Breakwater, Canoe Cove, Maple Bay, Lantzville, Horseshoe Bay, Brockton Point, Tsawassen, Friday Harbor, Turne Island, Port Townsend, Rosario Beach, Bremerton). The remaining seven patterns were unique to plants sampled from the respective sites Cape Beale (Variant B), Bordelais Islets (Variant C), Ucluelet (Variant D), Tofino (Variant E), Duke Point (Variant F), Kelsey Bay (Variant G), Coal Harbour (Variant H)). Geographically, Variant A was generally confined to the southern regions of Vancouver Island and northern Washington (Fig. 7). The distinctive patterns were found in plants originating in central Vancouver Island near the northern portion of the study area (except Coal Harbour).

To test for homogeneity of restriction patterns within populations, ten plants, originating from about 50 m of shoreline, were collected at both Cape Beale and Coal Harbour and these twenty plants individually analyzed with pCc18. All plants displayed population-specific banding patterns. There was, however, rDNA copy number variation as deduced from band intensities on autoradiographs. For example, though all Coal Harbour plants shared the same restriction pattern, when equal quantities of nuclear DNA from several plants were digested with Eco RI and analyzed with pCc18, the relative intensities of bands varied from plant to plant. It was verified that this copy number variation was not due to inconsistent collection of rDNA

Figure 10: Autoradiograph of Southern blot showing the eight variants of *Costaria* uncovered by probe pCc18. Nuclear DNAs were digested with Eco RI. The size of fragments is indicated in base pairs. Lanes 1 through 8 show Variants H, A, F, G, B, C, D and E respectively.

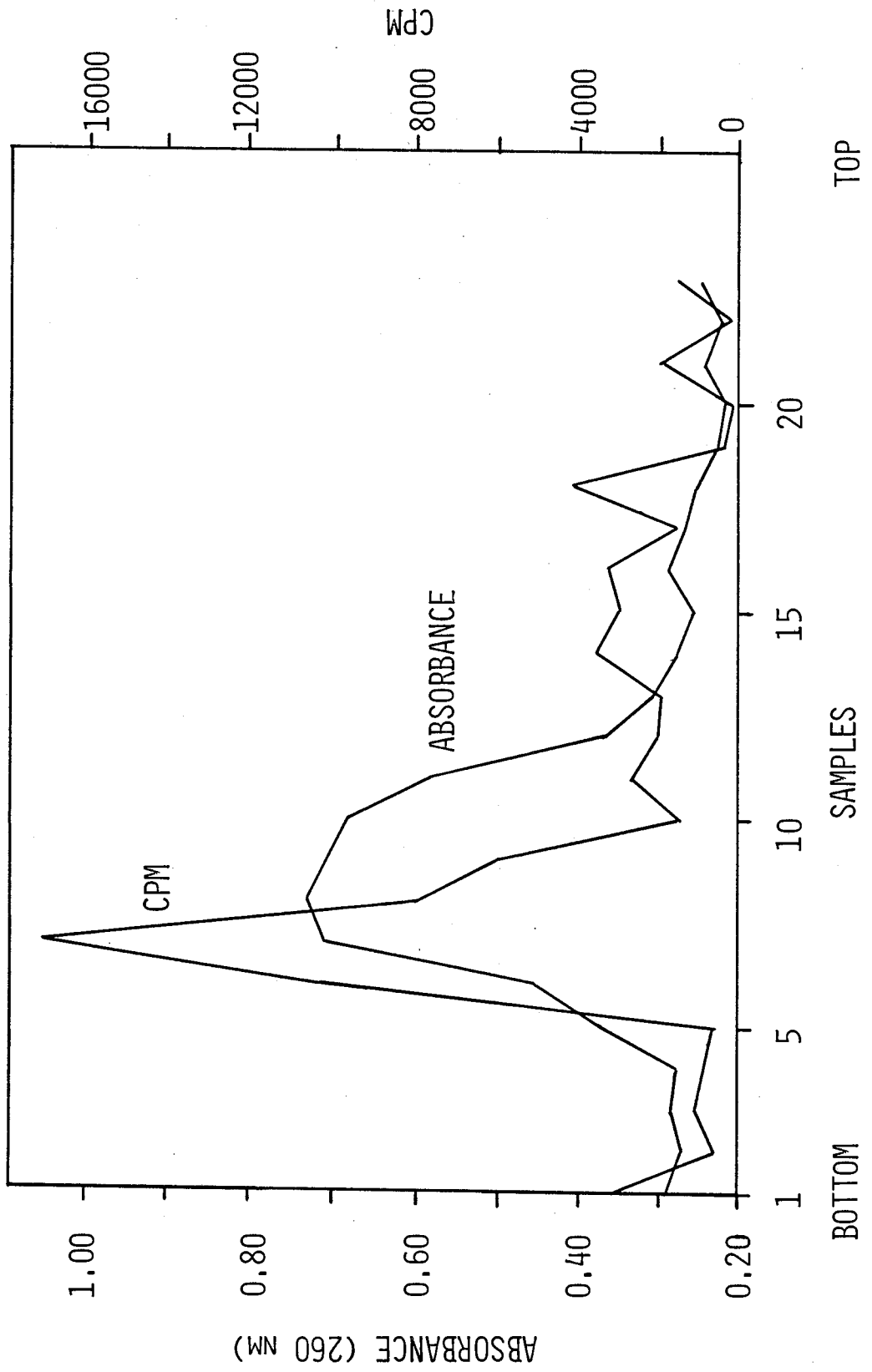


from Hoechst dye (H33258)-CsCl density gradients. Such inconsistency would result if the rDNA formed a satellite band distinct from the nuclear fraction and was unknowingly not collected in total.

To see if *Costaria* rDNA formed a satellite band, 500 microliter aliquots of CsCl were collected after ultracentrifugation of a total cellular DNA preparation of a plant from Tofino in a Hoechst dye-CsCl density gradient (Ingle et al. 1975). Twenty-three samples were taken from the 40 mL polyallomer tube starting before the visible nuclear band (Sample 1) and continuing after it had disappeared (Sample 23). The samples were brought up to 1 mL with addition of CsCl at the same density as in the gradient and the concentration of DNA estimated by measuring optical densities at 260 nm (Maniatis et al. 1982). DNA in each sample was then precipitated with ethanol, spun down, dried and dissolved in 10 mM Tris-Cl, 1 mM EDTA and dotted onto a nitrocellulose filter. The filter was then probed with pCc18 and the dots cut out, dried and assayed by liquid scintillation counting. The DNA concentrations/sample and cpm/sample were compared graphically (Fig. 11). It was clear that, though differing in G-C content, the rDNA of *Costaria* was essentially found within the main band comprising the nuclear fraction.

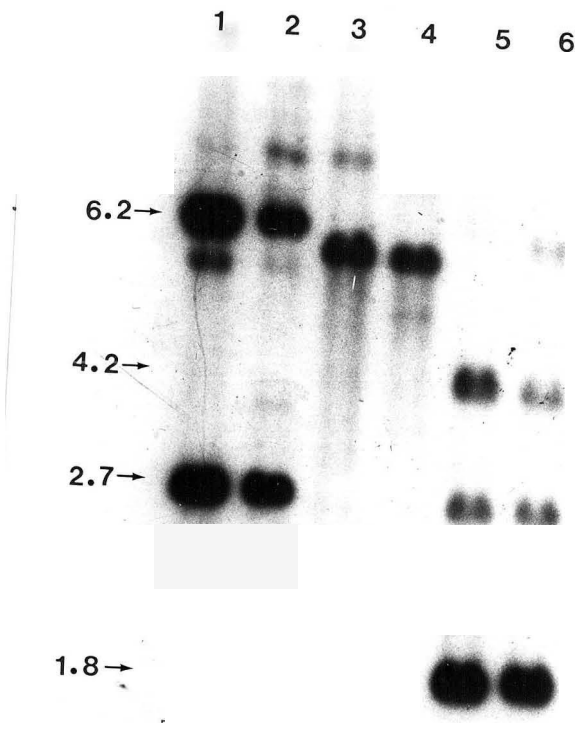
I postulate that the basis of the polymorphism uncovered by Eco RI is due to loss and gain of restriction sites in the IGS of *Costaria* rDNA. As stated earlier, there was no restriction

Figure 11: Identification of rDNAs in *Costaria* using a Hoechst dye-CsCl density gradient. Sample 1 is from near the bottom of the tube and Sample 23 is from near the top. Sample 7 contains the majority of the rDNAs while Samples 7-10 contain the greatest amount of total DNA.



site polymorphism found for genic sequences of rDNA in any of the plants from the sampled populations when the DNAs were digested with Dra I, Eco RI, Hind III, Pst I or Xba I using both pCc18 and pCes370 as probes (Fig. 12). Comparison of the DNA sequence of pCc18 for a plant each from Cape Beale and Coal Harbour also revealed no divergence (see Chapter II). Inspection of Figure 10 reveals that when Eco RI-digested DNAs were probed with pCc18 the major band resolved was that from which pCc18 was derived (1.8 kb). This band constitutes the major Eco RI fragment containing 1595 bp of small-subunit rDNA. The remainder of the higher molecular weight bands are polymorphic and, when compared together over all populations, appear to form an imperfect ladder based on multiples of about 200 bp insertions (Fig. 10). The 5' Eco RI site in pCc18 appears to be polymorphic with loss of this site in some of the repeats leading to a 2.2 kb or larger fragment. Since 1.8 kb is the smallest fragment visualized with pCc18 and since the 3' Eco RI site is in a conserved position within the small-subunit gene, I speculate that loss of the 5' site in the repeats of some plants would explain the polymorphic restriction pattern resolved by pCc18. The 200 bp repeats are believed to begin immediately upstream of the coding region of the small-subunit gene. Proof for this hypothesis would require a more detailed analysis of the IGS of *Costaria* rDNA relying on the results of both restriction mapping and sequence analysis. In this thesis I have limited the characterization of rDNA in *Costaria* and use pCc18 primarily as a tool for population analysis. The integrity of the banding

Figure 12: Autoradiograph of Southern blot showing conservation of restriction sites within the coding region of *Costaria* rDNA. Lanes 1,3 and 5 contain nuclear DNAs of a Cape Beale plant digested with Hpa I, Xba I and Eco RI respectively. Lanes 2,4 and 6 contain nuclear DNAs of a Coal Harbour plant also digested with Hpa I, Xba I and Eco RI respectively. The probe used in this hybridization encoded the genic sequence of rDNA in *Caenorhabditis elegans* (pCes370).



patterns presented here was, however, tested several times with DNAs digested to completion with two to threefold increases in Eco RI and Hind III concentrations and overnight incubations. Another possible explanation for the variant restriction patterns described here, which I have not accounted for, is due to polymorphic base modification within Eco RI restriction sites in the IGS within individuals. Supportive of the hypothesis of rDNA sequence heterogeneity in the IGS are, however, the finding of an absence of methylation of Eco RI restriction sites within probe pCc18 and the observation that cytosine methylation usually requires CG or CXG sequences as substrates (Jorgensen et al. 1987). Eco RI does not contain a suitable substrate in its recognition sequence.

The existence of subrepeats in the IGS of *Costaria* rDNA is not surprising since many plants share this trait. Subrepeat length may vary, for example, from 100 bp in radish to 200 bp in *Zea mays* to 325 bp in *Vicia faba* (Rogers and Bendich 1987 b). Furthermore, sequence analysis of the 204 bp immediately upstream of the coding region of the small-subunit gene in pCc18 suggests that the origin of the 200 bp subrepeats may have involved duplications of approximately 60 bp repeats (Fig. 13). The two 60 bp sequences shown in Figure 13 are 68.3% homologous. Such duplication events in the non-coding regions of rDNA have been postulated by Yakura et al. (1984) in their study of the 325 bp repetitive elements in the IGS of *Vicia faba* rDNA.

Figure 13: Alignment of two 60 base pair repeats found immediately upstream of the coding region of the small-subunit rDNA of *Costaria*. The nucleotides are numbered relative to the beginning of the coding region of this gene. Forty-one of the sixty base-pairs match giving a homology value of 68.3%.

- 144

CGGTGCATT^T CTTGCA T CG^ATTG GT TT G A^GTCCGAGAAAT^ATAAAAACATGACTCC^C TGTG^C
CGGTGC TT^CGGC GCACGTTGG^CT GCGTCCCTTCGGCA^CT AGA T_GTAAAC C CC_TGTGTGG

- 81

- 85

- 22

Results of the dot-blot analysis of percentage rDNA in *Costaria* of one plant each from Cape Beale, Duke Point and Friday Harbor are shown in Table VI. The percentage values of total rDNA per plant (summed over the two size-classes) varied by threefold with the individual from Cape Beale containing the least rDNA (0.10%). These data confirm the copy number variation suggested by results of the hybridization analysis and there appears, therefore, to be some variance in percentage rDNA in blades of *Costaria*. This trait may not be useful in resolving populations since there appears to be copy number variation within as well as between populations. An increased within population sample size would aid in resolving the nature of rDNA copy number variation in *Costaria*. Such an analysis may allow the differentiation of populations from Cape Beale and Coal Harbour which appear to have large differences in the number of rDNA repeats. Populations, which, however, contain equal numbers of rDNA repeats but vary in the distribution of restriction sites within the IGS would not be resolved with this technique. The mean percentage of rDNA over the three *Costaria* populations studied (0.20%) is similar to the average amount in *Vicia faba* leaves (0.22%, Rogers and Bendich 1987 a).

Discussion

Results presented here suggest that it is possible to resolve populations of the morphologically plastic kelp *Costaria* using variation in the IGS of rDNA as a molecular marker.

Table VI: Variation in populations of *Costaria*
of percentage rDNA in blade tissue.

POPULATION	SIZE - CLASS OF rDNA		TOTAL rDNA
	8.5 KB	9.3 KB	
CAPE BEALE	0.05	0.05	0.10
DUKE POINT	0.09	0.10	0.19
FRIDAY HARBOR	0.15	0.17	0.32

Costaria appears to display variation both in terms of copy numbers of rDNA genes and restriction site polymorphisms within the IGS. Of the twenty stands studied, all contained the same two size-classes of repeats (8.5 kb and 9.3 kb). Eco RI and Dra I restriction sites within the IGS were, however, polymorphic and could be used as suitable markers of populations. Polymorphisms within rDNA, largely due to repeat length variation, have been used in a similar fashion in other studies to infer genetic subdivision in domesticated lines or in wild plant populations (*Hordeum* spp., Saghai-Marroof et al. 1984; *Triticum dicoccoides*, Flavell et al. 1986; *Pisum sativum*, Polans et al. 1986; *Vicia faba*, Rogers and Bendich 1987 a; *Phlox divaricata*, Schaal et al. 1987).

Schaal et al. (1987) found considerable variation in rDNA repeat lengths and their frequencies within and between populations of subspecies of *Phlox divaricata*. Some populations were fixed for a particular variant while others exhibited varying frequencies of variants. Restriction sites within rDNA repeats were not polymorphic. Flavell et al. (1986) reported similar results in populations of *Triticum dicoccoides*. For one population which was homogenous for repeat length, Flavell et al. (1986) suggested that founder effects, rapid homogenization of rDNA length variants or selection as possible explanations. Though not variable in repeat length, homogeneity of rDNA IGS restriction patterns within Coal Harbour and Cape Beale populations of *Costaria* may also have resulted from such forces.

The geographic distribution of *Costaria* populations, within the study area at the time of collection, may be described as consisting of two distinct regions. In southern Vancouver Island and northern Washington there appears to be a large, clustered, interbreeding group with all individuals studied displaying the Variant A banding pattern. The more northern beds of *Costaria* are comprised of distinct populations with a high degree of genetic isolation.

I postulate that the population distribution data may have been established following the last glaciation (Wisconsin) which reached its maximum extent in the south (British Columbia) around 20,000 years ago (Matthews and Harris 1987). In particular, the western margin of the Cordilleran Ice Sheet extended from Puget Sound (northern Washington) northwards into the Aleutian Islands, covering the coastal shelf of the present study area (Matsch 1976) and presumably rendering it uninhabitable for kelp species. This ice sheet receded from the coastline of British Columbia (including Vancouver Island) and northern Washington by 12,000 years ago and may have opened it up to colonization by marine flora and fauna. The radiation of terrestrial plants and animals following the Wisconsin glaciation has been analysed (Karlstrom and Ball 1969). The major terrestrial refugia which are thought to have been sources of post-glaciation recolonization are south of the ice boundary, in central and northern Alaska, Yukon and the Northwest Territories (Ceska 1985). Randhwa and Beamish (1972) have, for

example, shown that the present distribution of diploid and polyploid *Saxifraga ferruginea* can be explained by correlating the positions of diploid populations with once-existing refugia. With respect to the marine environment, northern Washington and Alaska are likely locations for primary refugia in the northeast Pacific and may have served as centers of radiation once the ice sheet receded. Sites containing Variant A may, therefore, have been positioned at the center of radiation of *Costaria* populations northward. As stated earlier, dependence on founder events, rapid homogenization of variants or selection for superior variants within genetically isolated populations could also have led to the highly distinctive nature of rDNA variants in northern *Costaria* populations. This explanation is hypothetical with no direct proof presently available regarding ancestral and derived banding patterns to show northward radiation of *Costaria* or the gradual fixation of variants at sites. The distribution data is limited in that it is derived from a single point in time.

Supportive of such a hypothesis, however, is the oceanography of the study area. A mechanism by which the northward radiation of *Costaria* may be envisioned, for example, is through the action of tidal streams and currents. Based on Thomson (1981), the oceanography of surface waters in the majority of the study region may be generally described as having two major influences. First is the strong flow of water into and out of the region through the Strait of Juan de Fuca

due to the inward progressing ocean tide (Fig. 7). Once north of the San Juan Islands, the tidal stream is dramatically weakened as it moves northward. Superimposed on this northward flow of water are gyres between Vancouver Island and the mainland north of the San Juan Islands which lead to mixing of surface waters about as far north as Lantzville. Shores immediately north of here are influenced by weak tidal currents originating both from the south and from northern Vancouver Island where the southward progressing tidal stream enters. Floating *Costaria* plants may, therefore, be expected to be transported to all regions of southern Vancouver Island and northern Washington leading to genetic homogenization of these stands. The weak northward tidal stream would be expected to transport plants in this direction but the probability of gene flow between these populations and those south of them may decrease due to the rapid attenuation of this stream. Kelsey Bay is affected by the strong tidal stream entering from the north and may, therefore, be part of a northern breeding group distinct from the southern populations.

With respect to dispersal by surface currents, it should be noted that though *Costaria* does not have a gas bladder plants are capable of flotation due to the existence of bullations which trap air on the undersurface of detached individuals (as seen by the author). This trapped air allows some plants to float for a short time under calm wind and wave conditions. Plants from wave exposed sites such as Cape Beale and Bordelais Islets would likely not be able to disperse as well in this

manner due to the reduction or absence of bullations in plants originating from such harsh environments. Such an alteration in morphology may potentially lead to an increased chance of genetic isolation in open-coast, exposed stands.

Another possible explanation for the observed distribution of populations is due to the intervention of man through transport of tissue via ferry or pleasure boat traffic (Round 1981, van den Hoek 1987). The sites occupied by Variant A are areas of heavy marine traffic and homogenization of these stands may have resulted from the transportation of *Costaria* sporophytes or gametophytes on boat hulls from one port to another. Such an argument would account for the existence of Variant A in Lantzville, a site which is surrounded by distinctive populations north (Kelsey Bay) and south (Duke Point) of it. Lantzville is adjacent to a major ferry port (Nanaimo) which receives ferry traffic regularly from Horseshoe Bay (a Variant A site). This explanation is inconsistent with the existence of Variant H in Coal Harbour, a site which is necessarily influenced by boat traffic since it is adjacent to a yacht club in the middle of a busy port (Vancouver). Ten individuals analysed from Coal Harbour displayed an invariant, unique banding pattern. Furthermore, Brockton Point, which is about 1 km from the Coal Harbour site, contained plants which exhibited the Variant A banding pattern. Both sites are visibly exposed to marine traffic yet only plants from Brockton Point appear to outbreed. Physically, the two sites are very different

since Brockton Point is a rocky, moderately exposed shore while Coal Harbour is a very sheltered and muddy environment with plants clinging to scattered boulders. It is possible that the heavy sedimentation of the water column at Coal Harbour may inhibit dispersal of *Costaria* from this site and the entrance of plants from other populations (chiefly Variant A). In any case, the results of this study suggest that populations of *Costaria* in the study region may be comprised of outbreeding and genetically isolated groups. The distribution of these groups apparently cannot be described simply on the basis of morphology since plants possessing Variant A may display a range of phenotypes.

Until a cause and effect relationship can be established for dispersal of seaweeds it is unclear by which means *Costaria* outbreeds. Dispersal may be potentially accomplished by drifting sporophytes, meiospores or dislodged gametophytes with or without a developing sporophyte attached or via marine traffic. van den Hoek (1987) considers spore-dependent dispersal unlikely over great distances since chance events must bring two spores (giving rise to a male and a female gametophyte) close enough together on a foreign shore to allow fertilization. The survival time of kelp meiospores in the wild is unknown, though Kain (1964) found that some spores of the kelp *Laminaria* could remain suspended for days and some could survive for greater than 50 days in the dark (Kain 1969). Moss et al. (1981) considered dispersal of sporophytes, while still attached to female

gametophytes, as a potential means of spread of kelps onto North Sea oil platforms. To summarize, it appears that in spite of an inability to create a theory based on cause and effect, studies which utilize molecular biology to analyze attached macroscopic forms, may, presently, best be able to provide meaningful data regarding kelp dispersal. Such techniques are not limited by the difficulties involved in the tracing of often minute reproductive tissues in the oceanic system.

CHAPTER IV

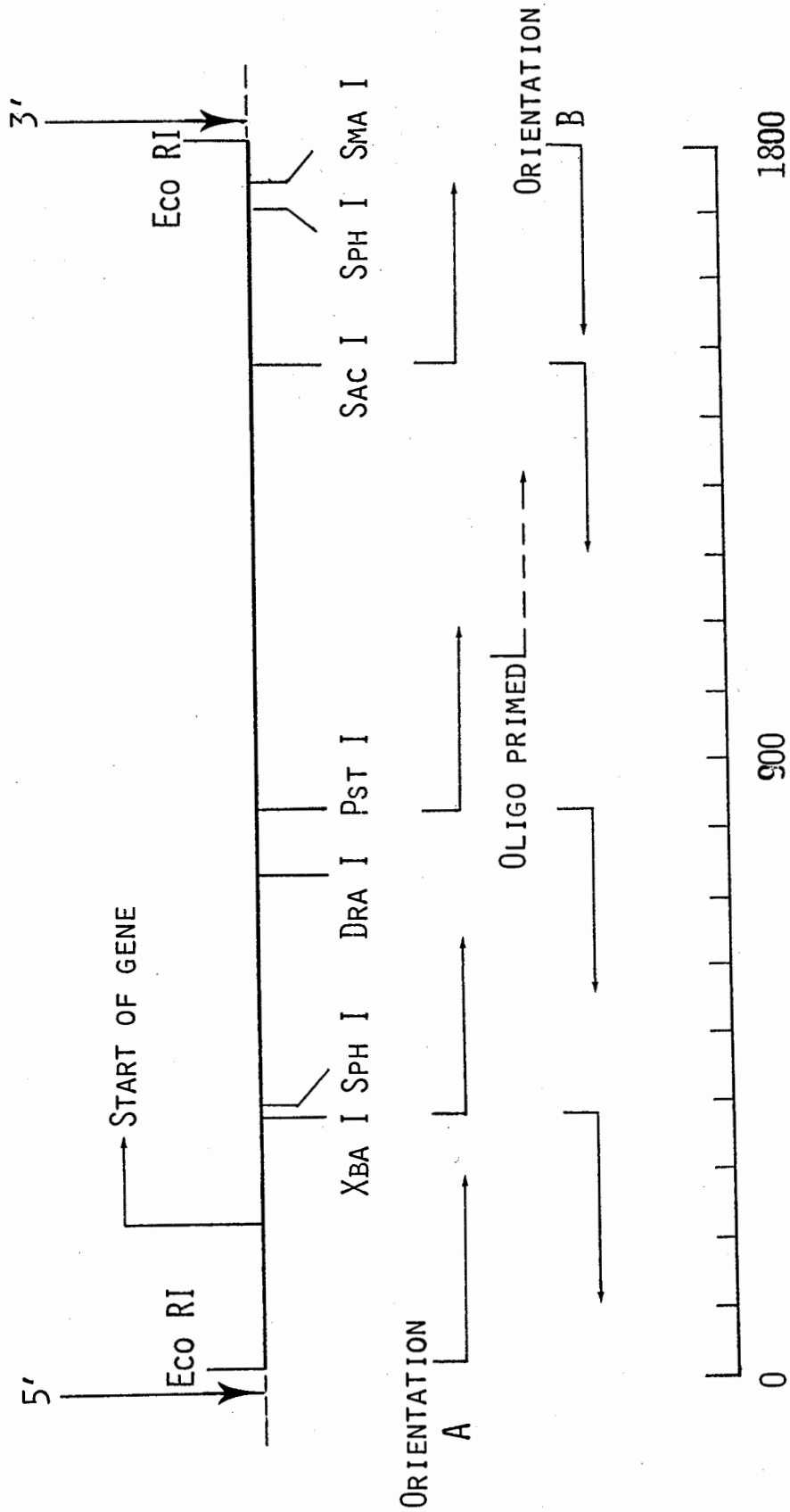
EVOLUTIONARY POSITION OF *COSTARIA*

In this chapter, the phylogenetic relationship of *Costaria* to other pigmented vascular and nonvascular plants is explored. In particular, the evolutionary position of *Costaria* relative to chlorophyll a+c plants and oomycetes is addressed in light of the small-subunit sequence data of Gunderson et al. (1987) which suggests that organisms from these two latter groups are closely related.

Materials and Methods

Nuclear DNA was prepared for a *Costaria* plant from Coal Harbour and used to create a shotgun genomic library with the restriction enzyme Eco RI (see Chapter II). Plasmid pCc18 of size 1.79 kb, which was identified as containing rDNA was hypothesized to encode a portion of the small-subunit gene after comparing its restriction map to those existing for *Vicia faba* (Yakura et al. 1984) and soybean (Varsanyi-Breiner et al. 1979). A restriction map of this Eco RI fragment is shown in Figure 14 using the following enzymes which all have six base recognition sites, Dra I, Eco RI, Pst I, Sac I, Sma I, Sph I and Xba I. As well, Figure 14 shows the sequencing strategy used with this clone.

Figure 14: Restriction map and strategy used to sequence the insert in plasmid pCc18 using restriction enzyme deletions and a synthetic oligonucleotide primer (designated by the broken line) on orientations A and B of the insert. pCc18 originated from a shotgun Eco RI genomic library of a Coal Harbour *Costaria* sporophyte.



Sequencing of the 1.79 kb insert was accomplished by the dideoxynucleotide chain termination method (Sanger et al. 1977) using denatured plasmid DNA as template (Hattori and Sakaki 1986). Approximately 92% of the insert was sequenced with forward and reverse primers with the aid of restriction site deletions in the insert (Baillie et al. 1985) which provided overlaps between restriction fragments (see Fig. 14). Primer sites to direct DNA strand synthesis by the Klenow fragment of DNA Polymerase I are located on either side of an insertion within the polylinker of pUC 19. Restriction enzyme deletions were generated on both orientations of the insert in plasmid pCc18 (ie. Orientation A and B). Deletions were obtained when a restriction enzyme cut within the insert as well as within the polylinker. Fragments spanning the restriction site in the polylinker to a site within the insert were lost and the plasmids carrying the deletions were allowed to reanneal and ligated with T4 DNA ligase. Only restriction enzymes Pst I, Sac I and Xba I were utilized to make deletions since sites for these enzymes were conveniently located in the fragment and were found in the polylinker region of pUC 19. A 150 bp gap in the middle of the insert was filled in using an 18 bp synthetic oligonucleotide primer from which DNA synthesis could be directed. This region of pCc18 is represented by a broken line in Figure 14. The sequence of the oligonucleotide primer was synthesized as complementary to 18 base pairs of the DNA immediately 5' to the gap (in Orientation A). The complete sequence of the insert in plasmid pCc18 is shown in Figure 15.

Figure 15: Nucleotide sequence of the insert in plasmid pCc18 encoding 204 base pairs of upstream sequence and 1595 base pairs of *Costaria* small-subunit rDNA. The insert is presented in a 5' to 3' direction and was sequenced as outlined in Figure 14.

GCTGCGTGCGAGTTCATGGCTGCTAAAGCCGTGTGTTTTCGTTCCGCAGTCTTACCTTTT 60
CGGTGCATTTCTTGCATCGATTGGTTTGAGTCGAGAAATATAAAACATGACTCCCTGTGC 120
GCGCGGTGCTTCGGCGCACGTTTCGCTGCGTCCCTTCGCAGTATGTAACCCCTGTG 180
TGCCCCGTGCGAAATCAATTTGGCAATCTGGTTGATTCTGCCAGTAGTCATACGCTTGTC 240
TCAAAGATTAAGCCATGCATGTCTAAGTATAAGGCCTTTATACTGTGAAACTGCGAATGG 300
CTCATTATATCAGTCATAGTTTATTTGAAAGTCCCTTACTACATGGATAACCGTAGTAAT 360
TCTAGAGCTAATACATGCATGCAAGGCCCAACTGCTTCGGCGGACGGGCTGCATTGATTA 420
GACCGAAACCAATGCGTCTTCGGAGGTTTTTGAATCATAATCACTTGCGGATCGCACGCT 480
TCGGCGGCGACGTTTCATTCAAGTTTCTGCCCTATCAGCTTTGGATGGTAGGGTATTGGC 540
CTACCATGGCTTTAACGGGTAACGGGGAATTGGGGTTCGATTCCGGAGAGGGAGCCTGAG 600
AAACGGCTACCACATCCAAGGAAGGAGCAGGCGCGTAAATTACCCAATCCTGACACAGGG 660
AGGTAGTGACAATAAATAACAATGCCGGGTTATACAAGTCTGGCAATTGGAATGAGAGCA 720
ATTTAAATCCATCATCGAGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAA 780
TTCCAGCTCAATAGCGTATATTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTGT 840
GGCGCGCCGTCCGGCGGGGCTCTTTCATTAGGGCCGTTTGTCCGGTTTTTCGGCCGCTC 900
CATTCTCGGGTAGCGTGTGCTGGCATTAGGTTGTCGGCTTCTTCGCGCCGTCTGTTGCT 960
GGGAAAAAATTAGAGTGTTCAAAGCAGGCTTAGGCGTTGGATACATTAGCATGGAATAAT 1020
GAGATAGGGCCACGACGGTCTATTTTGTGGTTTGACCGTTGTGGTAATGATTAACAGGA 1080
ACGGTTGGGGGTATTTCGTATTCAATTGTCAGAGGTGAAATTCTTGATTTATGGAAGACG 1140
AACTACTGCGAAACGTTTACCAAGGATGTTTTTCATTAATCAAGAACGAAAGTTAGGGGAT 1200
CGAAGATGATTAGATAACCATCGTAGTCTTAACCATAAACTATGCCGACTAGGGATTGGCG 1260
GTTTCGTAAATTTACAGGACTCCGTCAGCACCTTCCGAGAAATCAAAGTCTTTGGGTTCCG 1320
GGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGAG 1380
TGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCCGGACATAGTGA 1440
GGATTGACAGATTGAGAGCTCTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTA 1500
GTTGGTGGAGTGATTTGTCTGGTTAATTCGTTAACGAACGAGACCCCGCCTGCTAAATA 1560
GTGTGGCTTACGCTTCTGTGTAGGTGCTCGCTTCTTAGAGGGACTTTCGGTGACTAACCG 1620
AAGAAGTTGGGGGCAATAACAGGTCTGTGATGCCCTTAGATGTCCTGGGCCGCACGCGG 1680
CTACACTGATGCATGCAACGAGTTCTTTTTTTTCTGGTTCGAGAGGCCCGGGTAATCTGT 1740
TGAACGTGCATCGTGATAGGGATAGATCATTGCAATTATTGATCTTGAACGAGGAATTC 1799

Both DNA strands were sequenced for approximately 30% of the insert. No mismatches were found over this region.

It was verified that sequence divergence did not occur among the repeated copies of this gene within an individual genome. To accomplish this, five separate fragments of size 1.79 kb encoding the small-subunit rDNA were isolated from one individual. Comparison of approximately 600 bp of sequence in the upstream (5') region and within the gene showed no divergence. Additionally, comparison of the total (1.79 kb) sequence from two individuals of *Costaria* has also showed no divergence. The sequence data indicated that the 1.79 kb insert encoded 204 bp of 5' upstream sequence and 1595 bp of small-subunit rDNA coding sequence which could be used for phylogenetic analysis.

Results

Sequences encoding the small-subunit rRNA from *Chlamydomonas reinhardtii* (Gunderson et al. 1987), *Oryza sativa* (Takaiwa et al. 1984), *Glycine max* (Eckenrode et al. 1985), *Prorocentrum micans* (Herzog and Maroteaux 1986), *Ochromonas danica* (Gunderson et al. 1987), *Achlya bisexualis* (Gunderson et al. 1987), *Euglena gracilis* (Sogin et al. 1986) and the small-subunit rDNA from *Costaria* were aligned and nucleotides positioned on the basis of conserved eucaryotic primary and secondary structures (Elwood et al. 1985). The aligned sequences are shown in Appendix A with

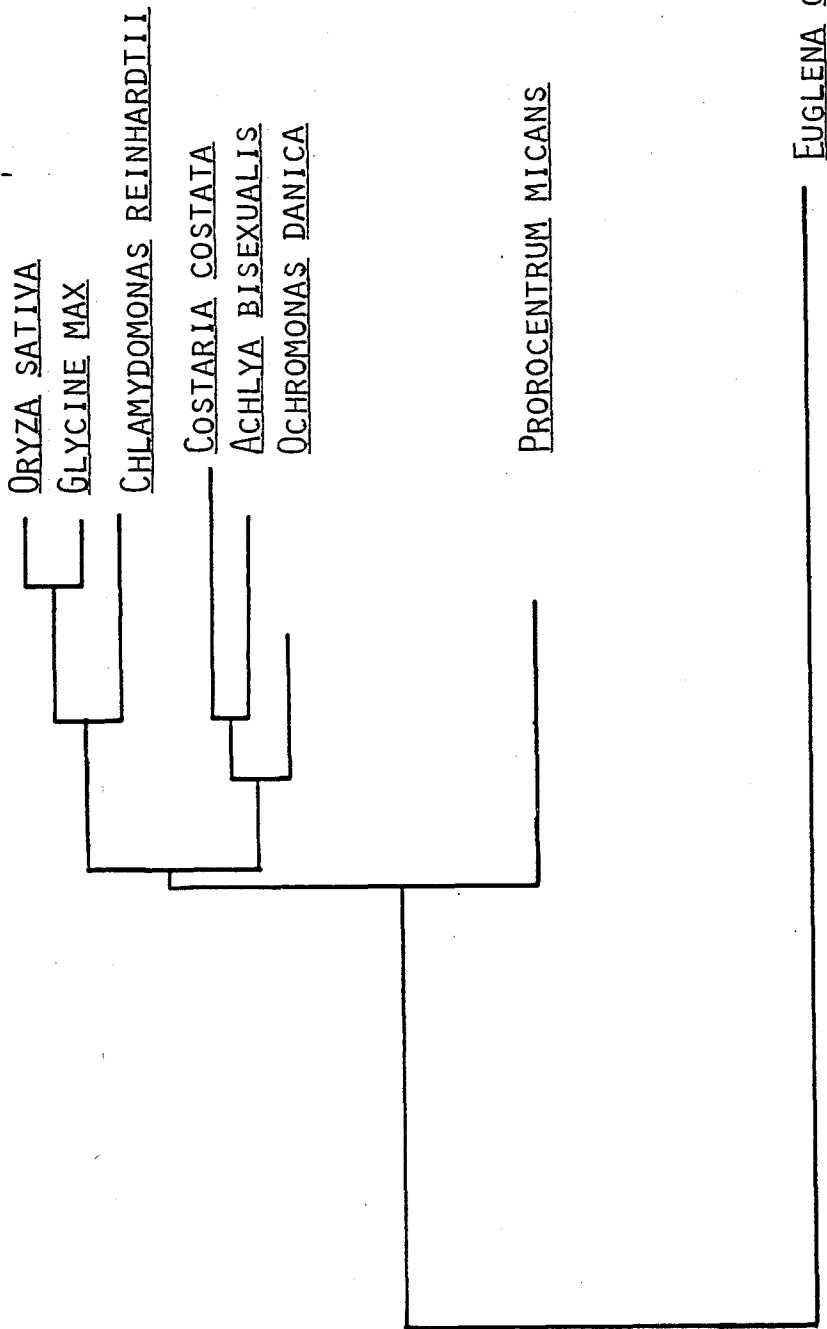
sites used for comparison designated by the number 1 in the "Mask" sequence. Derivation of structural similarity values were carried out as in Elwood et al. (1985). Pairwise sequence comparisons of approximately 1430 bases led to the calculation of percentage homology values and then to the construction of a distance matrix for all species. This matrix is shown in Table VII. Distance values ranged from a minimum of 0.032 between *O. sativa* and *G. max* and maxima ranging from 0.419 to 0.444 between *E. gracilis* and all other species. Structural distance values were converted to a phylogenetic tree using a form of the distance matrix method (see Fitch and Margoliash 1967 and Elwood et al. 1985). This tree, which represents relative macromolecular distance on the horizontal axis is shown in Figure 16.

On the basis of the present phylogenetic tree construction, four major diverging lines of host cells are suggested. *Euglena gracilis* represents the earliest divergence. Following this, a tripartite divergence occurs resulting in a chlorophyll a+b cluster (*C. reinhardtii*, *O. sativa*, *G. max*) and two chlorophyll a+c lines, one represented by *P. micans* and the other by *O. danica* and *Costaria*. The radiation of the chlorophyll a+b and chlorophyll a+c lines leading to vascular plants and the kelp, respectively, appears to occur simultaneously with *C. reinhardtii* and *O. danica* representing ancestral forms. The position of *P. micans* is less certain. Inspection of the distance matrix indicates that this dinoflagellate is least

Table VII: Structural distance data for study organisms based on small-subunit rRNA sequences.

	A.	B.	C.	D.	E.	F.	G.	H.
A. <i>C. REINHARDII</i>	-							
B. <i>O. SATIVA</i>	0.095	-						
C. <i>G. MAX</i>	0.101	0.032	-					
D. <i>O. DANICA</i>	0.128	0.136	0.136	-				
E. <i>P. MICANS</i>	0.146	0.145	0.142	0.137	-			
F. <i>E. GRACILIS</i>	0.424	0.419	0.437	0.422	0.435	-		
G. <i>C. COSTATA</i>	0.157	0.158	0.157	0.114	0.169	0.444	-	
H. <i>A. BISEXUALIS</i>	0.139	0.150	0.155	0.096	0.137	0.427	0.097	-

Figure 16: Phylogeny of plants bearing chlorophyll a+b and chlorophyll a+c on the basis of structural distance between small-subunit rRNA sequences. Relative evolutionary distance is represented by the separation of taxa on the horizontal axis.



distant from *O. danica* (0.137) and approximately equally distant from *C. reinhardtii* (0.146), *O. sativa* (0.145) and *G. max* (0.142). The aquatic oomycete, *A. bisexualis*, is least distant from *Costaria* (0.097) and *O. danica* (0.096). This result supports Gunderson et al.'s (1987) suggestion that this oomycete is closely related to chrysophytes.

Discussion

An understanding of the phylogenetic tree presented in this thesis may be gained by its comparison to existing trees based on pigment constitution and cellular data. There are a number of phylogenies which are based on RNA sequence data (5S and small-subunit) which may also be used for comparative purposes. The distinctiveness of the lineage leading to *E. gracilis*, relative to the other plant lineages, has been reported by Sogin et al. (1986) and Gunderson et al. (1987) in their studies using small-subunit rRNA sequences as well as by Kumazaki et al. (1982) on the basis of 5S RNA sequence data. The evolutionary position of *E. gracilis* is resolved with these nuclear DNA sequence divergence data. *Euglena gracilis* does not appear to be closely allied to green algae which presumably lead to vascular plants. Plastid characteristics are misleading in this case. There is clearly no necessary evolutionary pathway leading from this species to other chlorophyll a+b plants. Sogin et al. (1986) have pointed out that the plastid may have been acquired by *E. gracilis* long after it diverged from the main chlorophyll

a+b plant lineage. This contention is supported by *E. gracilis* plastid 16S rRNA sequence data (Graf et al. 1982) which indicates that the chloroplast of this species is on the chlorophyll a+b evolutionary line while the present data shows its nucleus to be closely allied with the most primitive eucaryotes (Palmer 1987).

The position of *C. reinhardtii* in relation to *O. sativa* and *G. max* agrees well with both RNA sequence and morphologically based phylogenies. *C. reinhardtii* is thought to be similar to some form of green flagellate which gave rise to vascular plants (Gunderson et al. 1987, Hori et al. 1985, Scagel et al. 1982). Similarly, an organism resembling *O. danica* may have given rise to morphologically complex chlorophyll a+c plants such as *Costaria*. Lim et al. (1986) positioned another unicellular Chrysophyte, *Hydrurus*, in a similar position in their phylogenetic study utilizing 5S RNA sequence data. The diatoms were also seen to fall into this group (chlorophyll a+c) leading to the kelps (Lim et al. 1986). The inclusion of *A. bisexualis* in the chlorophyll a+c lineage and its relatively small distance from *Costaria* (0.097) is strongly supported by the structural distance data even though this saprobic species has generally been classified with the "lower fungi" and not in association with chlorophyll a+c plants (Scagel et al. 1982). Gunderson et al. (1987) felt that oomycetes have evolved independently from the "higher fungi" with their close relationship to chrysophytes being supported by the shared traits of having heterokont

flagellated stages and similar kinetids.

Plastid characteristics are found to be misleading data in the case of *P. micans* as well. This dinoflagellate is not positioned within the lineage leading to the kelp. Gunderson et al. (1987), in a more extensive phylogenetic study based on small-subunit rRNA sequence data, found that *P. micans* grouped closely with ciliates and hypothesized that its peculiar nuclear organization may have arisen secondarily from one more typically eucaryotic. Furthermore, Hinnebusch et al. (1981) analysed the 5S RNA sequence of the dinoflagellate *Crypthecodinium cohnii* and compared it to existing sequences of diverse eucaryotic and procaryotic taxa. In their phylogenetic tree construction *C. cohnii* appeared near the point of the plant-animal divergence; an event more recent than the branching of the fungi. Hinnebusch et al. (1981) interpreted this result as indicating secondary loss of typical eucaryotic nuclear organization in *C. cohnii* and not of a state of primitiveness.

Four distinct, diverging lines of host cell evolution are suggested in this study:

- 1.) Euglenophyta (chlorophyll a+b),
- 2.) Pyrrophyta (chlorophyll a+c),
- 3.) Chrysophyta, Phaeophyta, Oomycota (chlorophyll a+c), and
- 4.) Chlorophyta, Spermatophyta (chlorophyll a+b).

In support of the molecular data, representatives within each line share cytological features unique to their line. These

include structure and arrangement of flagella, structure and behaviour of the nuclear envelop during cell division and storage products (Dodge 1974, Taylor 1976, Gibbs 1962). The structure of the chloroplast (when present), its pigmentation and positioning relative to the nucleus are also distinct (Gibbs 1962, 1970). These observations support the contention that photosynthetic eucaryotes arose from the union of several distinct photosynthetic symbionts and host cells (Ludwig and Gibbs 1985). Initially, this union involved a photosynthetic procaryote and a heterotrophic eucaryote, however, subsequent invasions may have involved a photosynthetic eucaryote and a heterotrophic eucaryote (Gibbs 1978, 1981, Ludwig and Gibbs 1985). Gibbs (1978) felt that this latter pathway resulted in species such as *E. gracilis* and *P. micans* which presently contain the plastid of an invading algal cell. As further evidence, cryptomonads have been shown to contain a vestigial nucleus (nucleomorph) present in the periplastidal compartment which likely originated from a red algal symbiont (Ludwig and Gibbs 1985).

In summary, the two unidivisional lines, Pyrrophyta (chlorophyll a+c) and Euglenophyta (chlorophyll a+b), are thought to represent relatively recent algal symbiont invasions (Gibbs 1978, 1981). The *E. gracilis* host cell is morphologically distinct from all other plant cells (Gibbs 1978) and on the basis of small-subunit rRNA sequence data, diverged from all major biotic assemblages early on (Gunderson et al. 1987). The

two remaining lines, containing the vast majority of photosynthetic organisms, may represent protracted symbiotic relationships and greater integration of the two biotas. Such integration may be inferred from the number and structure of chloroplast membranes (see Gibbs 1978). These lines diverged prior to the metazoa (Gunderson et al. 1987).

The positioning of the saprobic fungus *A. bisexualis* between the kelp *Costaria* and the chrysophyte *O. danica* supports Gunderson et al.'s (1987) finding of a close affinity between the chrysophyte and the fungus on the basis of small-subunit rRNA sequence data. The affinity of oomycetes to the Xanthophyta (chlorophyll a+c), a group considered closely associated with the Chrysophyta, has been suggested on the basis of similar flagellation - motile cells are biflagellate heterokonts with the leading flagellum tinselated (Cavalier-Smith 1978). This flagellar state is shared by the Oomycota, Chrysophyta, Xanthophyta and Phaeophyta.

CHAPTER V
GENERAL DISCUSSION

The results of this thesis lead to three major conclusions regarding *Costaria*. Firstly, individuals of this genus are characterized by a high degree of phenotypic plasticity. This trait may not be dependent on general sequence divergence. Environmental cues may be the key stimuli in controlling the qualitative nature of morphological differentiation. Secondly, stands of *Costaria* appear to exist in outbreeding or genetically isolated groups with the resolution of populations possible due to the existence of restriction site polymorphisms within the IGS of rDNA. Genetic homogeneity within stands may result from a dependence on founder events, rapid homogenization of rDNA variants or selection. And thirdly, comparison of terrestrial and aquatic plants on the basis of small-subunit rRNA sequence has indicated that *Costaria* is more closely related to Chrysophytes and Oomycetes than to morphologically complex chlorophytes.

These results may be integrated to allow a more complete assessment of variation, dispersal and phylogeny in *Costaria*. The absence of resolvable DNA sequence variation in *Costaria* (but by probe pCc18), on the basis of random clone RFLP analysis and the comparison of small-subunit rDNA sequences, is not surprising considering the 5S RNA sequence data of Lim et al. (1986). These authors compared five species of brown algae,

each from a distinct order, of varying morphologies and life-histories, and found 96-99% sequence similarity among them. In the same study, seven species of red algae were found to display 57-81% sequence homology for the 5S gene. Lim et al. (1986) postulated that, due to the observed high degree of 5S RNA sequence conservation, the brown algal species had separated from each other a relatively short time ago (approx. 0.2 billion years) and that their relative evolutionary positions could not be reliably estimated from these data. On another level, the results of interfertility studies by Sanbonsuga and Neushul (1978) and Yabu (1964) suggest a similar hypothesis. The former authors were able to successfully cross morphologically distinct, float-bearing kelps such as *Macrocystis angustifolia* with *Pelagophycus porra* and *M. angustifolia* with *Nereocystis luetkeana*. Sanbonsuga and Neushul (1978) found that progeny of the *M. angustifolia* X *P. porra* cross grew more readily than progeny of the second cross and that they were intermediate in morphology relative to the parents. Hybrid plants (*M. angustifolia* X *P. porra*) were found rarely in nature and formed sori on blades but viable spores were not formed. Yabu (1964) found interfertility between species of *Laminaria* (*L. religiosa*, *L. japonica*, *L. ochotensis*). Kelps appear to be a relatively homogenous group of organisms, on the basis of the interfertility and molecular data, even though they display great phenotypic differentiation. The divergence of the laminarialean line (Order Laminariales, Families Lessoniaceae, Alariaceae and Laminariaceae (containing *Costaria*)) is

approximated to have occurred about 80-100 million years ago on the basis of chloroplast DNA sequence divergence data (Fain 1986).

The above findings also suggest that the phylogenetic position of *Costaria* described in this thesis may be generalized to all kelps. In the comparison of 5S RNA sequences of *Eisenia bicyclis* (Subclass Phaeophycidae) and *Sargassum fulvellum* (Subclass Cyclosporidae), for example, Lim et al. (1986) found 97% homology even though these species are taxonomically distinct on the basis of life-history traits, vegetative construction and flagellation (Scagel et al. 1982). Such a contention would, of course, require further analyses of a more taxonomically informative DNA sequence such as that of the small-subunit rDNA from diverging lines within the Phaeophyta as proof.

And finally, results of the population analysis suggest the means by which speciation may occur in kelps. Populations of *Costaria* were strikingly homogenous with respect to their rDNA variants. All other variants but Variant A, which represents a relatively widespread population, appear to be genetically isolated. These distinct populations must ultimately depend on inbreeding for survival due to the obligate nature of the life-cycle of *Costaria*. It is conceivable that given time and an unchanging oceanography such populations may allow the genetic diversification of genera such as *Costaria* ultimately leading to the creation of novel species.

Dependence of marine algae on inbreeding as the primary form of reproduction has been previously documented. Innes and Yarish (1984) used enzyme electrophoresis to study stands of the green alga *Enteromorpha linza* in Long Island Sound. Distribution of enzyme phenotypes within stands indicated a predominantly asexual mode of reproduction. No clear relationship was found between geographic and genetic distance for stands even though this species is believed to have an effective dispersal stage (Innes 1987). Malinowski (1974) found a similar result for another green alga (*Codium fragile*) in Long Island Sound. Of fourteen enzyme loci studied by electrophoresis in 500 individuals, ten were monomorphic and four were fixed heterozygotes. These data suggest that founder events may be important contributors to the genetic structure of algal populations though no causal relationship between mode of dispersal and observed distribution exists. Presently, it appears that until the relationship between oceanography and dispersal in marine seaweeds is better understood, molecular biological analyses of populations may best lead to the generation of hypothesis regarding their origin and present distribution.

In summary, the results of this investigation have provided some initial insights into the evolutionary relationships of *Costaria*, both at the subspecific level and at higher taxonomic groupings. I conclude that molecular biological analyses are valuable tools in studies such as those which I have attempted

since they may provide: 1. insights into genomic organization, 2. unambiguous markers with which to tag populations, and 3. methods of evolutionary comparison between widely divergent taxa.

CHAPTER VI

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APPENDIX A

C. costata	RAUCUGUUGAUCUGCCAGUAGUC	AUACGGUUGUCUCAAGAUAUAGCC	AUGCAUGUCUAAGUAUAGGCG--CUU	UAUACUGUGAAACUCGGGAUUGGCUC	99
O. danica	AAACCGUUGAUCUGCCAGUAG-C	AUACGGUUGUCUCAAGAUAUAGCC	AUGCAUGUCUAAGUAUAGGCGAAUCU	UAUACUGUGAAACUCGGGAUUGGCUC	98
A. bisexualis	AAACCGUUGAUCUGCCAGUAGUC	AUACGGUUGUCUCAAGAUAUAGCC	AUGCAUGUCUAAGUAUAGGCGAAUCU	UAUACUGUGAAACUCGGGAUUGGCUC	100
O. sativa	UAACCGUUGAUCUGCCAGUAGUC	AUACGGUUGUCUCAAGAUAUAGCC	AUGCAUGUCUAAGUAUAGGCGAAUCU	UAUACUGUGAAACUCGGGAUUGGCUC	100
G. max	UAUCUGUUGAUCUGCCAGUAGUC	AUACGGUUGUCUCAAGAUAUAGCC	AUGCAUGUCUAAGUAUAGGCGAAUCU	UAUACUGUGAAACUCGGGAUUGGCUC	100
C. reinhardtii	UAACUGUUGAUCUGCCAGUAGUC	AUACGGUUGUCUCAAGAUAUAGCC	AUGCAUGUCUAAGUAUAGGCGAAUCU	UAUACUGUGAAACUCGGGAUUGGCUC	99
P. micans	AUCUGGUUGAUCUGCCAGUAGUC	AUACGGUUGUCUCAAGAUAUAGCC	AUGCAUGUCUAAGUAUAGGCGAAUCU	UAUACUGUGAAACUCGGGAUUGGCUC	98
E. gracilis	AAUCUGGUUGAUCUGCCAGUAGUC	AUACGGUUGUCUCAAGAUAUAGCC	AUGCAUGUCUAAGUAUAGGCGAAUCU	UAUACUGUGAAACUCGGGAUUGGCUC	98
Mask	111111111111111111111111	111111111111111111111111	111111111111111111111111	111111111111111111111111	
C. costata	AUUAUAUCAGUUAUAUUUUUGA	AGUUCGC--	UAUACUAUCAUGUAUAACCGUAGUA	AUUCUAGAGCUUAUAUCUAGUGCA	179
O. danica	AUUAUAUCAGUUAUAUUUUUGA	AGUUCGC--	UAUACUAUCAUGUAUAACCGUAGUA	AUUCUAGAGCUUAUAUCUAGUGCA	176
A. bisexualis	AUUAUAUCAGUUAUAUUUUUGA	UAGUAC--	UAUACUAUCAUGUAUAACCGUAGUA	AUUCUAGAGCUUAUAUCUAGUGCA	179
O. sativa	AUUAUAUCAGUUAUAUUUUUGA	UGGUACG--	UAUACUAUCAUGUAUAACCGUAGUA	AUUCUAGAGCUUAUAUCUAGUGCA	178
G. max	AUUAUAUCAGUUAUAUUUUUGA	UGGUUAUC--	UAUACUAUCAUGUAUAACCGUAGUA	AUUCUAGAGCUUAUAUCUAGUGCA	178
C. reinhardtii	AUUAUAUCAGUUAUAUUUUUGA	UGGUUAUC--	UAUACUAUCAUGUAUAACCGUAGUA	AUUCUAGAGCUUAUAUCUAGUGCA	176
P. micans	AUUAUAUCAGUUAUAUUUUUGA	UGGUUAUC--	UAUACUAUCAUGUAUAACCGUAGUA	AUUCUAGAGCUUAUAUCUAGUGCA	176
E. gracilis	CUUACUACAGCAUCUACUCGUGA	UAGAGUGUGUCUGGUCACCUGCAA	GGACCCCAUU--GGACAUCACCACAAA	ACCUUGUGGUUAUAUCUAGUGCA	197
Mask	111111111111111111111111	111111111111111111111111	111111111111111111111111	111111111111111111111111	
C. costata	AGGCCCAACUGCU--	-----	-----	UCGGCGGACGGGUGCAUUG	212
O. danica	AUCCUGACUUA-----	-----	-----	-----	203
A. bisexualis	AUACCCAACUGC-----	-----	-----	UUUCUGGACGGGUAGCAUUU	211
O. sativa	AAACCCGACUUC-----	-----	-----	-----	206
G. max	AAACCCGACUUC-----	-----	-----	UGGAAGGGAUGCAUUU	206
C. reinhardtii	CAACCCGACUUC-----	-----	-----	-----	204
P. micans	AAACCCGACUUC-----	-----	-----	UGGAAGGGAUGCAUUU	206
E. gracilis	CCAGUCAGCAUGCAACACUGGCA	GSGAUCCUGUCUGCCGACAGUCCU	UCACGGUGGUGGCGGAUGUAUGCC	CAGCUGAUAUGGAAGACACGAGCGCCG	297
Mask	111111111111111111111111	-----	-----	111111111111111111111111	
C. costata	AUUAAGACC--GAAACCAUUGGCUU	CG-----	GAGUUUUUGAAUCAUA	AUCACU-UGCGGAUCGC--AC-GC	296
O. danica	AUUAAGUAGAA--ACCAUUGGGGCA	A-----	CCUUUUUUGGUAUUCUAUA	GUAAUUUU--CGGAUCGA-----U--C	287
A. bisexualis	AUUAAGAUU--GAAACCAUUGGCGUU	CGGUGGUAUUG-UGUUGAGUCAUA	AUAAACUGUGCGGAUCGC--	UU-----UUAGCGAUACAUAUUU	295
O. sativa	AUUAAGAUAAAAGGCGUGACGGGGCU	CCGC CGCUGAUCCGUAUUCUUG	AUAACUCGACGGAUCGC--ACGGC	CCU-CGUGCCGGCGACGCAUAUUC	302
G. max	AUUAAGAUAAAAGGCGUGACGGGGCU	CUGCCUUGUUGCUUUGUAUUCUUG	AUAAACUCGUGGGAUCGC--ACGGC	CUU-UGUGCCGGCGACGCAUAUUC	302
C. reinhardtii	AUUAAGAUAAAAGGCGUGACGGGGCU	CUGCCUUGUUGCUUUGUAUUCUUG	AUAAACUCGUGGGAUCGC--ACGGC	CUU-UGUGCCGGCGACGCAUAUUC	297
P. micans	AUUAAGAUAAAAGGCGUGACGGGGCU	CUGCCUUGUUGCUUUGUAUUCUUG	AUAAACUCGUGGGAUCGC--ACGGC	CUU-UGUGCCGGCGACGCAUAUUC	300
E. gracilis	CAAGCCAGUGUUGGCGUUGUG	ACUG-CUGUUUUGUGUGUAUCAUA	AUAACCAAGCAUUCGC--AUGGC	AUC-----CCUGCCGCAUAUCAUUC	300
Mask	111111111111111111111111	111111111111111111111111	111111111111111111111111	111111111111111111111111	

C. costata	UUGGGCGGUCCAUUCUGGGUAGC	GUGUUGUGGCAUAGGUUC-GG	--CUUCUUCGGCGGUGUUUG--	754
O. danica	UUCGGA--AUCAUCCUCGAGAGGA-	ACACGUCUUCAGUUGAUGGG	--CGUGGAUUUCUGCUUUUA--	737
A. bisexualis	UUGGGC--C-AUUUUUGAGGGAU	GUUUUCUGCCAUUCAGUUGU-GG	UUGAGUAGACUUGCAUCGUUA--	747
O. sativa	CUCG-A-CCUUC-UGC-CGGCGAU	GCGUCUUGGCUUAACUGCCGGG	UUUGUCC-UCGCGCGGUUA--	754
G. max	CUCG-U-CCUUC-UGC-CGGCGAU	GCGUCUUGUUCUUAACUGCCGGG	U-CGUGCC-UCGCGCGGUUA--	751
C. reinhardtii	CUCG-A-CCUUC-UGC-CGGGAC	GGUCUCUGGGUUCACUGUCUGG	ACUCGGAG-UC-GGGGAGUUA--	745
P. micans	UGG--CAUCUUCU-UG---GAGAAC	GUA-GCUGGCACUUGACUGU-GG	U--GCGGUACCCAGACUUUA--	746
E. gracilis	GGACCUUGUUCGACCCUGUGUUG	GCUGAACGGGUGACUCAACCCCC	AGUGGUACGUCCUGCGCCACCUC	894
Mask			UACUGCGAUGGUGAGUUCUGCUCCU	
			1111111111	
C. costata	---	---	---	754
O. danica	---	---	---	737
A. bisexualis	---	---	---	747
O. sativa	---	---	---	754
G. max	---	---	---	751
C. reinhardtii	---	---	---	745
P. micans	---	---	---	746
E. gracilis	GCCAAAGUCUGUUCACUGCAGGC	CAAAAGCGUUUAUCCUCCGGCACU	GGCAACGGACACCAACAGGGGACCC	994
Mask			AGCCUCGAGCUGGGUAGUACUCCUC	
			1111111111	
C. costata	---	---	---	819
O. danica	---	---	---	805
A. bisexualis	---	---	---	816
O. sativa	---	---	---	820
G. max	---	---	---	816
C. reinhardtii	---	---	---	811
P. micans	---	---	---	811
E. gracilis	UUGUCCACCACCGGACCCACCUC	UUCGACACCCUGGAAACUCAGUGU	GCUCAAAGCAUCCCGCCACGGCUG	1094
Mask			11111111111111111111111111	
			11111111111111111111111111	
C. costata	UAGGGCCACGACGGUCUUAUUUGU	GGUUU-GCACGUU----	---	856
O. danica	UAGGACCUUGU----	CUAUUUUUGU	GGUUU-G-ACUCC----	838
A. bisexualis	UACGACCUUGGUGUCUAUUUGU	GGUUU-GCACACC	---	853
O. sativa	UAGGAUCCCGUC----	CUAUAUUGU	GGCCUUCGGAU-	856
G. max	CAGGAUUCUGAUC----	CUAUAUUGU	GGCCUUCGGAU-	852
C. reinhardtii	UAGGACUCUGG-C----	CUA-UCUGU	GGUCUGGGACC----	845
P. micans	UAGGACCUUGU----	CUAUAUUUGU	GGUUUCUAGACU----	847
E. gracilis	CAUCGACCAAGUGGCAUUGAGU	UGUGCUGCCUGGGGCCACUCUGG	ACAACUGGUGUGUUCUUCGAG	1194
Mask			GAUCAACAGGAUCUGUUGCCUGCCU1194	
			11111111111111111111111111	

