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An investigation of genetic variation within northwest Atlantic Porphyra (Bangiales, Rhodophyta) with specific phylogeographic analysis of the common, rocky intertidal species, Porphyra umbilicalis

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AN INVESTIGATION OF GENETIC VARIATION WITHIN NORTHWEST ATLANTIC *PORPHYRA* (BANGIALES, RHODOPHYTA) WITH SPECIFIC PHYLOGEOGRAPHIC ANALYSIS OF THE COMMON, ROCKY INTERTIDAL SPECIES, *PORPHYRA UMBILICALIS*

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ABSTRACT

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by

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To investigate the phylogeography of the rocky intertidal red alga, *Porphyra umbilicalis* Kützing, a restriction fragment polymorphism assay (RFLP) of the ribulose bisphosphate carboxlase large subunit (*rbc*L) was developed to accurately distinguish *P. umbilicalis* from the other morphologically similar species in the North Atlantic. Initial screening of ~800 *Porphyra* specimens resulted in the additional discovery of a cryptic *Porphyra* taxon.

The presence and variability of group-I introns of the ribosomal small subunit (SSU) were screened in North Atlantic species of *Porphyra* in order to assess whether they could be biogeographically informative. In an initial screening for the helix 50 intron, using flanking primers with the Polymerase Chain Reaction, the intron was detected in some, but not all, individuals within populations and across species. The amplified intron also exhibited variable sizes between and within species. Sequence

analysis of the helix 50 introns revealed conserved blocks of nucleotides between introns of different species and highly variable regions that were species-specific. Additional screenings of the ribosomal small subunit (SSU) from a collection of Northwest Atlantic *Porphyra* were conducted for the presence of the helices 21 and 50 introns. However, instead of using two flanking primers, the second screening used an internal primer (located within either the helix 50 or helix 21 intron) and a nearby flanking primer in the SSU.; Using these primers the frequency of detecting the intron in individual algal samples increased significantly (>90%). Although phylogenetic analysis of the helix 50 intron in select Northwest Atlantic *Porphyra* are generally similar to previously reported SSU phylogenies, some differences in topology suggest that horizontal transmission of the intron between species may have occurred. In contrast to previous studies in which the helix 50 intron was detected only in fraction of the accessions, an intraspecific survey using combined external and internal primers detected the helix 50 intron in all 28 samples of *Porphyra umbilicalis* collected across the geographic range of the species. A survey of *P. umbilicalis* also revealed that the helix 50 introns were present in two different sizes (710 bp and 1188 bp). The sequence of the larger version of the helix 50 intron encodes a His-Cys open reading frame that has been associated with mobility of group-I introns in other organisms.

The Helix 50 group-I intron and internal transcribed spacer (ITS) regions of the SSU were investigated in order to reconstruct the biogeographic history *P. umbilicalis* since the last glacial maximum. Statistical parsimony was used to estimate gene genealogies at the population level. Based on the assumption that the last glacial maximum caused extinction of Northwest Atlantic *P. umbilicalis* populations, the ITS

variation patterns are in congruence with a postglacial recolonization event from European refugia. The group-I intron polymorphisms appear to confirm the ITS patterns, but a greater divergence between intron haplotypes indicate that the locus has a higher mutation rate than ITS, which increases its biogeographic resolution. A non-coding intergenic region between the mitochondrial cytochrome oxidase (*cox*) 2 and *cox*3 genes was also investigated but exhibited extremely low levels of intraspecific variation.

Finally, a hybrid capture method was used to isolate sequences containing dinucleotide repeats in a search for microsatellite markers for *Porphyra umbilicalis*. Sixteen clones were selected because they contained between 16 and 49 dinucleotide repeats with sufficient flanking sequence to design primers for PCR amplification of the locus. An initial screening of 16 primer pairs using six geographically distant *P. umbilicalis* isolates demonstrated that all microsatellite-containing loci isolated were monomorphic. However, several primer sets supported amplification of size variants in four related species (*P. linearis, P. purpurea, P. leucosticta,* and *P. amplissima*).

INTRODUCTION

Phylogeography, a sub-discipline of biogeography, is an area of study concerned with the processes and principles governing the geographic distributions of genealogical lineages, especially those within and among closely related species (Avise, 2000). This dissertation investigates genetic variation within the red algal genus *Porphyra* C. Agardh (Bangiophycidae, Rhodophyta), with emphasis upon the endemic North Atlantic taxon *Porphyra umbilicalis* (L.) Kützing. Overall, the goal of this study was to determine if contemporary geographic distributions and intraspecific genealogies could be used to track evolutionary footprints of this common North Atlantic red alga.

Porphyra or "nori" is one of the most extensively studied seaweeds due to its economic importance as a valuable food resource. Currently, it represents the most profitable marine algal aquaculture product, with nori being used for wrapping the near-ubiquitous sushi. In Japan, the nori market yields about 10 billions sheets of *Porphyra* annually with estimated annual sales of 1.8 billion U.S. dollars (Jensen, 1993). With increased production of nori in the Republic of Korea and China for "fish feed," *Porphyra* makes up the majority of the 5.5-6.0 billion dollar seaweed industry (Saga and Kitade, 2002; McHugh, 2003). In addition to its food use, *Porphyra* has been shown to be a good source of taurine (Noda *et al.*, 1975), which controls blood cholesterol levels (Tsujii *et al*, 1983). Additionally, *Porphyra* has become a commercial source of the red pigment *r*-phycoerythrin that is used as a fluorescent "tag" for DNA and microscopic evaluations.

Recently, Saga and Kitade (2002) suggested that *Porphyra* be designated a model organism for genetic studies in the Rhodophyta, given that several of its biological features are typical of model organisms: *Porphyra* has a short, annual life cycle; a small number of chromosomes (2-7; Cole, 1990); and a small genome size (approximately 2.7-5.3×10^8 bp; Kapraun *et al*, 1991; Le Gall *et al*, 1993). The genome size is similar to that of the model angiosperm *Arabidopsis thaliana* (L.) Heynh.

Porphyra is classified in the family Bangiaceae, which includes only one other genus, *Bangia*. Approximately 140 species of *Porphyra* have been described (Silva, 1999; Yoshida *et al.*, 1997), but recent taxonomic investigations (Brodie and Irvine, 1997; Broom *et al.*, 2002; Klein *et al.*, 2003; Kornmann and Sahling, 1991; Neefus *et al.*, 2002) suggest that this number is an underestimation of the diversity in the genus. Despite its economic value, relatively little is known about the population genetics, phylogeny, and biogeography of *Porphyra* and other bangialean red algae.

Description of *Porphyra umbilicalis*

As it is unclear whether Friedrich T. Kützing or Carl Linnaeus first described *Porphyra umbilicalis*, different authorships of this species have been given including: *Porphyra umbilicalis* Kützing, *P. umbilicalis* (L.) Kützing, and *P. umbilicalis* (L.) J. Agardh. Based on Linneaus's incorrect assignment of *P. umbilicalis* to the unrelated taxon, *Ulva umbilicalis* Linnaeus, Kützing appears to have first described *P. umbilicalis*, with his description of the taxon in the 1843 publication of *Phycologia generalis* (Silva, 1999, Neefus, pers. comm.).

The geographical distribution of *Porphyra umbilicalis* includes the western North Atlantic coast from Greenland, Hudson Bay, and Labrador to Virginia (Taylor, 1957; Zaneveld, 1972; South and Tittley, 1986; Bird and McLachlan, 1992; Sears, 2002), and along the eastern North Atlantic coast from Iceland and Norway south to the Mediterranean Sea (Børgesen, 1903; Bird and McLachlan, 1992; Brodie *et al*, 1998; Brodie and Irvine, 2003). *Porphyra umbilicalis* has also been identified in the Caribbean (Taylor, 1960), southeast Atlantic (John *et al.*, 1979), southwest Atlantic (Taylor, 1960), northwest Pacific (Perestenko, 1994), and northeast Pacific (Scagel, 1957). Based on recent assessments of *P. umbilicalis* collections by S. Lindstrom (pers. comm.) and misidentification of some samples that were collected for this dissertation it is assumed that the range of *P. umbilicalis* is limited to the North Atlantic. Thus, all putative *P. umbilicalis* samples collected outside of the North Atlantic should be closely scrutinized and re-evaluated for species identification.

Porphyra umbilicalis is found from the high supralittoral zone downwards to the mid-eulittoral zone. It appears throughout the year as an aseasonal annual (Conway, 1964, Kornmann and Sahling, 1991; Mathieson and Hehre, 1986). The macroscopic haploid phase consists of a monostromatic blade with dark olive-green coloration of variable gradations. The specific epithet *umbilicalis* is a Latin derivative of *umbilicatus*, meaning navel-shaped or having a small central depression or hollow. The thallus is typically rounded, has a central holdfast and a rubbery texture, and measures 30-85 μm, thick and as much as 35 cm long or wide (Bird and McLachlan, 1992; Sears, 2002; see Figure 1).

Although most *Porphyra* species are generally considered to have a bi-phasic life history alternating between a gametophytic blade and a microscopic, shell-boring sporophytic "conchocelis" phase (Mitman and van der Meer, 1994), it has been suggested that *P. umbilicalis* might exhibit both a bi-phasic (Figure 2a) and a "direct" life history (Figure 2b). In the direct life history, blades are produced immediately after carpospore germination (Kornmann and Sahling, 1991; Yarish, pers. comm). Adding to this uncertainty about its life history, *P. umbilicalis* has been considered dioecious, with separate male and female blades. However, Taylor (1957) described both monoecious and dioecious fronds. I believe most of the uncertainty that has occurred with respect to incongruities about the cytology, life history, ecology, and morphology of *P. umbilicalis* can be attributed to misidentification of this taxon. For example, *P. umbilicalis* has been confused with *P. birdiae*, *P.leucosticta* and *P. purpurea* in descriptions ofNorthwest Atlantic material (Bird and McLachlan, 1992, Neefus *et al.*, 2002; Klein *et al.*, 2003). Such problems usually occur when the thallus being studied is vegetative, while reproductive plants are more easily identifiable to species by their marginal "whitish" fringe, which distinguishes *P. umbilicalis* from *P. purpurea* and *P. birdiae.*

This dissertation consists of a series of related projects designed to evaluate the phylogeography of *Porphyra umbilicalis* in the North Atlantic. Each chapter is intended to stand alone, yet collectively they aim to enhance our understanding of the historical processes that may have led to the divergence of *P. umbilicalis* populations in the North Atlantic.

Chapter I describes a molecular assay that was developed based upon species-specific DNA markers in the chloroplast genome to assist in the sorting and identification

of North Atlantic *Porphyra*. The content of this chapter was previously published in the Journal of Applied Phycology, volume 14, 2002. The methodology was essential to the phylogeographic studies that are outlined in Chapters II and III of this dissertation.

Chapter II describes an investigation of the distribution and evolution of group-I introns in the nuclear ribosomal small subunit gene (SSU) of North Atlantic *Porphyra* species, with particular emphasis upon intraspecific variation in *P. umbilicalis*. Initial studies of group-I introns in *Porphyra* (Oliveira and Ragan, 1994, Oliveira *et al.*, 1995) suggested that their higher rate of variation, relative to SSU, might prove useful for population-level investigations. Thus, chapter II provides an important understanding of intron identification and variation within *Porphyra*. The above information was required before using group-I introns and the variable ribosomal internal transcribed spacer (ITS) sequences for subsequent phylogeographic analysis.

Chapter III addresses two main biogeographic goals: first, by analyzing samples that represented the documented range of *Porphyra umbilicalis*, a molecular approach was used to evaluate the species genetic cohesiveness between the northeast and northwest Atlantic. Second, molecular data were compared with spatial and temporal characteristics to see whether the phylogenetic history of this species is consistent with its current biogeography. Both nuclear (Group-I Intron, ITS) and mitochondrial genomes (cytochrome oxidase spacer) were used for these analyses, based on their use in previous algal population studies and because they are easily accessible molecular markers.

Finally, Chapter IV details the isolation of microsatellite loci from *Porphyra umbilicalis*. Large numbers of hypervariable microsatellite markers would provide more resolution for population genetic and phylogeographic studies as compared to nuclear and

mitochondrial genes used in Chapter III. In this chapter, a size-selective hybridization method was used to enrich and isolate sequences containing CA, GA, or TA dinucleotide repeats. Microsatellite sequences with adequate flanking sequence for primer development and a repeat motif of ≥ 16 were screened against six *P. umbilicalis* individuals and four other *Porphyra* species for polymorphism.

Figure.1. Photographic example of foliose haploid blade of *Porphyra umbilicalis*.

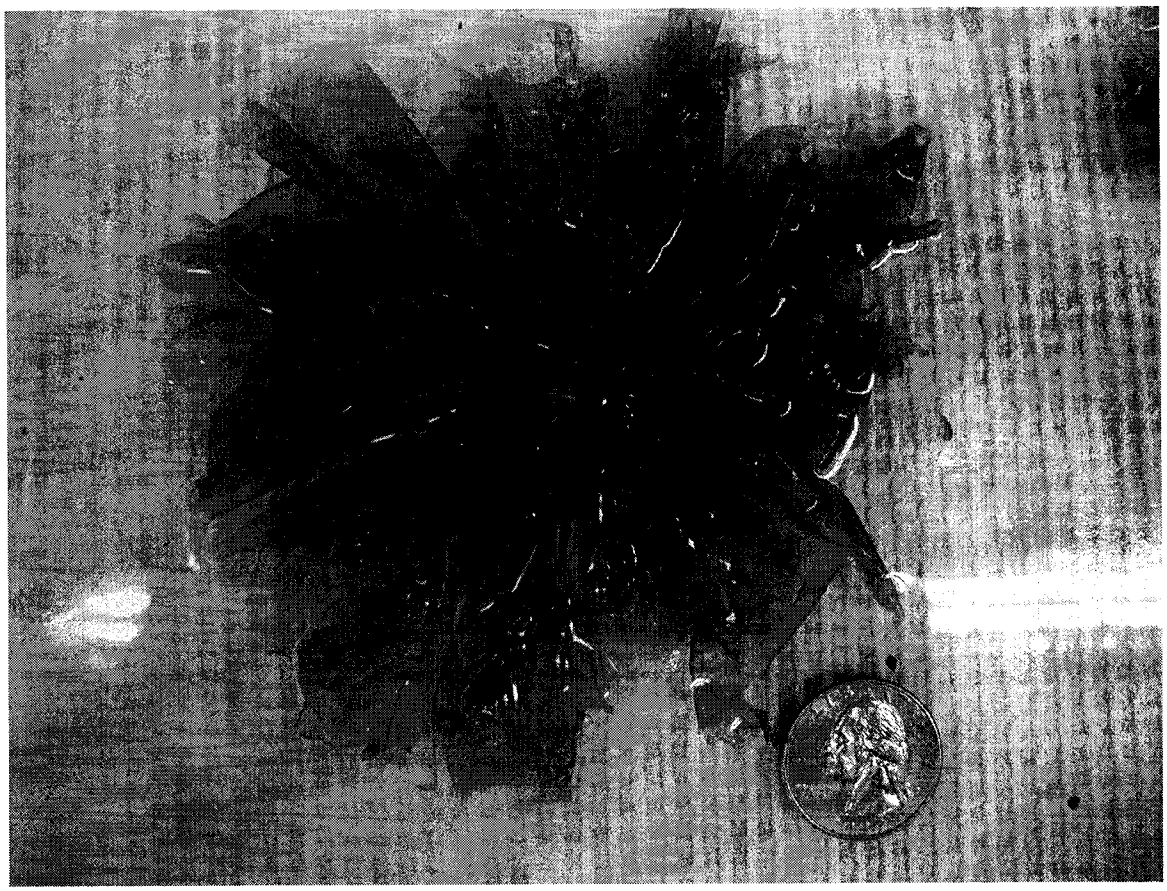
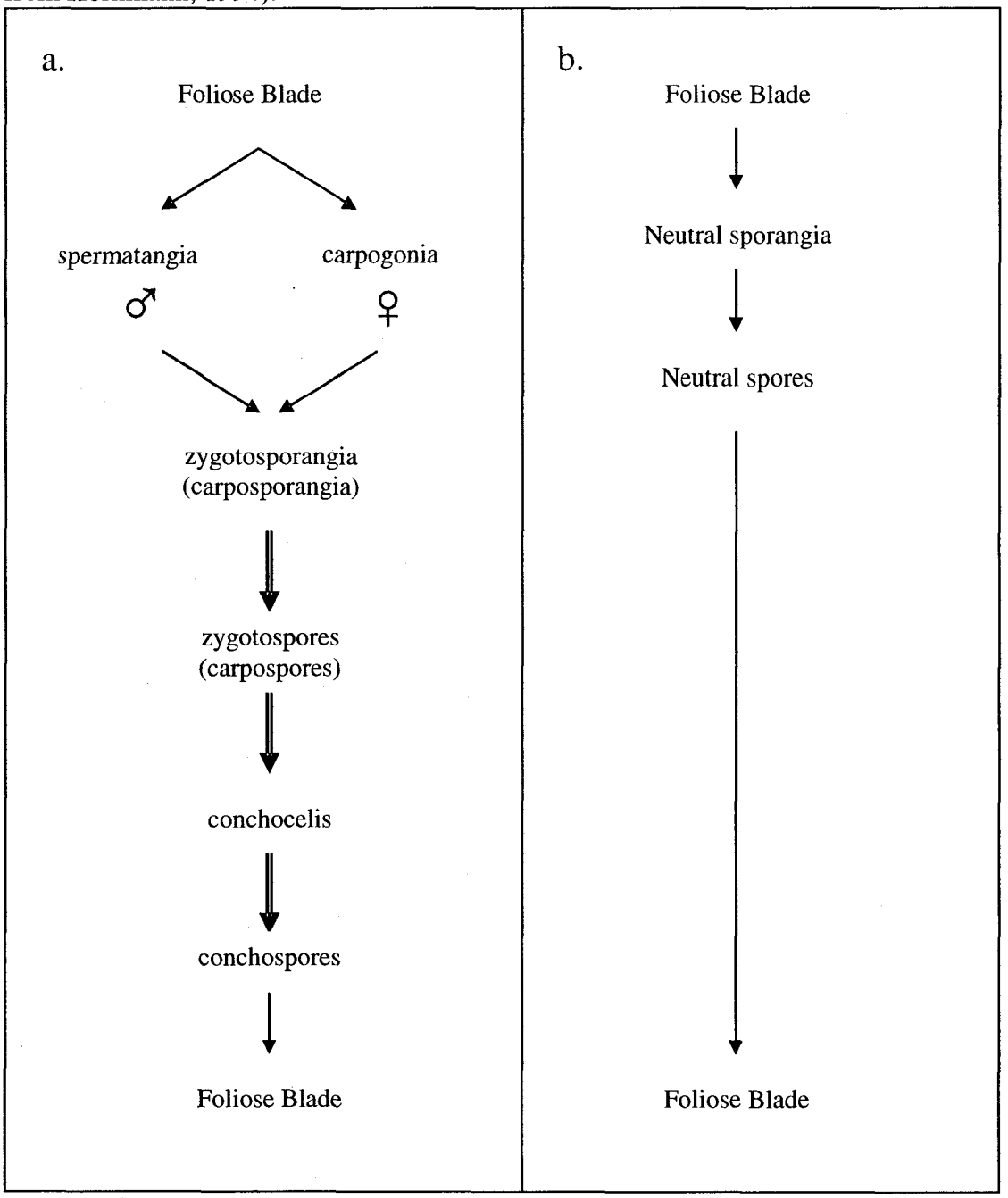


Figure 2. Schematics of two life-history types attributed to *Porphyra umbilicalis*. a. bi-phasic life history; b. direct life history. These two life histories correspond to Kornmann's (1994) Type 2 and Type 3 designations respectively. Single or double-lined arrows connect haploid or diploid parts of the life history, respectively. (Drawing adapted from Kornmann, 1994).



CHAPTER I.

A SIMPLE RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ASSAY TO DISCRIMINATE COMMON *PORPHYRA* (BANGIOPHYCEAE, RHODOPHYTA) TAXA FROM THE NORTHWEST ATLANTIC.[1]

Abstract

The identification of *Porphyra* species has historically been difficult because of the lack of distinguishing morphological and ecological characters. A restriction fragment length polymorphism (RFLP) assay was developed based on inter-specific sequence variation in the *ribulose bisphosphate carboxylase oxygenase large subunit* (*rbc*L) gene and *rbc*L-*rbc*S intergenic spacer, in order to provide a simple and effective tool for screening and sorting large collections of *Porphyra* from the Northwest Atlantic. A single restriction digest (*Hae* III) discriminates between multiple *Porphyra* species, including one cryptic taxon; an additional enzyme (*Hind* III) was necessary to distinguish between the closely related *P. leucosticta* and an introduced species *P. yezoensis.*

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[1] This chapter is revised from an article published in the *Journal of Applied Phycology* ©2002 Kluwer. Teasdale, B; West, A; Taylor, H. A.; Klein, A.S. A simple restriction fragment length polymorphism (RFLP) assay to discriminate common *Porphyra* (Bangiophycease, Rhodophyta) taxa from the Northwest Atlantic. Vol. 14, no. 4, pp.293-298.

Introduction

Species identification for the red algal genus *Porphyra* (Bangiophyceae, Rhodophyta) is difficult due to its simple morphology and lack of descriptive characters (Lindstrom and Cole, 1992). Although 133 species have been described for this genus, several recent accounts suggest that this is an underestimation (Yoshida *et al.*, 1997), particularly with *Porphyra* from the Northwest Atlantic (Bird and McLachlan, 1992).

Typically, the taxonomy of *Porphyra* has primarily relied upon morphological characters, including thallus size, shape, color, thallus thickness, cell dimensions, distribution of fertile tissues, and sequences of reproductive cell divisions. Although fertile Northwest Atlantic species can be recognized using these characters, vegetative specimens may be ambiguous and they are often misidentified. Such a morphology-based taxonomy has resulted in instances where both monoecious and dioecious fronds are attributed to the same species of *Porphyra* (Taylor 1957; Bird and McLachlan 1992) and different karyotypes have been recorded from a single taxon (Kapraun *et al.*, 1991; Lindstrom and Cole, 1992; Mitman 1992; Mitman and van der Meer, 1994; Wilkes *et al.*, 1999). The occurrence of such reproductive and cytological inconsistencies suggests the presence of significant variability within the taxon, either at an intra- or inter-specific level. Accordingly, Lindstrom and Cole (1993) recommend that at least one non-morphological diagnostic character should be used to verify the identification of individual specimens. DNA-based molecular markers are efficient and powerful tools for

providing the high-resolution diagnostic characters required for detailed taxonomic identifications.

The present study assessed the usefulness of a restriction fragment length polymorphism (RFLP) screen as a reliable and objective method for sorting vegetative and reproductive thalli of the genus *Porphyra* from the Northwest Atlantic. RFLPs have proven to be successful for this type of analysis in higher plants such as bamboo (Friar and Kochert, 1991), in fungi (Hibbett and Vilgalys, 1991) and more recently in delineating species of marine seaweeds (Goff and Coleman, 1988; Stiller and Waaland, 1993, 1996; González *et al.*, 1996; Candia *et al.*, 1999). The plastid-encoded gene, *ribulose bisphosphate carboxylase oxygenase* (*rbc*L) and the *rbc*L-*rbc*S intergenic spacer exhibit high levels of sequence divergence and they have been shown to be phylogenetically informative in the Rhodophyta at the family, genus, and species levels (Freshwater *et al.*, 1994, Brodie *et al.*, 1998, Müller *et al.*, 1998). In the present study we characterize RFLP patterns for *rbc*L and the *rbc*L-*rbc*S intergenic spacer in several common North Atlantic species of *Porphyra* including: *P. amplissima* (Kjell.) Setch. *Et* Hus *in* Hus, *P. suborbiculata* Kjellman, *P. linearis* Grev., *P. leucosticta* Thuret *in* Le Jol., *P. miniata* (C. Agardh) C. Agardh, *P. purpurea* (Roth) C. Agardh, and *P. umbilicalis* (L.) Kütz. In addition we describe the *rbc*L RFLP pattern for one cryptic Northwest Atlantic *Porphyra* taxon, *Porphyra* sp. Herring Cove, as well as one Asiatic species, *P. yezoensis* Ueda that was introduced to northern Maine for aquaculture in the mid 1990's, and an eastern North Atlantic species *P. dioica* J.Brodie *et* L.M.Irvine that can easily be confused with *P. purpurea.*

Most taxa examined in this study were originally described from European type material and there is uncertainty as to whether the names have been correctly applied in the Northwest Atlantic. Hence the accessions from this study are designated in quotes (e.g. *P. 'linearis'*) unless the sample has been compared by molecular means to type or epitype material (Brodie *et al.*, 1998).[2]

Materials and Methods

Taxa sampling.

All samples were obtained from attached individuals at ecologically different sites throughout New England and the Canadian Maritime Provinces (New Brunswick and Nova Scotia) with the exception of *Porphyra dioica* (Wales, United Kingdom). Site locations and corresponding herbarium accessions are described for each taxa in individual GenBank citations (Table 1). Provisional identifications to species were made based on morphology using a variety of taxonomic references: Bird and McLachlan (1992), Brodie and Irvine (1997), Coll and Cox (1977), Kornmann (1986, 1994), Kornmann and Sahling (1991), Schneider and Searles (1991), Sears (1998), and Taylor (1957). Additional information about the ecology and seasonal occurrence of different taxa helped in the initial sorting of field samples.

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[2] For convenience, the single quotes surrounding the specific epithet will be omitted in the remaining chapters.

Samples of tissue (0.1-.25 g) were ground in liquid nitrogen and genomic DNA was extracted using a standard CTAB method as modified in Stiller and Waaland (1993).

DNA amplification, sequencing and restriction site mapping.

A 1481 bp fragment, from position 67 (amino acid 23) of the large subunit of *rbc*L through the *rbc*L-*rbc*S intergenic spacer to the first codon of the small subunit, was amplified in a M. J. Research PTC-100 DNA Thermocycler (M. J. Research, Waltham, MA). Polymerase chain reactions (PCR) were performed in 50 μL volumes that contained 1-2 μL genomic DNA, 0.2 mM of each dNTP, 0.2 mM Mg^{2+}, 0.4 μL of Taq DNA polymerase ($5U \bullet \mu L^{-1}$, Promega, Madison, Wis.), and 1X Magnesium Free Reaction Buffer B (Promega) with 0.4 μM of the F67 and rbc-spc amplification primers (see below). The amplification profile began with an initial denaturation step of 93 °C for 3 min and was followed by 29 cycles of 30 sec at 93 °C, 1 min at 45 °C, and 1.5 min at 72 °C. The amplification concluded with a final extension at 72 °C for 10 min. For the amplification of the target sequence, a forward primer, F67 (5'-TACGCTAAAATGGGTTACTG) was developed from overlapping sequence of an earlier universal *rbc*L primer F57 outlined by Hommersand *et al.* (1994). Using the Lasergene™ suite of programs (DNASTAR Inc., Madison, WI), the reverse primer rbc-spc (5'-CACTATTCTATGCTCCTTATTKTTAT) was designed to selectively amplify *Porphyra* species (Table 2). PCR amplified products were sequenced with an ABI 373 Automated Sequencer, using standard procedures as outlined in Germano and Klein (1999). All sequences were submitted to the EMBL/GenBank Nucleotide Sequence database. Sequences were imported into Map Draw™ (DNASTAR Inc.) in order to

identify informative restriction sites for specific enzymes. Restriction digests using *Hae* III and *Hind* III were carried out according to the manufacturer's specifications. Twenty μL of PCR product were used in each 40 μL reaction. Fragments of all restriction digests were separated by electrophoresis on 2% agarose gels containing 1 μg/ml ethidium bromide. Both ΦX/*Hae*III marker (Promega) and uncut F67/rbc-spc PCR product (1481 bp fragment) were used as molecular weight standards to verify the size of the restriction fragments. All gels were visualized under UV light.

Results and Discussion

As *Porphyra* typically grows in association with a variety of other macroscopic and microscopic algae it is difficult to insure that contaminating organisms are removed prior to tissue extraction. To ensure that the PCR primers amplified the *rbc*L and *rbc*L-*rbc*S spacer from *Porphyra,* and not contaminating algal DNAs (where both *rbc*L and *rbc*S are encoded in a single transcriptional unit in the plastid), the rbc-spc reverse primer was designed. A sequence alignment of the *rbc*L-*rbc*S spacer from eleven *Porphyra taxa (P. 'amplissima', P. 'dioica', P. 'drachii'* Feldmann, *P. 'insolita'* Kornmann et Sahling, *P. 'leucosticta', P. 'linearis', P. 'miniata', P. 'pseudolinearis'* Ueda, *P. 'purpurea', P. 'umbilicalis', P. 'yezoensis'*), with three members of the Porphyridiales (*Galdieria palmate* Sentsova, *Cyanidium caldarium* (Tilden) Geitler, *Cyanidioschyzon merolae* De Luca, Taddei *et* Varano); the advanced red alga *Palmaria palmata* (L.) Kuntze (Palmariales); and a pennate diatom, *Cylindrotheca* sp. Rabenh. (Bacilariales) were used in the rbc-spc primer development (Table 2). A summary of the spacer sequence alignment and the reverse complement of the genus specific primer rbc-spc are given in

Table 2. A degenerate base was incorporated into the primer sequence to compensate for the variation between *Porphyra* taxa at position 59 in the spacer. Although highly specific for *Porphyra*, the F67 and rbc-spc primers should also amplify the target region in the filamentous red algae *Bangia*, a sister genus that is paraphyletic to *Porphyra* (Müller *et al.*, 1998). To increase amplification efficiency, the forward primer (F67) was developed as an alternative to the universal F57 primer described by Hommersand *et.al.* (1994). With all *Porphyra* templates tested to date, PCR amplification using the F67 and rbc-spc primers produced a single amplicon of ca. 1481 bp.

An initial version of the RFLP assay was based on predicted *Hae* III restriction sites of 1000 bp *rbc*L fragments. With the development of new amplification primers (F67, rbc-spc), the assay was modified to use the larger *rbc*L amplification product. The modification produced larger restriction fragments that were easier to resolve on conventional agarose gels. The *rbc*L sequences were extended to at least 1381 bp of the 1481bp PCR product in order to verify that the sizes of the observed restriction fragments corresponded to polymorphisms predicted by DNA sequence. Because template DNAs were not available for some of the original accessions, the *rbc*L sequences of some species were extended using additional individuals of the same species. Species identities were confirmed for each new algal sample by single pass sequencing over the common region between the new and old PCR products (Table 1).

Restriction enzymes were evaluated on the basis of their ability to discriminate between the *Porphyra* species of interest and the production of size fragments that could be easily resolved using standard agarose gel separation. The fragment sizes produced in each species from two restriction enzymes are summarized in Table 1. All *rbc*L PCR

products were initially screened with the *Hae* III enzyme where the sizes of the restriction fragments for each species were confirmed to those predicted by sequence analysis (Table 1, Figure 1). In order to distinguish all of the various taxa in this study, a second *Hind* III restriction digest was used to distinguish closely related taxa (i.e. *Porphyra 'leucosticta'* and *P. 'yezoensis'*).

The *rbc*L RFLP assay was used to screen *Porphyra* DNA templates from Northwest Atlantic samples that were initially identified morphologically. Of these samples, a collection of *Porphyra* samples identified as *P. 'umbilicalis'* from Herring Cove, Nova Scotia produced a unique *rbc*L *Hae* III restriction pattern as compared to other taxa (Table 1, Figure 1). The *rbc*L and *rbc*L-*rbc*S spacer from the Herring Cove sample was sequenced (GenBank Accession AF319460) and used to verify the fragment sizes from the *rbcL Hae* III digest. The sequence of *Porphyra* sp. Herring Cove was distinct when compared to all *Porphyra* taxa for which *rbcL* sequence was available on GenBank [BLASTN 2.2.1;(April 13, 2001); Altschul *et al.*, 1997]. Whether *Porphyra* sp. Herring Cove is a new species or is a new record of a species previously described in other geographical regions requires additional taxonomic comparisons and sequence information.[3]

Application of RFLPs for species-level comparisons has been employed in several studies of the Rhodophyta using both nuclear and plastid DNA. Goff and Coleman (1988) used total plastid DNA to demonstrate the effectiveness and utility of whole plastid genome RFLP patterns to distinguish red algal genera and species. However, the

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[3] *Porphyra* sp. *Herring Cove* has since been recognized as a new species, *Porphyra birdiae* (Neefus *et al.*, 2002).

separation of plastid DNA from nuclear DNA is a time-intensive and expensive process that will often include contaminating mitochondrial or plasmid DNA. Stiller and Waaland (1993) utilized RFLPs of the PCR amplified small subunit ribosomal RNA gene from fifteen *Porphyra* species to show how species-specific RFLP patterns were useful in phylogenetic analysis; these RFLP patterns later helped distinguish a new species, *Porphyra rediviva* (Stiller and Waaland, 1996). Recently, the internal transcribed spacer (ITS) of the nuclear ribosomal cistron has been utilized with RFLP analysis to delineate species within the Gracilariales (Goff *et al.*, 1994) and more specifically to distinguish between morphotypes of *Gracilaria chilensis* (Candia *et al.*, 1999).

I believe the high level of inter-specific sequence variation within the *rbc*L gene, combined with the specificity of restriction enzymes provide another important tool for distinguishing morphologically similar taxa. The *rbc*L restriction fragment patterns from this assay accurately identified all previously recorded Northwest Atlantic *Porphyra* taxa (Bird and McLachlan, 1992, Klein *et al.*, 2003), plus the Asiatic species *P. 'yezoensis'* and the European *P. 'dioica'* (Figure 1). Concurrent research (West, 2001) has shown how the use of this molecular screen using high throughput DNA extraction and PCR amplification method facilitates DNA identification methods in conjunction with field ecology studies. Thus, the *rbc*L RFLP assay allows reliable identification of immature or vegetative *Porphyra* specimens, plus cryptic species, resulting in a more accurate assessment of ecological data.

Table 1. Restriction size fragments (in base pairs) for *Hae* III and *Hind* III enzymes[1]

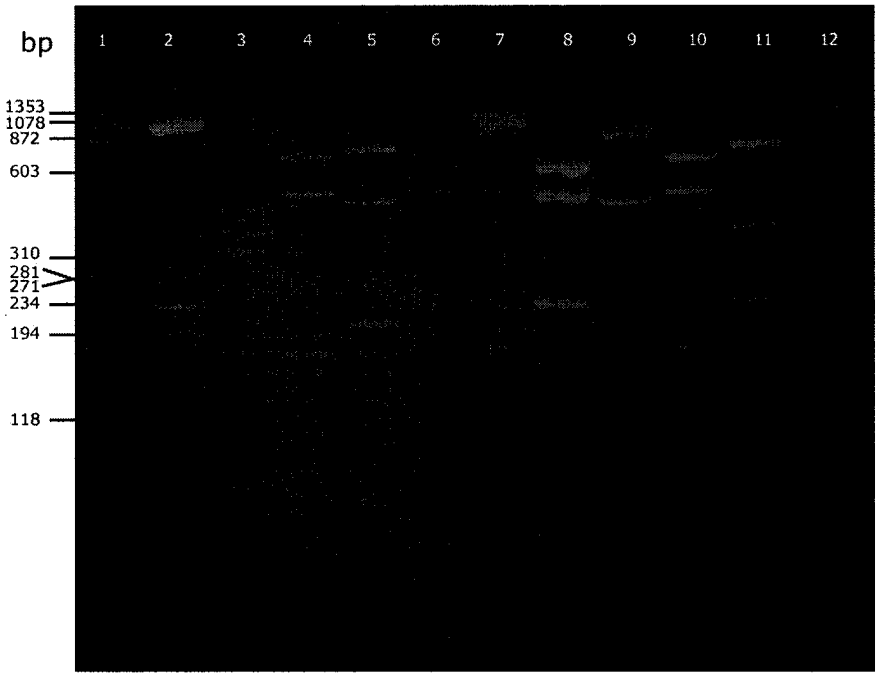
Taxon	GenBank accession numbers	*Hae* III					*Hind* III	
Porphyra 'amplissima'	AF021034	237	1244				1481	
Porphyra suborbiculata	AF078743, AF414593, AF414594	237	533	711			194	1287
Porphyra 'dioica'	AF081291	99	216	337	382	447	101	1380
Porphyra 'leucosticta'	AF078744, AF414597, AF414598	58	179	521	723		1481	
Porphyra 'linearis'	AF078745, AF414597, AF414598	216	482	783			101	1380
Porphyra 'miniata'	AF021033, AF414599, AF414600	237	521	723			626	855
Porphyra 'purpurea'	AY028536, AF414603, AF414604	179	1302				101	1380
Porphyra 'umbilicalis'	AF078747, AF414601, AF414602	482	999				101	1380
Porphyra 'yezoensis'	AF021032	58	179	521	723		538	943
Porphyra sp. *Herring Cove*	AF319460	237	395	849			1481	

[1]Predicted from the combined sequences.

Table 2. The rbc-spc primer location across different algal divisions. Shaded areas represent differences from the consensus sequence. The underlined bases of the primer correspond to the first codon of the *rbc*S gene.

Species	GenBank#	
Porphyra 'umbilicalis'	AJ010782	CTTA..TAAAAAT..AAGGAGCATA.GAATAGTG
Porphyra 'yezoensis'	AJ010783	CTTA..TAACAAT..AAGGAGCATA.GAATAGTG
Porphyra 'leucosticta'	AJ010789	CTTA..TAACAAT..AAGGAGCATA.GAATAGTG
Porphyra 'miniata'	AJ010786	CTTA..TAACAAT..AAGGAGCACA.GAATAGTG
Porphyra 'linearis'	AJ010781	CTTA..TAAAAAT..AAGGAGCATA.GAATAGTG
Porphyra 'amplissima'	AJ010780	CTTA..TAAAAAT..AAGGAGCATA.GAATAGTG
Porphyra 'purpurea'	AJ010776	CTTA..TAAAAAT..AAGGAGCATA.GAATAGTG
Porphyra 'drachii'	AJ010788	CTTA..TAACAAT..AAGGAGCATA.GAATAGTG
Porphyra 'pseudolinearis'	AJ010787	CTTA..TAACAAT..AAGGAGCATA.GAATAGTG
Porphyra 'insolita'	AJ010778	CTTA..TAACAAT..AAGGAGCATA.GAATAGTG
Porphyra 'dioica'	AJ010779	CTTA..TAAAAAT..AAGGAGCATA.GAATAGTG
Galdieria palmate	AB018008	TTTGT..ATAACCAAAGGAGTATA.AAACAGTG
Cyanidium caldarium	Z21723	TTT..CTAA.....AAAGGAGGATCTCAA..GTG
Cyanidioschyzon merolae	D63675	CGTATAAAACTATCAGAGTCAAACTATGAGAGTG
Cylindrotheca sp.	M59080	CTTTTAAAAAAATTAAAGGAGTATTTGAATAGTG
Palmaria palmata	U28421	CTTGCCTAATCATTAAAGGAGTATA.GAATAGTG
***RBC*-SPC PRIMER**		
COMPLEMENT		A..TAAMAAT..AAGGAGCATA.GAATAGTG
		5' ⟶ 3'

Figure 1. RFLP patterns of the *Porphyra* rbcL gene and rbcL-rbcS spacer (1481 bp) using the Hae III restriction enzyme. Lane 1= standard of ΦX174 DNA cut with *Hae* III (ordinate numbers indicate DNA size). Lane 2= *Porphyra 'amplissima'*; 3= *P. 'dioica'*; 4= *P. 'leucosticta'*; 5= *P. 'linearis'*; 6= *P. 'miniata'*; 7= P. *'purpurea'*; 8= *P. 'suborbiculata'*; 9= *P. 'umbilicalis'*; 10= *P. 'yezoensis'*; 11. cryptic *Porphyra* taxa from Herring Cove,Nova Scotia, 12. uncut DNA. The diffuse band at the end of each lane represents excess primer.



CHAPTER II

DISTRIBUTION AND EVOLUTION OF VARIABLE GROUP I INTRONS IN THE SMALL RIBOSOMAL SUBUNIT OF NORTH ATLANTIC *PORPHYRA*.

Abstract

Several studies of the red algal order Bangiales have identified putative group-I introns at helices 21 and 50 of the nuclear ribosomal small subunit (SSU). To examine the utility of these introns for biogeographic studies in the red alga *Porphyra,* several populations of Northwest Atlantic species were screened for the presence of group-I introns in the SSU rDNA. In an initial screening for the helix 50 intron, using flanking primers with the Polymerase Chain Reaction, the intron was detected in some but not all individuals within populations and across species. The amplified intron also exhibited variable sizes between and within species. Sequence analysis of the helix 50 introns revealed conserved blocks of nucleotides between introns of different species and highly variable regions that were species-specific. Additional screenings of *Porphyra* SSUs were conducted for the presence of the helices 21 and 50 introns. Instead of two flanking primers, an internal primer (located within either the helix 50 or the helix 21 intron) and a nearby flanking primer in the SSU were used for PCR amplification. In these second screenings, the frequency of detecting the intron in individual algal samples increased significantly (>90%). Although phylogenetic analyses of the helix 50 intron in select Northwest Atlantic *Porphyra* are generally similar to previously reported SSU

phylogenies, some differences in topology suggest that horizontal transmission of the intron between species may have occurred. In contrast to several previous studies, an intraspecific survey using combined external and internal primers detected the helix 50 intron in all 28 specimens of *Porphyra umbilicalis* collected across the geographic range of the species. The survey also revealed that the helix 50 introns were present in two different sizes (710 bp and 1188 bp). The sequence of the larger version of the helix 50 intron encodes a His-Cys open reading frame that has been associated with mobility of group-I introns in other organisms.

Introduction

Porphyra (C. Agardh) is a red algal genus within the subclass Bangiophycidae. The genus includes several economically important species that are used extensively as a food source for humans. *Porphyra* has a wide geographic range that includes most cold temperate marine waters in both the Northern and Southern hemispheres (Yoshida *et al.*, 1997). Because of a limited range of morphological variability, the systematic relationships of *Porphyra* species are still unclear. The genus, as currently circumscribed, consists of 140 species (Silva, 1999; Yoshida *et al.*, 1997), with only two examples of subgenera classification (*i.e., Displastidia* and *Diploderma*; Krishnamurthy, 1972; Kurogi, 1972). *Porphyra* has also been shown to be paraphyletic with the genus *Bangia (*Müller *et al.*, 1998), suggesting the need for an overall re-evaluation of the phylogeny in the Bangiophycidae.

Recent molecular systematic studies using the nuclear ribosomal small subunit (SSU) and the chloroplasts ribulose bisphosphate carboxylase (*rbc*L) genes have been used to clarify phylogenetic relationships for many *Porphyra* species (Oliveira *et al.*, 1995; Kunimoto *et al.* 1999; Broom *et al.* 2002; Klein *et al.*, 2003; Lindstrom and Fredericq, 2003). Additional studies have examined more variable regions of DNA, thereby providing useful information regarding the phylogenetic relationships between closely related species. In previous phylogenetic studies of *Porphyra* the nuclear internal transcribed spacer (ITS) and/or ribosomal introns have been utilized (Kunimoto *et al.*, 1999; Müller *et al.*, 2001; Broom *et al.*, 2002). Introns represent variable noncoding regions that are flanked by conserved coding regions. A recent study of volvocine green

algae estimated an intron substitution rate of approximately 3 X 10^{-8} per bp position per year, which is about 10-fold higher than the synonymous substitution rate in protein-coding regions and about 150-fold higher than in the more conserved rRNA genes (Liss *et al.*, 1997). Thus, introns may provide useful information about intraspecific phylogenetic relationships.

Using primers designed to anneal to the conserved coding regions, polymerase chain reaction (PCR) amplification can be directed across the more variable intron regions (Slade *et al.*, 1993). The method has been called Exon Primed Intron Crossing PCR or EPIC-PCR (Palumbi and Baker, 1994) and has been the primary method employed for intron detection and amplification.

Ribosomal RNA genes, which are found in all eukaryotes, are present in multi-copy repeats and localized in one or more chromosomal regions known as the nucleolar organizer (Long and Dawid, 1980). Each tandem repeat is composed of an 18S (SSU), 5.8S, 2S, and 28S (LSU) rRNA gene organized as a single transcription unit. The "rDNA" loci are subject to concerted evolution, a process that preserves sequence homogeneity within the array, but allows the sequence of the entire array to change over time (Arnheim, 1983). Concerted evolution in the rDNA locus is believed to be driven by the recombinational mechanism of unequal crossing over and gene conversion (Szostak and Wu, 1980; Coen *et al.*, 1982; Dover *et al.*, 1982; Dvôrak *et al.*, 1987). When concerted evolution occurs, the actual mutation rate of the multi-copy locus would be masked by this homogenizing mechanism. Additionally, some organisms exhibit incomplete homogenization of the repeats at the intra- and inter- individual levels. Factors that can interfere with the homogenization process include polyploidy,

hybridization, asexual reproduction, and the presence of the ribosomal cistrons on more than one chromosome. When the rDNA occurs on different chromosomes, as multiple nucleolar organizers, the rates of concerted evolution across non-homologous chromosomes are much slower and lead to more intra-individual heterogeneity. All of these factors have been documented in a diverse array of organisms (Bobbola *et al.*, 1992; Sang *et al.*, 1995; Wendel *et al.*, 1995), as well as in algal species (Serrão *et al.*, 1999; Famà *et al.*, 2000). Examples of incomplete homogenization of the ribosomal repeats, including the organization of the ribosomal repeats in one or more nucleolar organizers have yet to be established in *Porphyra.*

The red algal order Bangiales contains introns in the nuclear-encoded small subunit ribosomal RNA (SSU) genes (Oliveira and Ragan, 1994; Oliveira *et al.*, 1995; Müller *et al.*, 1998, 2001). Self-splicing RNAs (also known as ribozymes or Group-I introns) have been extensively studied in diverse organisms, including eubacteria, fungi, plants, and protists (Belfort, 1991; Damberger and Gutell, 1994; Gargas *et al.*, 1995; Hibbett, 1996; Yamada *et al.*, 1994). Although group-I introns have been found in different genes (De Wachter *et al.* 1992; Gargas *et al.* 1995), the majority of more than 1000 group-I introns interrupt the SSU rRNA gene of protists and fungi at common sites within conserved sequence regions (Haugen *et al.*, 2003)[4]. The two most frequently found intron insertion sites of the SSU gene are at base pairs 516 and 1506 [according to SSU rDNA gene sequence numbering of *Escherichia coli* (Migula).Castellani and Chalmers], which also correspond to the predicted secondary structure of *E. coli* at helices 21 and 50. Group-I introns are characterized by a conserved primary and

secondary RNA structure (Kruger *et al.*, 1982; Cech, 1990), which has allowed further classification of these introns into sub-groups (Michel and Westhof, 1990; Suh *et al.*, 1999).

The secondary structure of group-I introns has primarily been investigated in the ciliated protozoa *Tetrahymena thermophila* Nanney and McCoy, which contains a group-I intron in the large subunit (LSU) of its rRNA (Michel *et al.*, 1982; Michel and Westhof, 1990; Cech *et al.*, 1994). Figure 1 illustrates the secondary structure of a "typical" group-I intron. All group-I introns are characterized by nine base-paired regions (P1-P9) along with four (P, Q, R, S) highly conserved internal elements (Burke, 1988; Burke *et al.*, 1987). The sub-groups are based on conserved similarities, which are usually characterized by additional sequences or secondary structures. Müller *et al.* (2001) characterized the bangialean ribosomal introns as belonging to the IC1 subgroup, the most common type of group-I introns. Sub-group IC1 introns are characterized by a lack of stems between the P7 and P3' regions, the presence of a composite P9 region, a sequence conservation at the edge of P2.1, P3, and P8, and a segment connecting P7 and P8 stems that starts with an extra U (Myllys *et al.*, 1999). Since group-I introns are not found in all organisms, considerable research has been conducted to understand the phylogenetic history and inheritance patterns of these introns.

The origin and evolution of group-I introns in nuclear ribosomal rDNA genes has been widely studied. The sporadic and broad distribution of group-I introns in the small and large subunits of the rDNA repeats and organelle genes of a wide variety of organisms, including *Porphyra,* suggests that intron sequences are highly successful at

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[4] The higher incidence of group-I introns in rDNA genes may be biased towards the popular use of rDNA

invading and maintaining themselves in eukaryotic genomes (Battacharya *et al.*, 1994, 1996a; Battacharya 1998, Friedl *et al.*, 2000). A study by Battacharya *et al.* (2001) showed through a diverse phylogeny of eukaryotic group-IC1 introns that the origin of a helix 21 intron in bangiophyte red algae and a brown alga (*Aureoumbra lagunensis* Stockwell, DeYoe, Hargraves *et* Johnson) were specifically related. They concluded that it is unlikely that the group-IC1 introns in the SSU were vertically inherited from a common ancestor of the red algae and brown algae because these algal lineages do not share a close evolutionary relationship (Battacharya *et al.*, 2001). Further, their analyses suggested that lateral transfer may establish new intron lineages in different organisms and that, over time, these sequences may evolve into distinct secondary structure variants. Battacharya *et al.* (2001) concluded that more detailed analysis of group-I introns will provide valuable comparative data to elucidate intron transfer mechanisms and to understand group-I intron evolutionary history.

Several studies of group-I introns in the SSU rDNA of algae have been conducted with the following taxa: the brown alga *Aureoumbra lagunensis* (Bhattacharya *et al.*, 2001); the green algae *Ankistrodesmus stipitatus* (Chodat) Komárková-Legnerová (Davila-Aponte *et al.*, 1991), *Characium saccatum* Filarsky, *Dunaliella parva* Lerche, *D. salina* (Dunal) Teodoresco (Wilcox *et al.*, 1992), *Chlorella ellipsoidea* Gerneck (Huss, Seidel, and Kessler, unpublished), and *Scenedesmus pupukensis* (Haugen *et al.*, 1999), including numerous members of the Zygnematales (Bhattacharya *et al.*, 1994, 1996b). In the red algae (Rhodophyta), the Bangiales are the only order besides the Florideophycidae taxon *Hildenbrandia rubra* (Sommerfelt) Meneghini in which the

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sequences in phylogenetic analysis.

presence of SSU group-I introns has been documented (Ragan *et al.*, 1993). Oliveira and Ragan (1994) described the occurrence of a group-IC1 intron in the 18S ribosomal RNA genes at helix 50 of *Porphyra spiralis* var. *amplifolia* Oliveira *et* Coll that differed in size based on three distinct populations. Both a flanking primer set and a combination of external and internal (intron) primer sets were used to amplify the intron and determine its size. Oliveira and Ragan (1994) also showed that by manipulating amplification conditions, the group-I intron insertion was detected less frequently and the occurrence of a minor "intronless" band could be seen; they further suggested that this was due to differing levels or "copies" of the intron in the SSU rDNA repeats of some individuals.

Using EPIC PCR, Kunimoto *et al.* (1999) detected a helix 50 group-I intron during their analysis of the 18S rRNA gene in wild and cultured strains of *Porphyra yezoensis* Ueda. However, only seven of the fifteen specimens sampled showed an intron. Of those introns detected, there were some instances in which the intron sequences were identical to other individuals at the same collection site (*i.e.*, collection sites at Nanaehama and Shinori), while in others they were different (*i.e.*, Ogatsu site) based on variable numbers of base substitutions and insertions/deletions. Kunimoto *et al.* (1999) also found intron size variants between individuals of the same taxon, much like the size variants described in *P. spiralis* var. *amplifolia* (Oliviera and Ragan, 1994). Kunimoto et al. (1999) suggested that the size variants might be useful for delineating strains of *P. yezoensis*. However, the low-level detection rate of the intron (7/15 of helix 50) implied a limited utility of introns for strain identification.

Broom *et al.* (2002) amplified the helix 21 and helix 50 introns from *Porphyra suborbiculata* Kjellman using EPIC primers. The helix 21 intron was present in all eight

individuals examined, whereas the helix 50 intron was not detected in four samples from Mexico and Japan; they also found a size variant of the helix 21 intron in one of their populations. Broom *et al.* (2002) also observed intraspecific sequence variation in the helix 50 intron (nine gaps and five nucleotide substitutions); this was less sequence variation than that found by Kunimoto *et al.* (1999) in isolates of *P. yezoensis*[5] (16 gaps and eight substitutions).

Müller *et al.* (2001) surveyed the helices 21 and 50 group-ICI introns in a large number of species of the Bangiales to evaluate their phylogenetic utility and use for biogeographic studies; EPIC primers were used to amplify both introns, which were subsequently sequenced for phylogenetic analysis. The results of their survey showed that certain individuals lacked either one or both of the two group-I introns and that when detected there were size variants at both inter- and intraspecific levels. The interspecific sequence variation between taxa for both introns ranged from 0-44.3%. Based on the intron and rRNA phylogenies of the same accessions, Müller *et al.* (2001) hypothesized that the ribosomal group-I introns were vertically inherited and frequently lost in recently radiated taxa. In contrast to the intraspecific or biogeographic utility of the rDNA introns proposed by both Oliveira and Ragan (1994) and Kunimoto *et al.* (1999), Müller *et al.* (2001) concluded that they were not useful for biogeographic analysis between or within species of *Porphyra* or *Bangia*.

Hibbett (1996) examined the utility of internal primers when amplifying group-I introns in members of the homobasidiomycetes (mushroom forming fungi). While developing a PCR assay for intron detection, he found that by using a combination of

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[5] Unequal sample sizes between the two studies may have had an effect on the number of difference

internal intron primers and flanking primers in the SSU, introns that would otherwise go undetected by EPIC PCR were amplified. Hibbett (1996) described two instances of false negative results in his assay of *Lentinellus ursinus* (Fries) R. Kühner and *L. montanus* O. K. Miller. He attributed the failure of the intron amplification (using EPIC primers) to low copy numbers of intron-containing rDNA repeats and an artifact of PCR called template preference. Artifacts such as short-allele dominace[6] have been well-documented in studies of microsatellite DNAs, specifically in the red alga *Gracilaria gracilis* Steentoft, L.M. Irvine and Farnham (Wattier, *et al.*, 1998). Hibbett (1996) concluded that for any study focusing on the distribution of introns, it is essential to use intron-specific PCR primers to assess their presence or absence, especially for related taxa that are known to contain the intron.

The present study builds on earlier work of North Atlantic *Porphyra* population genetics (Klein *et al.*, 2003) by surveying a large collection of *Porphyra* for the presence of group-I introns in the SSU and characterizing their size, structure, and utility for phylogeographic studies.[7] Because Hibbett (1996) showed that rDNA primers may fail to reveal intron-containing rDNA repeats when they are present at low copy number, both flanking (EPIC) and internal "intron-specific" PCR primers were developed and used to screen for the helix 50 (*Escherichia coli* numbering position 1506; *cf.* Müller *et al.*, 2001) 18S nuclear ribosomal intron that have previously been characterized in *Porphyra*. An additional amplification of the helix 21 intron (*Escherichia coli* numbering position 516;

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detected.

[6] Sefc *et al.* (2003) found: 1) PCR fragment size had a strong influence on microsatellite amplification and 2) PCR primers designed to amplify smaller fragments had a higher chance of success and repeatability.

[7] An initial survey of group-I introns was carried out between 1998-1999 by Andrew West, a M.S. student in Genetics at the University of New Hampshire.

cf. Müller *et al.*, 2001) was performed using both a flanking and internal (intron) primer to screen for intron presence and the usefulness of the internal primer for intron detection.

This paper describes a survey of ribosomal introns among several North Atlantic *Porphyra* taxa and the Asiatic species *P. yezoensis*; it also shows how the application of intron-specific primers enhances the detection of the introns within the SSU ribosomal gene. Additionally, helix 50 group-I introns were sequenced from representative species to evaluate their phylogenetic value. A widespread biogeographical sampling was done in one species, *Porphyra umbilicalis* Kützing, in order to examine the intraspecific variation within the intron and to determine whether variation within the helix 50 intron would be useful for phylogeographic studies.

Materials and Methods

Blades of *Porphyra* were collected[8] from various locations (Appendix A) along the coast of New England and the Canadian Maritime Provinces. All samples were visually identified to species following methods detailed in Klein *et al.* (2003) and morphological identifications were confirmed using molecular methods, including direct sequencing, species-specific PCR, or an RFLP assay using the chloroplast *ribulose bisphosphate carboxylase* (*rbc*L) gene (Chapter 1).

DNA extraction and amplification, plus sequencing of introns

Individual blades of *Porphyra* were ground separately in liquid nitrogen and total DNA was extracted in one of two different ways. In most cases, a modified

cetyltrimethlammonium bromide (CTAB) method (Stiller and Waaland, 1993) was employed. Unless otherwise specified, all reagents and steps were at room temperature, buffers were at pH 8.0, and centrifugation was at 16,000 X *g* in a microcentrifuge. That is, 0.1-0.25g of frozen tissue was ground with liquid N_2 in a 1.5 ml Eppendorf tube using a micropestle. A CTAB isolation buffer (0.75 ml; 2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl, 0.2% β mercaptoethanol) was added to the tube, mixed and incubated at 60° C for 30 minutes. The DNA was then extracted with a 25:24:1 phenol, chloroform, isoamyl alcohol solution and centrifuged for 10 minutes. The aqueous phase was then mixed with 24:1 chloroform, isoamyl alcohol, and again centrifuged for 10 minutes. The aqueous phase was then transferred to a fresh tube containing an equal volume of isopropanol, mixed, incubated for 30 minutes, and centrifuged for 5 minutes. The resulting pellet was carefully drained, washed with 1 ml 76% ethanol, 10 mM ammonium acetate, and resuspended in 50 µl of 10 mM Tris/1 mM EDTA. A second DNA extraction method was performed using the Puregene® DNA Isolation Kit for plant tissue (#D5500A; Gentra Systems, Minneapolis, MN) to extract all *P. umbilicalis* samples (see Chapter III; Table 1) used in the intraspecific analysis.

Purified DNA samples (5-10 ng/µl) were either aliquoted into 96 well microtiter plates for high throughput analysis or individually in 0.2 µL thin-walled tubes for polymerase chain reaction (PCR). High throughput screening was obtained by transferring 4 µL of template DNA to separate 96 well plates and performing PCR using primers specific for the locus to be amplified (SSU group-I introns or the *rbc*L gene).

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[8] Except for the *Porphyra umbilicalis* intraspecific analysis, all samples for this study were collected by Dr. Arthur Mathieson and Dr. Chris Neefus of the University of New Hampshire, as well as Dr. Charles Yarish of the University of Connecticut.

Samples were screened for SSU introns using either a flanking (*porint1; porint2*) set of primers or a combined flanking and internal primer (*porint5; porint6*) set (Table 1; Figure 2). Reagents for each 50μL reaction contained 1X Magnesium Free Reaction Buffer B (Promega, Madison WI), 0.2 mM each dNTP (Promega), 0.5 units of *Taq* DNA polymerase (Promega), 0.4 μM each primer, and variable $MgCl_2$ concentrations (depending on primer set) given in Table 2. All amplifications were carried out in an MJ Research PTC-100 Programmable Thermal Controller (Watertown MA) with amplification profiles for each primer set given in Table 2. PCR products were visualized using gel electrophoresis. Flanking primers for the helix 50 intron were designed in Primer Select (Lasergene™, DNASTAR, Madison, WI) from approximately 950 bp of SSU rDNA sequence previously generated from *Porphyra leucosticta* Thuret in Le Jol., *P. umbilicalis, P. linearis* Grev., *P. dioica* J. Brodie et L. Irvine, *P. suborbiculata* Kjellman, *P. miniata* (C. Agardh) C. Agardh, *P. purpurea* (Roth) C. Agardh (Table 3; Klein *et al.*, 2003), and *P. spiralis* var. *amplifolia* Oliveira and Coll (GenBank Accessions L26175-L26177; Oliveira and Ragan, 1994).

The sequences of the helix 50 intron for individual accessions of *Porphyra linearis, P. leucosticta, P. miniata, P. suborbiculata, P. purpurea* and *P. dioica,* plus 28 different geographic isolates of *P. umbilicalis* were determined. The collection site of each sample, representative herbarium specimen (if available) and the corresponding GenBank Accession information are given in Table 3, except for the *P. umbilicalis* samples that are given in Table 1 of Chapter 3. Each intron sequenced was amplified twice in independent PCR reactions. The PCR products were separated on 0.9% low melting point agarose (Life Technologies, Gaithersburg, MD) by gel electrophoresis,

excised from the gel, and digested with agarase according to the manufacturer's directions (Sigma, St. Louis, MO). Cycle sequencing was done according to manufacturer's instructions using the ABI PRISM Taq DyeDeoxy Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase (P/N 401384; Perkin Elmer, Foster City, CA) using 10 pmol of primer and 30-90 ng of template DNA. The reaction products were separated and analyzed on an ABI PRISM 377 Automated Sequencer (UNH Sequencing Facility). Sequences were generated with ABI DNA Sequencing Software version 2.1.1 and edited using SeqEd Software (version 1.0.2; ABI, Foster City, CA). Both strands were sequenced for the helix 50 intron for each species. Contiguous sequences were assembled and aligned using SeqMan (DNASTAR) algorithms. GenBank Accessions for sequenced samples are given in Table 3.

Alignment of helix 50 group-I introns and phylogenetic analyses

Initial alignments of the helix 50 group-I intron sequences from select *Porphyra* taxa were made using the Clustal algorithm in Megalign (DNASTAR). The intron sequences were manually aligned in Bioedit Vers. 5.0.6 (North Carolina State University), which is an alignment program that can incorporate secondary structure information. The computer alignments were compared to secondary-structure models with the aid of a group-I intron database (Damberger and Gutell, 1994), conserved base-paired segments or "P" domains (Michel and Westhof, 1990) and the secondary structure folding program *mfold* version 3.0 (Mathews *et al.*, 1999). The group-IC1 intron of *Hildenbrandia rubra* (Sommerfelt) Meneghini (GenBank Accession #L19345, Ragan *et al.*, 1993) was used as an outgroup in the phylogenetic analyses.

Phylogenetic inference by maximum parsimony analyses was performed using PAUP (Swofford *et al.,* 1998; vers. 4.0b10, Sinauer Associates, 2002), with a full heuristic search strategy that first involved 200 replicates with RANDOM taxon addition with the tree-bisection-reconnection (TBR) branch-swapping algorithm. Initial searches found the 10 shortest trees; which were then used as starting trees in searches that used TBR branch-swapping from which only the most parsimonious tree was saved. Gaps were treated as missing data. To assess support for the inferred relationships, bootstrap analyses (Felsenstein, 1985) were conducted with 1000 replicates.

Based on prior work by Wheeler and Honeycutt (1988), who showed that paired nucleotides forming stem regions appear to undergo compensatory mutations that maintain secondary structure, a weighted parsimony analysis was done using the stem/loop definitions described in Soltis and Soltis (1998) with stem bases assigned a weight of 1 and loop bases a weight of 2. A weighted bootstrap parsimony search was conducted as previously detailed with the equally weighted characters.

To test for data set incongruence between the Helix 50 group I intron dataset and a previously published SSU phylogeny of North Atlantic *Porphyra* (Klein *et al.*, 2003),. The SSU dataset was re-analyzed using the same taxa used in the group-I phylogeny for comparison purposes. A partition homogeneity test (PHT; Swofford, 1998), also known as an incongruence-length difference (ILD; Farris *et al.*, 1994, 1995), was performed using PAUP*. Only those taxa that were available for both data sets were examined. A random addition heuristic search was conducted using 100 replicates; the resulting *P*-value determined the probability of rejecting the null hypothesis of congruence (or homogeneity) of the two data sets.

Intraspecific Survey of the helix 50 Intron and Alignment

An additional survey of twenty-eight *Porphyra umbilicalis* individuals was done using a flanking primer (H50-1) and an internal intron primer (H50-2), which was designed to be exactly complimentary to this taxon for the Helix 50 intron. To determine the exact intron insertion points, which are located near the 3' end of SSU (Table 1, Figure 2), a second set of flanking amplification primers (H50-FL and UMB-ITS-1) were developed to extend the amplified region into the adjacent internal transcribed spacer. The rDNA-intron regions of all individuals were amplified by PCR, they were than sequenced[9], and aligned as described previously with the interspecific alignment of the helix 50 introns. A secondary structure diagram was created for *P. umbilicalis* using the computer drawing program Adobe Illustrator (Adobe Systems Inc., San Jose, CA). The diagram was based on the secondary structure information obtained manually from *P. umbilicalis* sequence using comparative sequence analysis (Cech, 1988), which is based on the covariance involved in maintaining Watson-Crick base-pairing within a potential secondary structure helix. The secondary structure features from ambiguous regions of the *P. umbilicalis* sequence were resolved using the secondary structure folding program *mfold* version 3.0 (Mathew *et al.*, 1999). The secondary structure was than compared to the published alignments of conserved "P" domains from group-I introns (Michel and Westhof, 1990), and finally compared to secondary structure diagrams of similar *Porphyra* group-IC1 introns described in the previously mentioned intron database (Damberger and Gutell, 1994).

Results

Amplification survey of introns

A total of 715 individuals representing 88 collection sites and nine different species were screened for the presence of the helix 50 intron with flanking primers *porint 1+2* (EPIC). All samples were scored according to the size of the intron after flanking sequences were subtracted (*i.e.*, the 730 bp amplification product contained a 555 bp intron). Table 4 shows the results of the primer screen with data for each helix 50 intron size class. *Porphyra dioica* and *P. yezoensis*[10] both have unique size variants of the intron, while other species seem to share size variants or have multiple size introns in the helix 50 position. Species that were not heavily sampled (n <6: *P. dioica, P. suborbiculata*, and *P. yezoensis*) are also listed in Table 4. There were no apparent patterns (size, presence or absence of the helix 50 intron) differentiating populations of the same species. Figure 3 represents a subset of 96 samples from the *Porphyra* survey. Several different amplification products were observed from various templates. Variable size introns occurred (Figure 3), where the amplified fragments (including flanking 18S sequence) were ~730 bp, 820 bp, and 1250 bp. The 820 bp fragment was specific to *Porphyra purpurea* (lanes 1, 27-28, 30, 32-33), whereas the 730 and 1250 bp fragments amplified only in *P. linearis*. In two samples (Figure 3; lanes 50 and 74), flanking primers amplified three different size bands from the individual *P. linearis* template, including common fragment sizes and a weak 1000 bp amplification product. Interestingly, the EPIC amplification of *P. umbilicalis* in this subset (Figure 3; lanes 11-

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[9]Because of the internal primer location, the introns amplified by the H50-1 and H50-2 primer pair lack ~100 bp at the 3' end.

[10] *Porphyra yezoensis* has both unique, shared and multiple size variants of the Helix 50 intron.

14; 17-19; 52-54; 56-58; 84-85; 87-91) produced fragments only in the 174 bp size range, the size predicted if no introns were present at the helix 50 position in the 18S rRNA gene. The low molecular weight fragment (~174 bp) appears as part of a doublet (see arrow in Figure 3), while the other band beneath it represents a primer dimer. To confirm that the amplified fragments were in fact products of the 18S gene, the ends of the fragments were sequenced. The sequence data confirmed placement of the intron at the expected insertion site, with complementary 18S sequence at the ends of the amplified fragments (Figure 4).

Subsequently, a subset of the original *Porphyra* collection (Table 4; Appendix A) was examined with an internal and flanking primer pair (*porint 5+6*) to evaluate whether the original screen had detected all helix 50 introns. Figure 5 shows the number of samples that screened positive for the intron with either primer set. Of the three species that showed low intron occurrence in the EPIC screen (*i.e., P. umbilicalis, P. miniata,* and *P. amplissima*) there was a substantial increase in the detection of the helix 50 intron using an internal intron primer: 2⇒7 *P. amplissima* (an increased detection of five introns using the internal intron primer); 1⇒4 *P. miniata*; 1⇒27 *P. umbilicalis*. *Porphyra umbilicalis* appears to have very low occurrence of the helix 50 intron in rDNA genes as detected by flanking primers (1/200), plus relatively high occurrence with the internal/flanking primer pair (81.8%). By contrast, the combination of flanking and internal primers, *porint5* and *porint6*, failed to amplify the helix 50 intron from a single sample of *P. suborbiculata.*

Using the same individuals from the initial helix 50 screen, an additional subset of 88 individuals from all nine *Porphyra* species was screened for the helix 21 intron; the

PCR primers again represented an intron-specific and a rDNA-specific primer (*int5pr-1* and *int5pr-2*, Figure 2). The helix 21 intron was detected at high levels in all species, except *P. miniata*. In five of the nine species (*P. leucosticta, P. yezoensis, P. umbilicalis, P. dioica,* and *P. suborbiculata*) the helix 21 intron was detected in every individual screened (see Figure 6).

Intron characteristics and Phylogenetic analyses

The helix 50 introns were sequenced for samples representing each species and compared to published data. Sequence data indicated that similarly sized introns could vary at the sequence level, with highly variable loop domains and highly conserved stem domains. The variable and conserved regions were identified by aligning the helix 50 intron sequences according to secondary structure; 544 bases could be aligned in conserved domains. The P, Q, R, and S functional core domains were highly conserved across all *Porphyra* species and only slightly different for the outgroup *Hildenbrandia rubra*. The conserved P domains or "stem" regions showed minimal variation between species and were easily aligned by eye, except for the P8 region that showed high levels of variation between taxa (Figure 4).

Of the 544 nucleotide positions aligned, 143 were parsimony-informative, primarily from the variable loop domains. Heuristic searches using equal weighting of all characters found 1 minimal-length tree of 495 steps (consistency index [CI] =.8566; retention index [RI] = 0.690). The strict consensus of the most parsimonious tree is given in Figure 7a. Using the red alga *Hildenbrandia rubra* as an outgroup for phylogenetic analysis, the seven North Atlantic *Porphyra* species form two well-supported clades.

Porphyra suborbiculata, *P. purpurea*, and *P. miniata* form the first clade (bootstrap value= 99%). The sister clade includes all other *Porphyra* taxa in the analysis: *P. umbilicalis*, *P. dioica*, *P. linearis*, and *P. leucosticta* (bootstrap value= 80%).

Parsimony analyses that differentially weighted stem versus loop bases did not change the overall topology of the phylogenetic trees. The major difference between the bootstrap trees of the weighted analysis and that of equal weighting was stronger bootstrap support of species relationships in the weighted phylogram (see Figure 7a). In general, differential weighting of stems versus loops did not substantially alter the results obtained using equal weighting of characters.

Figure 7b is a phylogenetic analysis of 910 bases of the SSU for the same *Porphyra* taxa that were analyzed for the helix 50 group-I intron (Figure 7a). The SSU phylogeny showed strong bootstrap support for two main clades; the first clade containing *P. carolinensis, P. leucosticta*, and *P. miniata*; and a second clade that includes *P. dioica, P. linearis, P. umbilicalis*, and *P. purpurea*. The partition homogeneity test between the group I intron and SSU datasets found the two datasets to be incongruent with $P = 0.010000$.

Sequence divergence within the Helix 50 intron of Porphyra umbilicalis

A fragment of approximately 548 bp from the 5′ half of the helix 50 group-I ribosomal intron was amplified from all 28 of the *Porphyra umbilicalis* collected for intraspecific analysis (see Chapter 3). These partial intron amplification products were sequenced (GenBank Accessions: AY347883-AY347910). The intron sequences (18 European, 10 North American) were easy to align with considerable sequence

conservation being shown in the computer-generated alignment (with CLUSTAL; Appendix 4). The only size variation between amplification products resulted from the presence of a 1bp insertion at alignment position 510 in isolate IRE1a and NHP3. Since all twenty-eight amplification products represented only a fragment of the total intron size, full length intron sequences for *P. umbilicalis* were obtained from four of the taxa using a reverse 3′ primer in the adjacent internal transcribed spacer (ITS) region. In addition, gel-band sizing was used in tandem with other *Porphyra* (*P. umbilicalis* and *P. purpurea*, as referenced previously) group-I Helix 50 introns and 18S ribosomal sequences for accurate size and positioning of all *P. umbilicalis* isolates. The full size of the intron was ~710 bp. Although all *P. umbilicalis* sequences were easily aligned, there was a significant degree of sequence variation between different *Porphyra umbilicalis* isolates. Analysis of this variation revealed sequence divergence between the North American and European populations (Appendix 4, Chapter 3).

Amplification of the helix 50 intron in four Northeast Atlantic *Porphyra umbilicalis* specimens using the *H50-1* and *H50-2* primers produced a second larger amplification product (1188 bp) for the intron (GenBank Accessions: AY613848-AY613851). Alignment of both the smaller and larger intron sequences showed that the difference was due to the addition of a 478 bp insertion near the 5' end relative to the smaller intron. Three of these four individuals (AY613849-AY613851) contained both size classes of the helix 50 intron, which are aligned in Figure 8 (the 710 bp helix 50 amplification product was not detected in the fourth individual). In addition to the 478 bp insert in the larger helix 50 intron, there were sequence similarities between the different size classes (see shading, Figure 8). A pairwise sequence comparison between both

introns in all three individuals showed that the smaller intron (Intron 1) had 96.2-100% sequence similarity among the 710 bp size introns and the larger intron had 99.8-100% sequence similarity among the 1188 bp introns. Strikingly, there was only 93.1-94.2% sequence similarity between the two size classes (Table 6).

The proposed secondary structure model for the 710 bp *Porphyra umbilicalis* helix 50 group-I intron is shown in Figure 9 and was based on isolate IRE1a. It does not include the 478 bp insertion found in the 5' region of the larger version of the helix 50 intron

His-Cys Box

The large size variant of the group-I intron found during the intraspecific survey of *Porphyra umbilicalis* populations appears to contain a homing-endonuclease open reading frame (ORF) as described in Haugen *et al.* (1999) and Müller *et al.* (2001). After conducting a search of the sequences using the National Center for Biotechnology Information (NCBI) ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), the 1188 bp introns were found to contain an open reading frame (ORF) on the complementary strand to that encoding the SSU rRNA (Figure 10). The 155 amino acid ORF was compared to other protein coding sequences in GenBank using BLASTP (Altschul *et al.*, 1997; vers. 2.2.8; 3-21-2004), with all search results suggesting that this extended region encodes a putative His-Cys Box endonuclease. The amino acid sequences from all four of the large size variants were aligned to similar sequences predicted from the Helix 50 ribosomal intron in the Bangiophyceae (*Porphyra spiralis*, Acc. L26177; and *Bangia atropurpurea*, Acc. 36066) as well as the endonuclease I-*Ppo*I (Acc. L03183). The

alignment (Figure 11) showed that one of the *P. umbilicalis* samples (IRE-2) contained the His-Cys Box motif and conserved residues proposed to be directly involved in zinc binding and the active site of the endonuclease (Haugen *et al.*, 1991; Figure 11). The amino acid sequence for IRE-2 was identical in 14 amino acids with that of the I-*Ppo* I endonuclease. The amino-acid sequence for the His-Cys box in the other three *P. umbilicalis* samples were less conserved compared to I-*Ppo*I with only 12 amino acids being identical. In addition, the three less-conserved sequences terminated prematurely at amino acid position 105 (Figure 12) relative to IRE-2 because of nucleotide change that resulted in the formation of a stop codon (TAG) instead of the amino acid Glutamine (GAG). However, sequence information from all three revealed retention of the His-Cys box motif and the putative endonuclease stop codon further downstream (Figures 11 and 12).

Discussion

The distributions of helices 50 and 21 group-I ribosomal introns within North Atlantic *Porphyra* were assessed in the present study using PCR amplification and sequencing. The results support the following assertions: 1) EPIC and/or non-species specific primer design underestimates the presence of group-I introns in members of the Bangiales; 2) the rDNA repeats within an individual *Porphyra* appear to be heterogeneous for the presence or absence of the Helix 50 group-I intron; and 3) rDNA group-I introns may exist in all species of *Porphyra*. The results are strikingly different from an earlier assessment of ribosomal group-I intron distribution in the Bangiales by Müller *et al.* (2001), suggesting the need for a re-evaluation of their results.

Helix 50 and Helix 21 Intron Distribution in North Atlantic Porphyra taxa

The results of the surveys for helices 50 and 21 group-I intron in Northwest Atlantic *Porphyra* confirm the importance of using an internal primer with a corresponding flanking primer for detection of ribosomal introns as documented in fungi by Hibbett (1996). Several previous studies of introns in *Porphyra* relied primarily on amplification using flanking or EPIC primers. For example, in their study of *Bangia* and *Porphyra* rDNA Müller *et al.* (2001) detected the helix 50 intron in 39 individuals from a total sample size of 60 (about 65%). Similarly, Kunimoto *et al.* (1999) detected the helix 50 intron in seven of the 15 (46%) *P. yezoensis* isolates they sampled. By contrast, my data indicated that incorporating an internal primer in combination with a flanking primer increased detection of the helix 50 intron in eight of nine *Porphyra* species screened; the difference in detection rates suggests that previous studies likely encountered false negatives when they failed to detect the helix 50 intron in samples (Stiller and Waaland, 1993). Most noticeable from my results was the increased detection of the intron using the combination of an intron-internal primer and an external primer as compared to using only flanking (EPIC) primers; detection increased >75% for *P. umbilicalis* and >45% for *P. amplissima*. *Porphyra suborbiculata* was an exception as the single sample tested with the internal/flanking primer combination showed no amplification. A more extensive intron survey is needed to evaluate the effectiveness of the *porint5/porint6* primer set in this and other species. Alternatively, the internal primer used in this study for multiple species may not have been optimized for individual species and therefore resulted in false negatives.

Interestingly, Hibbett's (1996) studies of Basidiomycetes showed that even with the internal intron amplification primers, 34 of 39 species surveyed still appear to lack a ribosomal intron. Of the five species showing presence of an intron, three species were from a single genus, *Lentinellus*, and a fourth from a closely related species *Clavicornia pyxidata* (see Figure 5 in Hibbett, 1996). Based on these observations a potential drawback to Hibbett's intron assay may have been the use of only one set of flanking (*SR1c* and *NS6*) and intron-specific primers (*943a* and *943b*) for all 39 species of Homobasidiomycetes instead of optimizing an intron-specific primer for each species or genus.

The results presented here suggest that successful amplification of the intron is highly dependent on where the internal primer is placed within the intron. Although the internal *porint 6* primer showed a substantial increase in intron detection and amplification, the results of the intraspecific survey of the helix 50 group-I intron using the more specific H50-2 internal primer in *Porphyra umbilicalis* showed that all individuals were positive for the intron and in some individuals more than one size variant of the helix 50 intron existed. Hence, my results contrast those of Müller *et al.* (2001) where only 25% of the *P. umbilicalis* samples were found to contain the helix 50 intron. Although most internal intron primers have been developed in the conserved core regions (*i.e.*, P, Q, R, S) to be used with multiple species, it appears that these may not be the optimal locations for primer design and successful PCR amplification; thus, the conserved regions for each "species" should be considered when developing each primer set.

Additional support for intron detection using both flanking and internal intron primers was seen by the results of the helix 21 intron screen. Successful amplification of the helix 21 intron occurred in 100% of the samples for five species and approximately a 50-60% increase in detection for all remaining species with the exception of *Porphyra miniata*. In the four individuals of *P. miniata* screened, there was no detection of a helix 21 intron. Based on previous studies that have detected the helix 21 group-I intron in *P. miniata* (Müller *et al.*, 2001), the lack of the intron's detection in this study may be due to an incompatible internal intron primer and reiterates the earlier suggestion of the need to develop specific intron primers for each species.

The present observations lead to the hypothesis that introns may exist in every *Porphyra* individual but detecting the introns may be hindered by factors such as short allele dominance[11] (e.g. for EPIC PCR; Wattier *et al.*, 1998; Sefc *et al.*, 2003), and lack of a perfect match between the primer and template may hinder the successful "capture" of these introns by PCR as shown in this study.. Although no quantitative analysis was done to determine how many copies of the small ribosomal subunit in any one individual might contain a ribosomal intron, it can be speculated that increased intron detection in certain individuals DNAs may indicate that certain *Porphyra* isolates have a higher level of introns in copies of their rDNA. As suggested by Hibbett (1996) it would be useful to determine the relative frequency of intron-containing and intron-lacking rDNA repeats, possibly by quantitative densitometry of Southern blots of genomic DNA probed with rDNA sequences that flank the intron.

Intraspecific intron variation

Oliveira and Ragan (1994) suggested that ribosomal introns maybe a potential species and biogeographic marker, while Müller *et al.* (2001) dismissed this idea because many of their samples had lacked an intron. The present study suggests that the conclusion of Müller *et al.* (2001) may have been premature; my intraspecific survey demonstrated that the helix 50 group-I intron was found in all *Porphyra umbilicalis* samples across a large geographic range. Successful amplification of the intron within all members of a species or population may therefore have biogeographic utility based on the higher mutation rate found within self-splicing group-I introns as compared to rDNA and the internal transcribed spacer (see Chapter 3). Even more interesting is the occurrence of multiple size introns within the same *P. umbilicalis* individual. The three examples from this study suggest that multiple forms of the intron within an individual may be a common occurrence in *P. umbilicalis* and that gene conversion mechanisms may not be homogenizing all rDNA cistrons. The observations that helix 50 introns of similar size are more similar at a sequence level between different algal accessions than within an individual could be used to model mechanisms of both horizontal transmission and pseudogene evolution as outlined below.

ORFs Coding for His-Cys Box Endonucleases

For horizontal transmission of introns to occur there must be mechanisms at either the RNA or DNA level to promote intron insertion. Currently, intron movement is hypothesized to occur at both levels (Belfort and Perlman, 1995). At the RNA level, a

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[11] Where smaller fragments have been shown to have a higher rate of successful amplification during the

reversal of the self-splicing process could cause reinsertion of the intron into an intronless allele of the gene (in this case a copy of rDNA) or at a heterologous site of the same gene (Roman and Woodson 1995; Roman *et al.*, 1999; Woodson and Cech, 1989). Intron spread at the DNA level (and specific to my analysis of ORF containing introns in *Porphyra umbilicalis*) depends on the expression of intron-encoded enzymes, called homing endonucleases (Lambowitz and Belfort, 1993; Johansen *et al.,* 1997; Belfort and Roberts 1997). The homing activity refers to the intron's ability to move horizontally into an intron-less allele of the same genome or into a new genome. One group or "family" of these endonucleases that are made up exclusively of nuclear-encoded enzymes are characterized by a conserved His-Cys box motif (Johansen *et al.,* 1993). Finally, if horizontal transfer is occurring within *Porphyra* or Eukaryota in general it must have a vector to allow such a transmission. Bhattacharya, Friedl, and Damberger (1996a) reported that based on phylogenetic evidence, the 1512 ribosomal intron of the SSU in green algae may be of viral origin. It is unclear how widespread viruses are among marine algae, but it has long been known that they may act as potential vectors of nucleic acids. If a virus is capable of infecting very closely related taxa such as the *Porphyra* in this study, it is most likely also capable of transferring nucleic acids horizontally between various hosts.

The analysis of the 1188 bp introns in some populations (GenBank Accessions AY613848-AY613851) of *Porphyra umbilicalis* revealed ORFs for His-Cys box endonucleases. Similar to the results of other studies on bangialean group-I introns, the ORF-containing introns only appear to contain the insertion within the P1 region (Haugen

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initial cycles of the PCR, and hence outcompete the longer template throughout the overall reaction.

et al., 1999; Müller *et al.*, 2001). The sporadic distribution of ORF-containing and ORF-free introns within monophyletic groups suggest strong selection against ORFs coding for endonucleases thereby resulting in increased loss of the ORFs (De Jonchheere 1994; Muscarella and Vogt 1993). The ORFs found in the four individuals from this study show two different types of His-Cys box retentions. Only one (IRE-2; Accession AY613848) of the four ORF-containing *P. umbilicalis* individuals contained all of the conserved zinc binding and active site residues associated with the I-*Ppo*I endonuclease. Isolate IRE-2 also appears to have an extended C-terminal end when compared to other endonuclease-like sequences (Haugen *et al.*, 1999) from Bangiophyceae helix 50 group-I introns. Unlike in Haugen *et al.* (1999), no frameshifts were apparent in any of the *P. umbilicalis* ORF sequences. However, the remaining three *P. umbilicalis* isolates did show a higher degree of polymorphism in their sequence compared to both IRE-2 and the His-Cys box motif of the endonuclease I-*PpoI*, resulting in a premature stop codon and loss of two I-*PpoI* conserved amino acids (*H110, N119*).

Based on reports that nuclear homing endonucleases generate double-strand breaks at intron-lacking rDNA alleles (Johansen *et al.*, 1997; Elde *et al.*, 1999) and are lethal when expressed in yeast (Lin and Vogt, 1998), Haugen *et al.* (1999) suggested that selection was occurring against functional forms of these genes. The observed truncation at the C-terminus and polymorphisms I found in the less-conserved *Porphyra umbilicalis* sequences (Figures 11 and 12) may therefore represent nonfunctional pseudogenes.

Additional analysis is needed to investigate whether all *Porphyra* taxa contain copies of the ORF containing introns. If the His-Cys ORF represented a pseudogene in the sampled individuals, a lack of functional constraints would suggest a region that

would show high rates of mutation. However, the amino acid sequence of the His-Cys box motif is well conserved in *Porphyra* and other taxa in which they have been found (Müller *et al.,* 2001; Haugen *et al.,* 1999). The pairwise comparison between the different intra-individual intron types (containing the ORF or not) in Table 6 showed a higher sequence similarity (99.8-100%) between the ORF-containing introns when compared to the ORF-lacking introns. The lack of variation found between the ORF-containing introns may indicate that these sequences have functional constraints acting upon them compared to the more variable introns that do not contain or have lost the homing endonuclease ORF region. Unfortunately, intron size variants were not found in IRE-2, the only individual exhibiting an ORF region highly conserved with the *Ppo-I* endonuclease gene. It could be speculated that group-I introns such as the one found in IRE-2 may show the highest sequence conservation because of the high retention of its endonuclease active sites.

Introns as sources of phylogenetic information

Before introns can be evaluated for their phylogenetic informativeness in the Bangiophyceae, there must be a consistent and reliable method for identifying a taxon at the species level. Since members of the genus *Porphyra* are difficult to identify in the field due to the lack of distinguishing morphological characters and cryptic diversity, samples can be misidentified (Klein *et al.*, 2003; Lindstrom and Cole, 1993). The impact of incorrect identification can have significant implications in phylogenetics. For example, when the consensus sequence for the *P. umbilicalis* helix 50 intron from the 28 geographically different isolates in this study were aligned with the two published

examples of the *P. umbilicalis* helix 50 introns in the GenBank database (GenBank Accessions: AF172602, AF318959), both sequences showed a low level of similarity (21% and 30%, respectively) to those obtained in this study (GenBank Accessions: AY347883-AY347909). The degree of differentiation suggests that AF172602 and AF318959 came from other *Porphyra* species and that these algal accessions from which the introns were amplified were incorrectly identified. One of these sequences (AF172602) was used in the phylogenetic analysis of the SSU and ribosomal group-I introns resulting in a discordant relationship with other *P. umbilicalis* isolates used in that study (see Müller *et al.*, 2001). In contrast to the work of Müller and coworkers, my study used an independent molecular assay (RFLP Assay, Chapter 1) to confirm the *Porphyra* species identification. Accurate species identification increases the confidence of further extrapolation of the submitted sequences for phylogenetic comparisons.

The helix 50 intron phylogeny from this study was compared to the SSU gene tree constructed previously for these species (see Figure 3 in Klein *et al.*, 2003). The intron phylogeny is more limited but the overall topology is similar to the SSU gene tree (Figure 7b). However, in the intron phylogeny *Porphyra purpurea* and *P. leucosticta* are grouped with different clades. Although other minor changes in topology occur, these two taxa "jump" highly supported branches (100% bootstrap) in the SSU phylogeny. Another contradiction is the relationship of a *P. umbilicalis—P. dioica—P. linearis* clade. The SSU phylogenetic analysis by Klein *et al.* (2003) suggested that *P. linearis* and *P. umbilicalis* shared a recent common ancestor. However, the helix 50 intron phylogeny contrasts with the other gene tree phylogenies, indicating that *P. dioica* and *P. umbilicalis* are more closely related than *P. linearis* and *P. umbilicalis*.

The disagreements between the different gene trees might be explained by the theory of horizontal transfer (Oliveira and Ragan, 1994; Li, 1997). Müller *et al.* (2001) suggested that the ribosomal introns in the Bangiales were present prior to species radiation and therefore vertical inheritance was exhibited by the intron phylogenies. Although the results from this study also show similarity between the SSU and intron phylogenies they are not identical and suggest some instances of horizontal transmission may have occurred. The results of the partition homogeneity test suggest that the SSU and group I intron data sets are exhibiting different phylogenetic signals. Although, the two data sets may be evolving at different mutation rates, a paraphyletic phylogeny was found in the group-I intron data set that is consistent with datasets from which horizontal transmission has occurred. An alternative explanation to the lack of congruence found between the two data sets is saturation (phylogenetic signal is overwhelmed by multiple changes at each site), where saturated data could increase variance in branch lengths potentially increasing the chances of long-branch attraction and an incorrect phylogeny (Felsenstein, 1978).

The mobility and/or immobility of introns may be related to the putative endonuclease ORFs found within group-I introns. A phylogenetic comparison (Haugen *et al.*, 1999) of ORF-containing Helix 50 group-I introns from four *Porphyra* and *Bangia* individuals found that the endonuclease-like protein sequences showed a pattern of horizontal transfer whereas the remaining intron core sequence showed a pattern consistent with long-term vertical transmission. Therefore, group-I introns containing an endonuclease motif may show patterns of mobility and a phylogenetic history linked to the origin of the intron and putative endonuclease instead of the host organism (*i.e.*,

Porphyra), whereas non-functioning forms or ORF-lacking forms of the intron could provide phylogenetic information for the host species such as recent biogeographical radiation. In other words, if some group-I introns have lost self-splicing and re-insertion capabilities because of selective pressure, the intron may exhibit long-term retention within the rDNA. If immobile forms of the intron exist within *Porphyra* in tandem with putative mobile forms (IRE2) than it would be consistent with predominantly vertical and rare instances of horizontal transmission.

Müller *et al.* (2001) also believed that the lack of introns in some individuals was due to multiple losses over time and that the more "derived" a taxon was, the higher the incidence of intron absence. Again, the intron surveys from my study suggest that their lack of detection may be an artifact of the PCR amplification procedure, which would explain the inconsistent detection of the intron in previous studies (Kunimoto *et al.*, 1999; Müller *et al.*, 2001), and that presence or absence of introns is probably not a phylogenetically informative character. Another important observation from my study that suggest the phylogenetic results of Müller *et al.* (2001) may be incorrect is the amplification of genetically different forms of the Helix 50 intron within an individual. The group-I intron phylogenies of Müller et al. (2001) did not discriminate between the different forms that may be found within a single individual. The results from the *Porphyra umbilicalis* study suggest that group-I introns lacking the putative endonuclease ORFs may not be suitable for phylogenetic studies based upon two factors. First, the majority of ribosomal introns amplified in the *Porphyra umbilicalis* study lacked an endonuclease-like ORF region. Whether or not short- allele dominance plays a role in the amplification of the shorter intron is still unclear and ORF-containing introns may

still exist within the same individual. Second, ORF-containing introns are more genetically similar to other ORF-containing introns than they are to an ORF-lacking intron within the same isolate, which indicates that the two forms of group-I introns are diverging at different rates. If phylogenies are constructed using ribosomal intron sequence without discriminating between the two forms of the intron, a skewed topology will result. A possible example of this artifact may be illustrated with the *Bangia fuscopurpurea* (Dillwyn) Lyngbye helix 21 intron phylogeny (see 516 intron phylogeny in Fig. 5 from Müller *et al.,* 2001), where the two His-Cys Box containing intron sequences (GenBank Accessions AF172560; AF172561) group together and differentiate them from the other five *B. fuscopurpurea* specimens in the phylogeny.

Conclusions

The existence and catalytic properties of group-I introns have been well-documented throughout the Eukaryota along with the observation that group-I introns are lost frequently in nature based on their presence or absence in closely related taxa (Müller *et al.,* 2001; Bhattacharya *et al.*, 1996a; Van Oppen, *et al.*, 1993). The subgroup IC1 (Michel and Westhof, 1990) nuclear-encoded rDNA group-I introns are the most widely studied form of these introns and they were chosen for this study to investigate their distributional patterns in North Atlantic members of the red algal genus *Porphyra.* Hence a primary goal of this study was to confirm previous distributional patterns and to evaluate the usefulness of the ribosomal group-I intron for determining intraspecific phylogenetic relationships.

My studies confirm the earlier observations of several investigators that group-I introns are widespread in the small ribosomal subunit of *Porphyra* (Oliveira and Ragan 1994; Kunimoto *et al.* 1999; Oliveira *et al.* 1995; Müller *et al.* 1998, 2001; Broom *et al* 2003). However, screening for these introns with a combination of internal and flanking primers detected them at higher frequencies than previously documented. My study also gives new insights for detecting these introns and how they may increase our ability to utilize and understand these loci in the Bangiales. The intraspecific analysis of *P. umbilicalis* also points to strong evidence that specimens thought to lack introns may actually have them. That is, the introns may exist in low copy number and therefore go undetected in conventional PCR screens because of template preference or because primers have not been optimized for intron detection. Although this study uncovered only four examples of helix 50 introns containing the His-Cys box ORF, the incidence of their detection might also be improved by more specific amplification methods (*i.e.*, developing a primer in the His-Cys box region). Finally, the presence or absence of introns is probably not an informative character for phylogenetic studies as previously hypothesized by Müller *et al.* (2001). Hence intron-based phylogenies within the Bangiophycidae should be re-evaluated and the use of introns for biogeographic and intra-specific investigations still may be possible if the introns are present in all specimens and if only sequences without the His-Cys regions are used.

Figure 1. General diagrammatic model of a conserved stem-loop secondary structure derived from the RNA sequence of self-splicing group-I introns. Italicized letters represent the core conserved regions (P, Q, R, S) found in all group-I introns. (P= stem regions, L= loop regions.) Illustration adapted from Cech (1990).

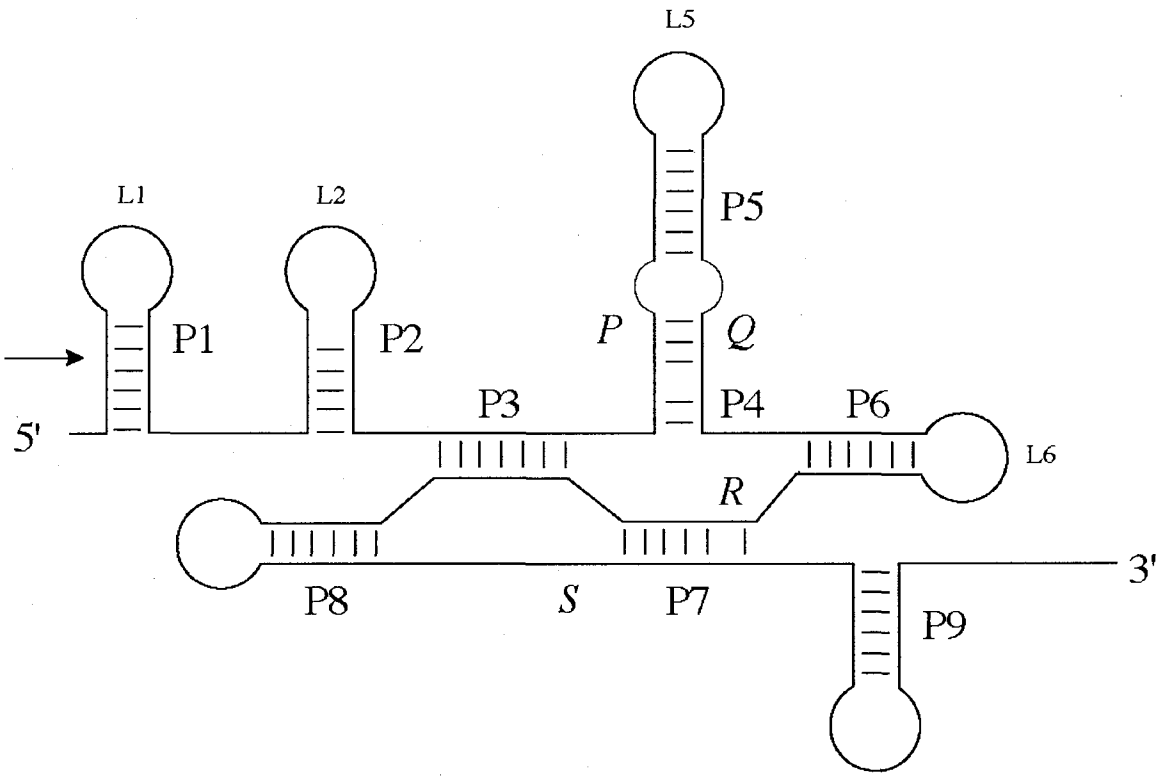

Table 1. List of primers (label; sequence) used for ribosomal group I intron amplification and DNA sequencing in species of *Porphyra.*

Primer Label	**5'-Primer sequence-3'**
Helix 50 INTRON	
Porint 1	TGTACACACCGCCCGTC
Porint 2	CTGATCCTTCTGCAGGTTCACCTAC
Porint 5	TTGGGGGCATTCGTATTTCAT
Porint 6	GGCTGCAAAGGCTTCGGTA
H50-1	GAAGGAGAAGTCGTAACAAGGTTT
H50-2	CAGGGGACCGACTGTCTCTTA
H50-FL	GAGGAAGGAGAAGTCGTAACAA
UMB-ITS1-R	TATCCACCGTTAAGAGTTGTAT
Helix 21 INTRON	
Int5pr1	AGGCGAACCTTCAGAGACT
Int5pr2	CAGACAAATCACTCCACCAA

Table 2. PCR profiles.

Region	Primers	Fragment size	Reaction volume (µL)	Taq units	MgCl2 (mM)	Annealing temperature °C	Annealing time	Extension time	Cycles
Helix 50	*porint1&porint2*	variable	50	4	2.5	56.0	1 min	1 min	35
Helix 50	*porint5&porint6*	variable	50	1	2.25	54.3	1 min	1 min	30
Helix 50	*H501&H502*	~471/949	50	2	1.75	56.5	1 min	1 min	36
Helix50 and ITS1	*H50FL&UMBITSR*	~1016/1494	50	2	2.0	55.0	1 min	1 min, 30 sec	30
Helix 21	*Int5pr1&int5pr2*	variable	50	1	2.0	56.0	1 min	1 min	35

Figure 2. The 18S ribosomal subunit showing the relative positions of coding regions (white boxed areas), noncoding regions (double lines), and PCR amplification and sequencing primers. Dotted arrows represent internal (intron) PCR primers. Triangles represent group I introns.

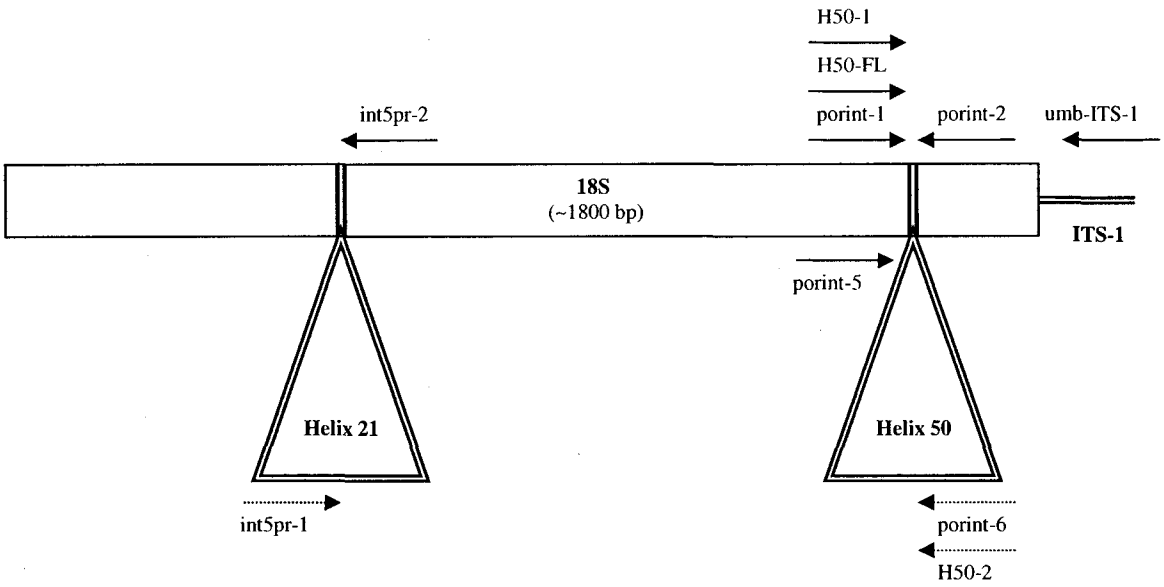

Table 3. *Porphyra* helix 50 ribosomal introns sequenced for phylogenetic and intraspecific analysis. 1=Used in phylogenetic analysis; 2= Used in intraspecific analysis; 3= contains His-Cys ORF motif.

Genus, species	Hodgdon Herbarium #	Date Collected	Geographic origin	GenBank Accession Numbers	
				18S rDNA	Helix 50 Intron
P. dioica[1]	N.A.	4/4/1998	North of Aberystwyth, Wales, UK	-	AY573850
P. leucosticta[1]	67142	5/17/1997	Montauk Pt., NY	-	AY573853
P. linearis[1]	60868;	2/18/1996	South Bristol, ME;	Klein et. al.,2003	AY573852
P. miniata[1]	65301	6/23/1996	Fink Cove, Nova Scotia, Canada	Klein *et al.*, 2003	AY573855
P. suborbiculata[1]	N.A.	5/20/1998	Masonboro, North Carolina	-	AY573851
P. purpurea[1]	65186	11/1/1996	Ross Island, New Brunswick, Canada	Klein *et al.*, 2003	AY573854
P. umbilicalis[1,2]	76580	8/28/2000	Clare Island, County Mayo, Ireland	-	AY347910
P.umbilicalis[2]	N.A.	Variable	Variable (See Chapter 3)	-	AY347883-AY347909
P. umbilicalis[3]	N.A.	Variable	Variable (See Chapter 3)	-	AY613848-AY613851

Table 4. Initial screening for the Helix 50 intron from North Atlantic *Porphyra* collections using flanking primers *porint1* and *porint2*. A dash (-) signifies no intron amplification.

			Intron Size Classes (bp)						
	#Sites	N	425	725	825	1075	645	555	% w/intron
Porphyra umbilicalis	25	200	-	-	-	-	-	1	0.5
Porphyra leucosticta	12	143	-	-	-	-	4	80	58.7
Porphyra purpurea	19	127	-	-	3	3	62	-	53.5
Porphyra linearis	13	109	-	-	4	9	-	85	89.9
Porphyra amplissima	6	65	-	-	-	-	-	1	1.5
Porphyra miniata	3	31	-	-	-	-	1	1	6..5
Porphyra sp.	3	18	-	-	-	-	8	8	88.9
Porphyra yezoensis	3	11	4	-	-	-	-	4	72.7
Porphyra dioica	2	6	-	6	-	-	-	-	100.0
Porphyra suborbiculata	2	5	-	-	-	-	4	-	80.0
Total	88	715	4	6	7	12	79	180	40.3

Figure 3. An agarose gel (Plate #6) of 96 *Porphyra* samples illustrating an initial amplification of Helix 50 introns with flanking primers *porint1* and *porint2* from 96 *Porphyra* samples (see Table 4 for reference). The lanes show variable size introns and templates that lacked intron amplification (appearing as the top band of a doublet). The first lane (M) in each series is a *Phi*X/*HAE* III DNA size marker. Numbers attached to arrows represent amplification size class and actual intron size (parentheses) in bp.

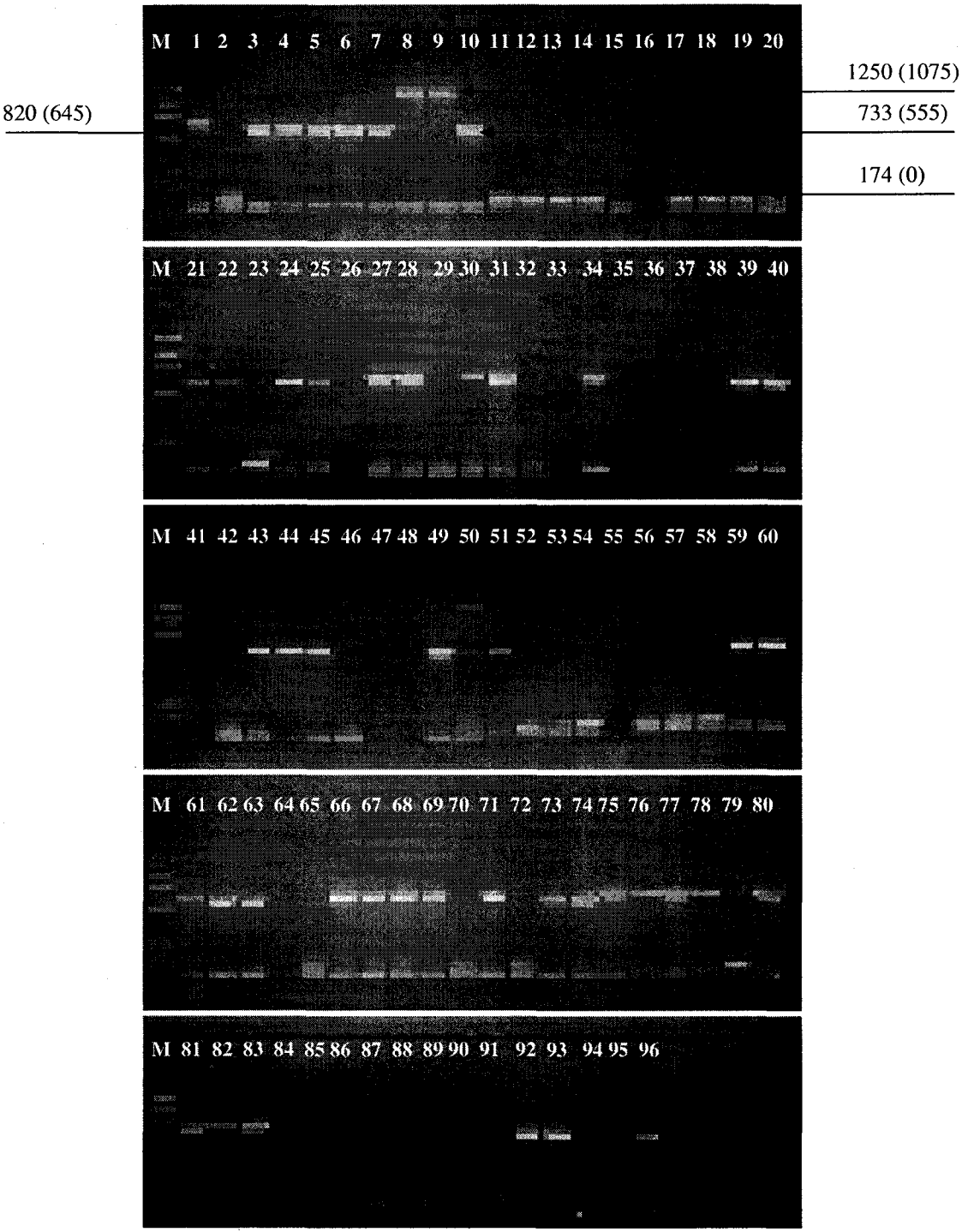

Table 5. Sample information for Plate 6 (see Figure 4) extracted from Appendix A including corresponding DNA number, species designation, collection site, and intron amplification size(s).

Plate #	DNA ID	Species	Collection Site	Date Collected	Insertion Size
1	606	*P. 'purpurea'*	Orr's Island, ME	2/3/96	820
2	608	*P. 'purpurea'*	Orr's Island, ME	2/3/96	
3	609	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96	730
4	610	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96	730
5	611	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96	730
6	612	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96	730
7	613	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96	730
8	614	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96	1250
9	615	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96	1250
10	616	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96	730
11	617	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96	
12	618	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96	
13	619	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96	
14	620	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96	
15	621	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96	
16	NA	+ control	-	-	
17	622	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96	
18	623	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96	
19	624	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96	
20	625	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96	
21	626	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96	730
22	627	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96	730
23	628	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96	
24	629	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96	730
25	630	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96	730
26	632	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96	
27	633	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
28	634	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
29	635	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	
30	636	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
31	637	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	
32	638	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
33	639	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
34	640	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	
35	641	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96	
36	642	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96	
37	NA	+ control	-	-	
38	647	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96	
39	646	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96	730
40	645	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96	730
41	644	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96	
42	643	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96	
43	648	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96	730
44	649	*P. 'linearis'*	Reid State Park, ME	2/10/96	730
45	650	*P. 'linearis'*	Reid State Park, ME	2/10/96	730
46	651	*P. 'linearis'*	Reid State Park, ME	2/10/96	
47	652	*P. 'linearis'*	Reid State Park, ME	2/10/96	
48	653	*P. 'linearis'*	Reid State Park, ME	2/10/96	
49	654	*P. 'linearis'*	Reid State Park, ME	2/10/96	730
50	655	*P. 'linearis'*	Reid State Park, ME	2/10/96	1250-1000-730
51	656	*P. 'linearis'*	Reid State Park, ME	2/10/96	730
52	657	*P. 'umbilicalis'*	Pine Point, ME	2/3/96	
53	658	*P. 'umbilicalis'*	Pine Point, ME	2/3/96	
54	659	*P. 'umbilicalis'*	Pine Point, ME	2/3/96	
55	660	*P. 'umbilicalis'*	Pine Point, ME	2/3/96	
56	661	*P. 'umbilicalis'*	Pine Point, ME	2/3/96	
57	662	*P. 'umbilicalis'*	Pine Point, ME	2/3/96	
58	664	*P. 'umbilicalis'*	Pine Point, ME	2/3/96	
59	665	*P. 'linearis'*	Rye Harbor, NH	2/3/96	730
60	667	*P. 'linearis'*	Rye Harbor, NH	2/3/96	730
61	668	*P. 'linearis'*	Rye Harbor, NH	2/3/96	730

62	669	*P. 'linearis'*	Rye Harbor, NH	2/3/96	730
63	670	*P. 'linearis'*	Rye Harbor, NH	2/3/96	730
64	NA	+ control	-	-	
65	671	*P. 'linearis'*	Rye Harbor, NH	2/3/96	
66	672	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96	820
67	673	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96	820
68	674	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96	820
69	675	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96	820
70	676	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96	
71	677	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96	820
72	678	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96	
73	679	*P. 'linearis'*	Rye Harbor, NH	2/3/96	730
74	680	*P. 'linearis'*	Rye Harbor, NH	2/3/96	1250-1000-730
75	681	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
76	682	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
77	683	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	
78	663	*P. umbilicalis*	Pine Point, ME	2/3/96	
79	684	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	
80	685	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
81	686	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
82	867	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
83	688	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96	820
84	689	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95	
85	690	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95	
86	691	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95	
87	692	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95	
88	693	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95	
89	694	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95	
90	695	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95	
91	696	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95	
92	697	*P. 'linearis'*	Rye Harbor, NH		730
93	698	*P. 'linearis'*	Rye Harbor, NH		730
94	699	*P. 'linearis'*	Rye Harbor, NH		
95	700	*P. 'linearis'*	Rye Harbor, NH		
96	701	*P. 'linearis'*	Rye Harbor, NH		730

Figure 4. Multiple sequence alignment of the Helix 50 group-I intron in New England *Porphyra* species using ClustalW. Locations of conserved P (1-9) regions are annotated above alignment and core elements are shaded. *lowercase* = SSU ribosomal sequence, • = intron insertion point.

```
                                    P1
Porphyra umbilicalis    --tna---tcgtaacaagnt•TT-GCTA------------AGGCAACAGAAAACAGACTATCATGGACATGCAACCAATCGACCACCCATGTCGGCCACCA
Porphyra dioica         ----------gtaacaaggt•TT-GCTA------------AGGCAACAGAAA-----------------------------------CGTCGAT----A
Porphyra linearis       --gagaagtcgtaacaaggt•TT-GCTA------------AGGCAACAGAA-------------------------------------------GTCTCTT
Porphyra leucosticta    --gagaagtcgtaacaaggt•TT--CCA------------AG--TATAGAAGA-----------GAACATGCA-------------TGATGT---------
Porphyra suborbiculata a-----agtcgtaacaaggt•TTTGCCGTATCC---------ACAC---------AGT------------GCGA--A---------------CGTTTCTC-
Porphyra purpurea       ----gaagtcgtaacaaggt•TT--CCGTAATTTGTGGATAGACACC--AAACCGAATCGTCAAAGACG-ACGA--AGTT---------TG----------
Porphyra miniata        aggagaagtcgtaacaaggt•TTTGCCGTATTTTGTGGATGGAC--------------TGGCAAGGA-G-GC------CC---------CGCCGCCCCCCT
Hildenbrandia rubra     -----------------gt•TTT-----ATCCTATGGA-------------------------------------------------------------

                                                                  P1'      P2
Porphyra umbilicalis    GCGTTGTCCATGAAGAATGGCCTTCTGTGGGAAACACTTAC-CGAAGCCTTTGCAGCCCGA--AAGGGTG-GCGAT-CGCGACTTAT-ATAAACAA--A-
Porphyra dioica         GCGTTGTCCATGAAGAATGACCTTCTGTGGGAAACACTTAC-CGAAGCCTTTGCAGCCCGA--AAGGGTG-GCGAT-CGCGACTTAT-ATAAACAA--A-
Porphyra linearis       GCATCATC-ATGA---ATGGCCTTCTGTGGGAAACGCTTAC-CGAAGCCTTTGCAGCCCGA--AAGGGTG-GCGAT-CGCGACTTGTCAAAAACAA--T-
Porphyra leucosticta    --------------GAATGTCCTTCTGTGGGAAGCGCTTAC-CGAAGCCTTTGCAGCCCGA--AAGGGTGCGCGAT-CGCGACT-AT--AAAACAA--AA
Porphyra suborbiculata ------------G------ATCCTTTCGCAGGAAA-ACGTACACGAAGCCTTTGCAGCCCGAT-AAGGGTGCACAGTACGCGACT--------CTAA--AA
Porphyra purpurea       ---------GTG------GTCCTTCCGTGGGAAG-ACGTACACGAAGCCTTTGCAGCCCGA--AAGGGTGCACAGACCGCGACT--------ATAA--A-
Porphyra miniata        GC------CACG------CTCCTTCCGTAGGAAA-ACGTACATGAAGCCTTTGCGGCCCGA--AAGGGTGCACAGTGCGCGACT--------ATAA--A-
Hildenbrandia rubra     ---------------------------------------------AGCCTTTGCAGCCCGATGAAGGGTGGGCGCT-CGCGACTAG--ATAAATAATGAC
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Porphyra umbilicalis   GAATCGACTTG-----AATGCAAGTGGCGGGCGAGCTCATCGGACCGGAGAGAAGAATCTGGCGCCATTCTACATTGAGTGAGTTGTCTGCACGCCCTTT
Porphyra dioica        GAATCGACTTG-----AATGCAAGTGGCGGGCGCGCTCATCGGACCGGAGAGAACAATCTGGCGCCATTTTATATTGAGTGACTTGTCTGCACGCCCTTT
Porphyra linearis      GAATCGGCTTG-----AATGCAAGCAGCGGAC--------------------------------------------------------------------
Porphyra leucosticta   GAATCGACTTG-----AATGCAAGCGGCGGATA---------------GAAGCACA-------GCCA------------------GCCAGCCCGCTTCTT
Porphyra suborbiculata CACTGAGACTGTGGTCAATGCAAGCGGCGG----------T-G--TGGAGAG------------------------------TTTGCCT---CGCTTC--
Porphyra purpurea      TACTGTTTCTGTGGTCAATGCAAGCAGCGG----------TGG--TGG-------------------------------------TCC------GCCCGA
Porphyra miniata       TACTGATCCTGTGGTCAACGCAAGCAGCGG----------TAG--TGG-------------------------------------GCCT---TGGC----
Hildenbrandia rubra    GAAT--GCCTGA----AATGCAAGCGGCGGA----------------------------------------ACATC------------------------

                                                                                                   P2'      P3         P4
Porphyra umbilicalis   AAAATGGCGGGATGGCAATTTT-----GCACAAGCATTAGGGTGCCTTATCCTTCCGGTTTGTCGAACTGCGTGTCAGCTGCGACTTTCTCAAATTGCGG
Porphyra dioica        AAAATGGCGGGACGGCATTTTT-----GCACAAGTATTAGGGTGCCTTGTCCTTCCGGTTCGTCGAACTGCGTGTCAGCTGCAACTTTCTCAAATTGCGG
Porphyra linearis      ------------------TTTT-----G---------------------------------------------GTCAGCTGCGACTTTCTCAAATTGCGG
Porphyra leucosticta   ---------------------------------------------------------------------------CAGCCGCGACTTTCTCAAATTGCGG
Porphyra suborbiculata AACGGGGCGG------------CACACACACACACA--------------C-----A------------------CAGCTGCGACTTTCTCAAATTGCGG
Porphyra purpurea      AAAAGGGCGGGAGAA------------GC-C-------------------------A------------------CAGCTGCGACTTTCTCAAATTGCGG
Porphyra miniata       -----GGCGGGGGACTTTTTCCCCCGC-CGC-CG----------------CCCTCTA------------------CAGCTGCAACTTTCTCAAATTGCGG
Hildenbrandia rubra    ------------------TCTT--------------TTAGGGTG--------------TTTG---------------GCCGCGATCTTCTCAAATTGCGG
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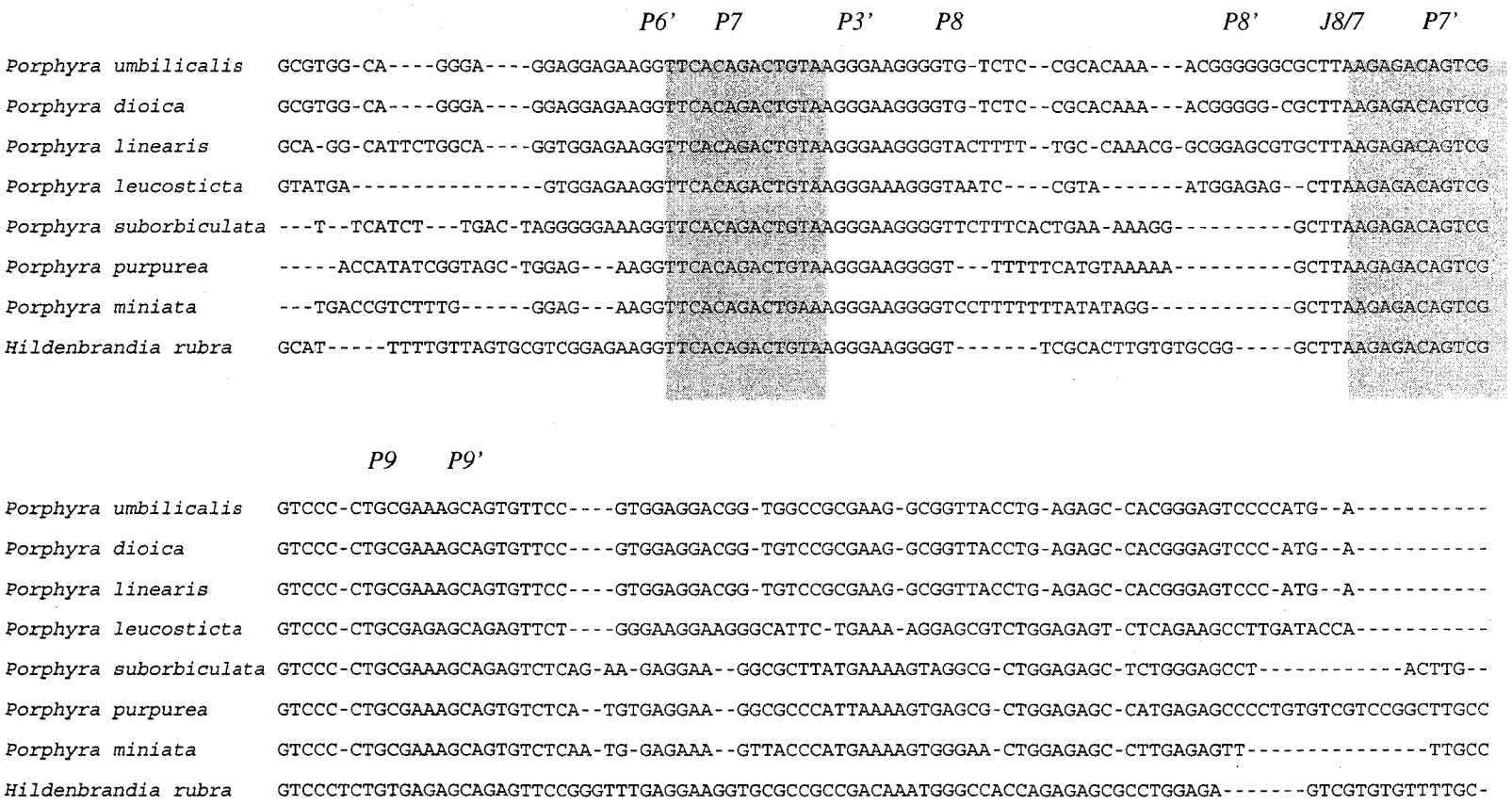
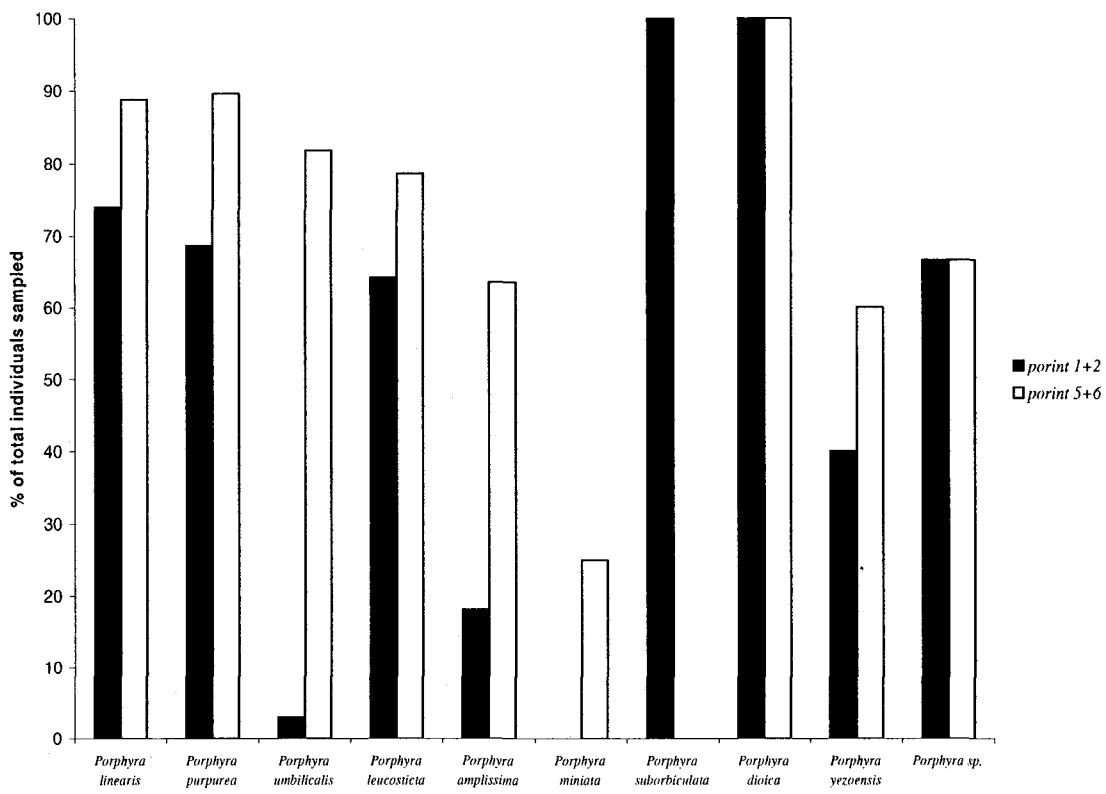
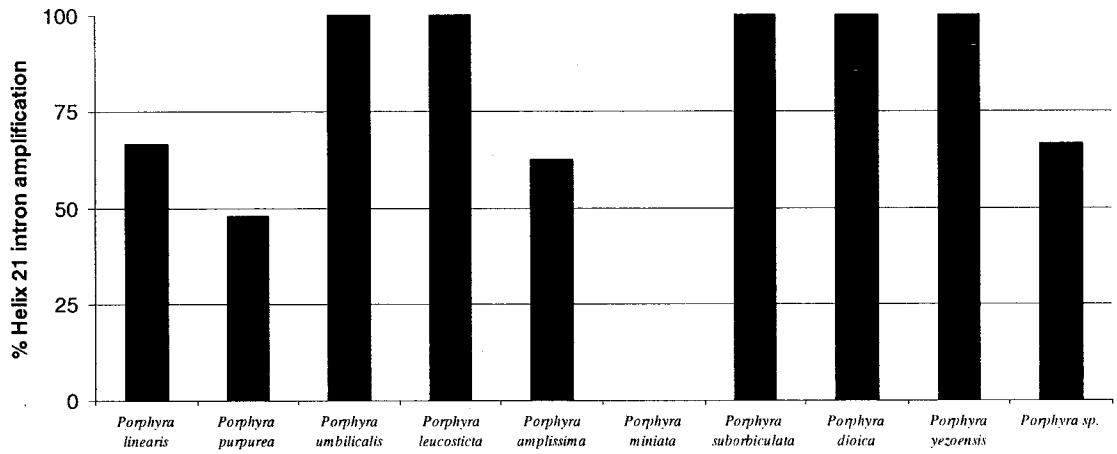


Figure 5. Percent comparison of successful H50 Intron Amplification in a sub-sample (Total N=181) of the *Porphyra* Survey Using Flanking Primers (*porint*1+2) and Intron Specific Primers (*porint* 5+6). *Table* represents number of samples screened for each taxon.



porint 1+2	40	33	1	9	2	0	1	3	4	2
porint 5+6	48	43	27	11	7	1	0	3	6	2
Total (N)	54	48	33	14	11	4	1	3	10	3

Figure 6. Amplification results of Helix 21 Group-I introns using the *Int5pr1* and *Int5pr2* primer pair from a survey of 88 *Porphyra* individuals. *Table* represents actual data.



Int5pr1&2 (H21)	10	10	9	14	5	0	1	3	10	2
Total (N)	15	21	9	14	8	4	1	3	10	3

Figure 7a. Phylogram of Helix 50 Group-I Intron using Maximum Parsimony analysis. Numbers represent bootstrap support from resampling of 1000 replicates. *Parentheses*= bootstrap support from weighted analysis.

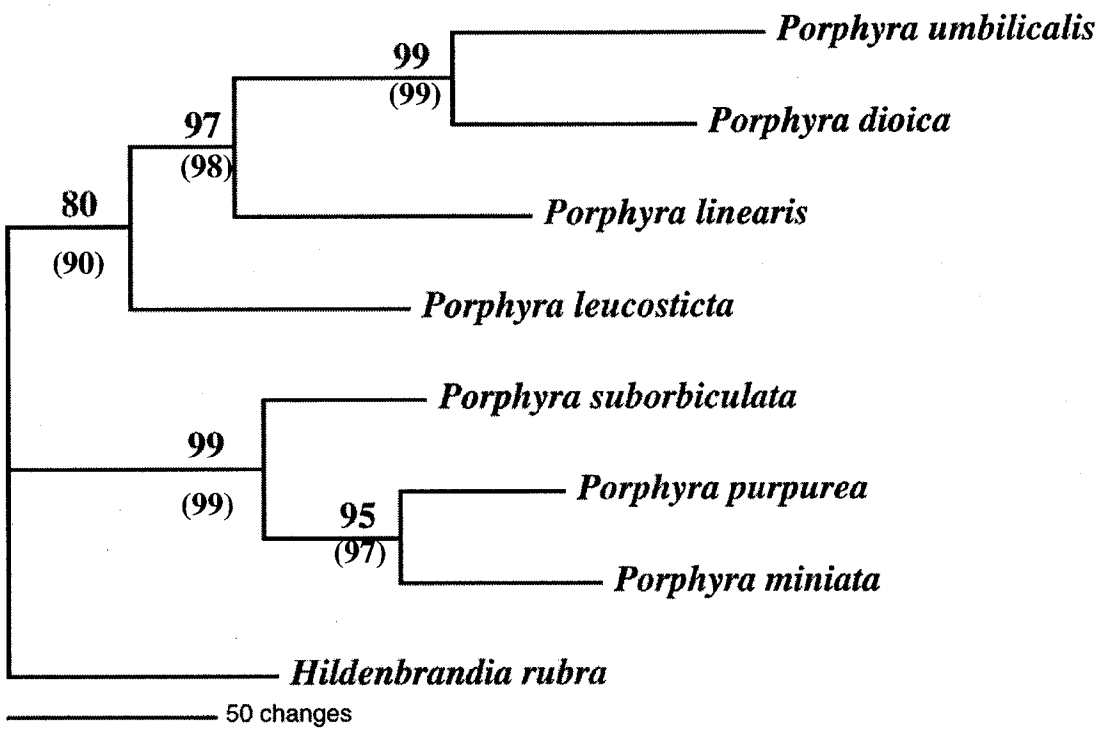


Figure 7b. Phylogram generated from 910 bp from the SSU of the rRNA gene in *Porphyra* using Maximum Parsimony analysis. Numbers represent bootstrap support from resampling of 1000 replicates (recalculated from data given in Klein *et al.*, 2003).

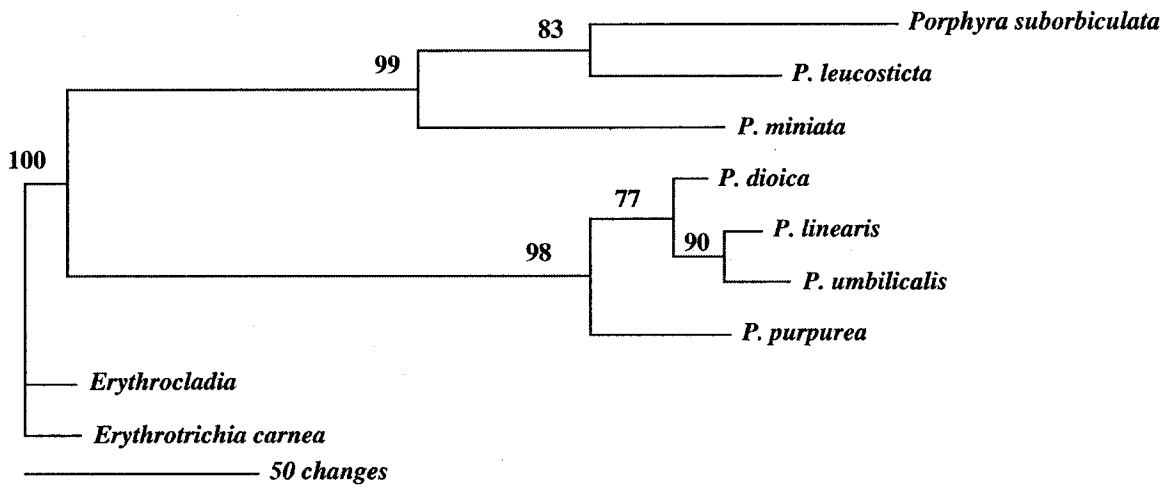
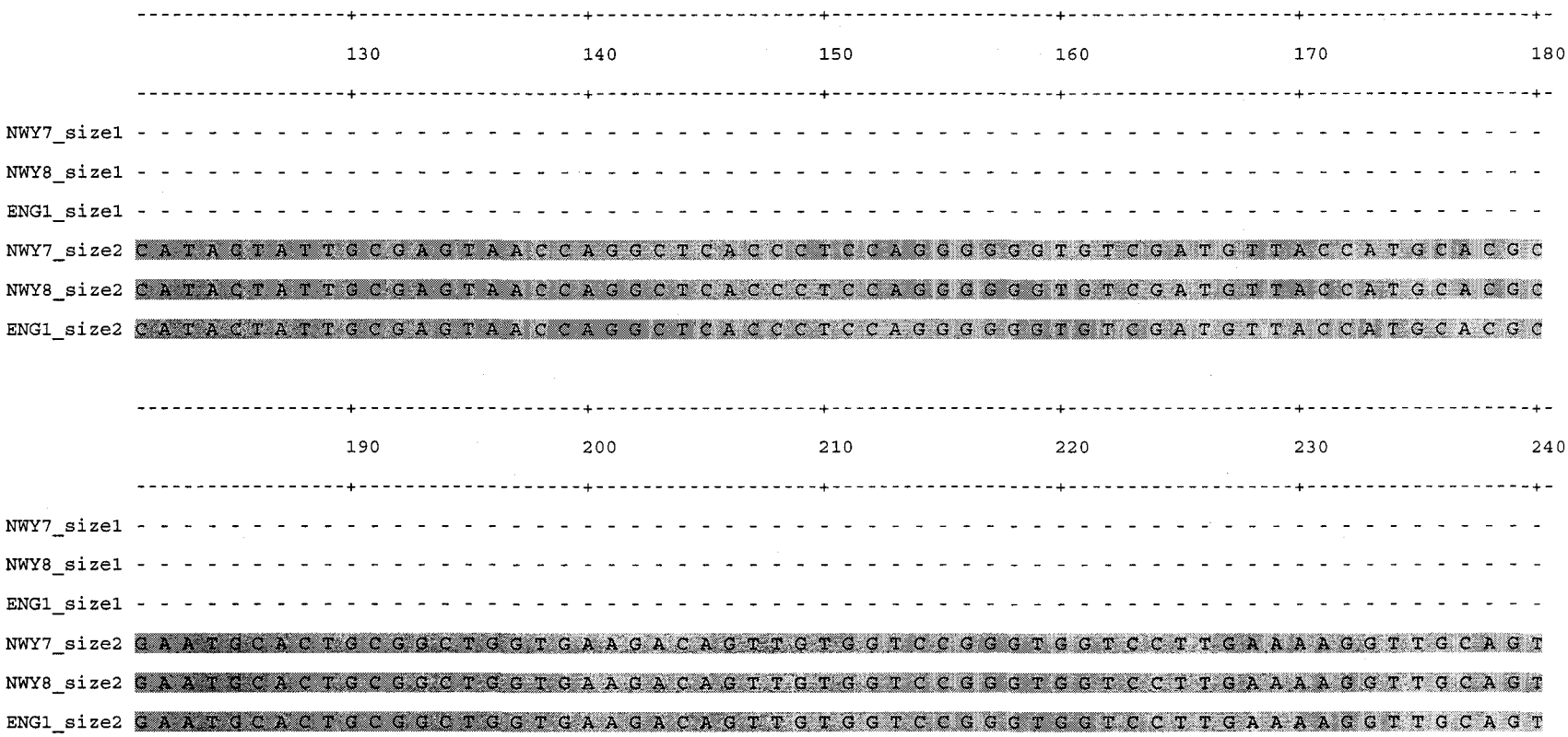
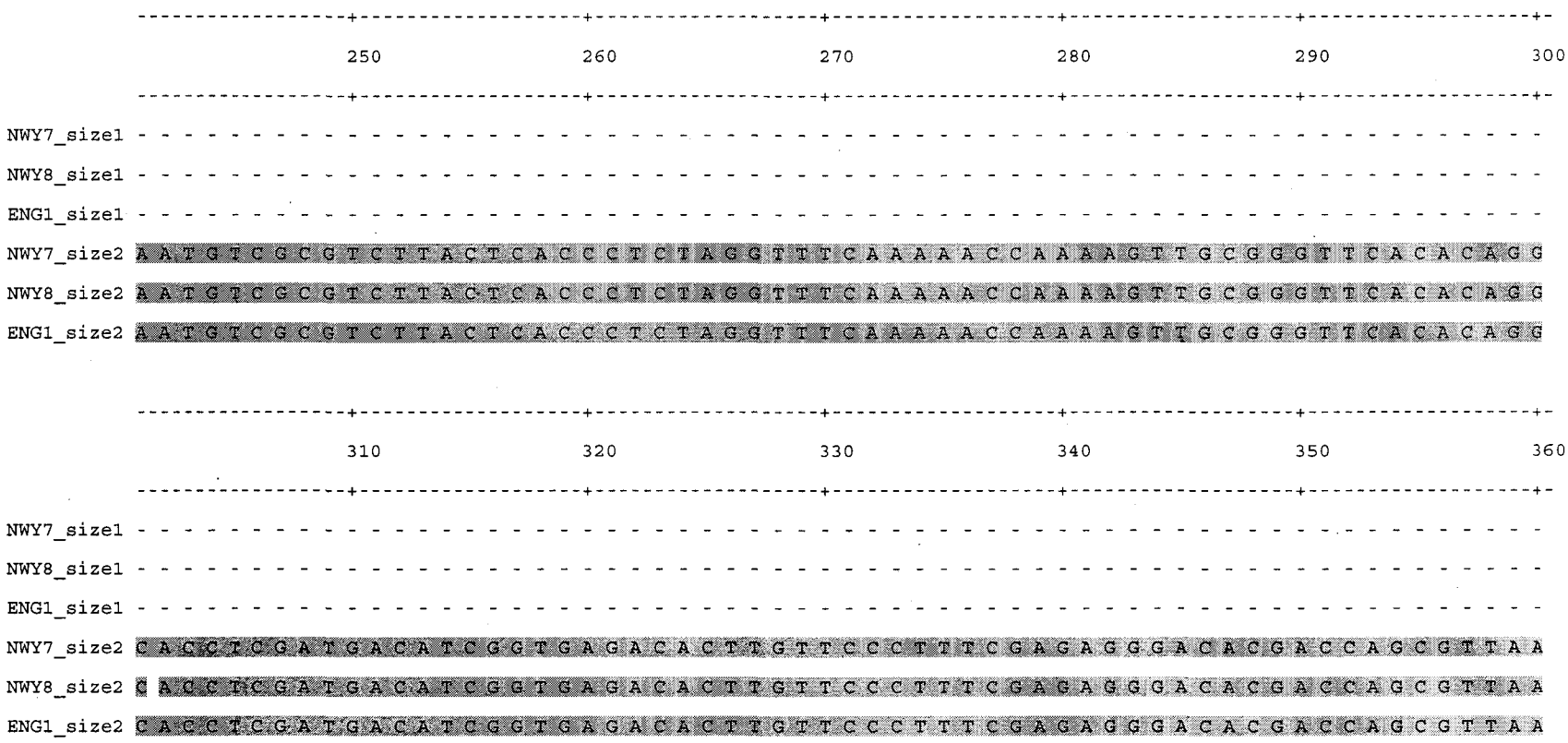


Figure 8. Sequence alignment of the Helix 50 Group-I intron in three *Porphyra umbilicalis* individuals. Each individual contained two different size variations of the inton based on the presence and absence of a His-Cys homing endonculease ORF near the 5' end of the intron (see bp position 88).

```
                    10        20        30        40        50        60
            ---------+---------+---------+---------+---------+---------+-
NWY7_size1  ttttaggagtcgtaacaaggttTGCTAAGGCNACAGAAAACAGACTATGATGGACATGCA
NWY8_size1  -----------------------------------------AGACTATGATGGACATGCA
ENG1_size1  ---------------------------------------------------------GCA
NWY7_size2  ---------------------------AGGCAACTGAAAACAGACTATCAGGGACGTGCA
NWY8_size2  ------------------------------------------------------CGTGCA
ENG1_size2  -------------------------------------------------AGGGACGTGCA

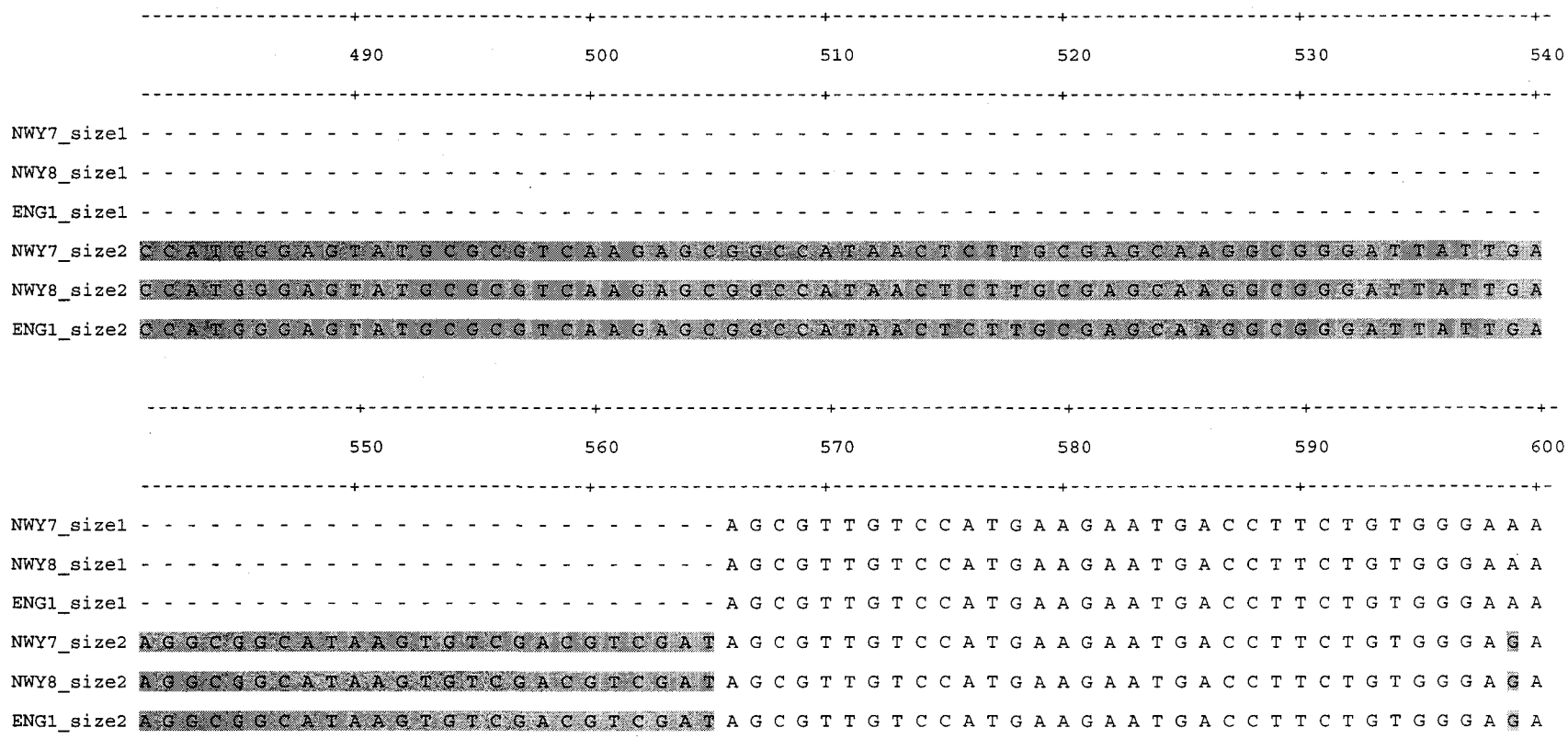
            ---------+---------+---------+---------+---------+---------+-
                    70        80        90       100       110       120
            ---------+---------+---------+---------+---------+---------+-
NWY7_size1  ACCAATCGACCACTCATGTCGGCCACC---------------------------------
NWY8_size1  ACCAATCGACCACTCATGTCGGCCACC---------------------------------
ENG1_size1  ACCAATCGACCACTCATGTCGGCCACC---------------------------------
NWY7_size2  ACCAATCGACCACTCATGTCGTCCACCCTCAAGCGTATCCCTCGTTTACTATATATCGTT
NWY8_size2  ACCAATCGACCACTCATGTCGTCCACCCTCAAGCGTATCCCTCGTTTACTATATATCGTT
ENG1_size2  ACCAATCGACCACTCATGTCGTCCACCCTCAAGCGTATCCCTCGTTTACTATATATCGTT
```

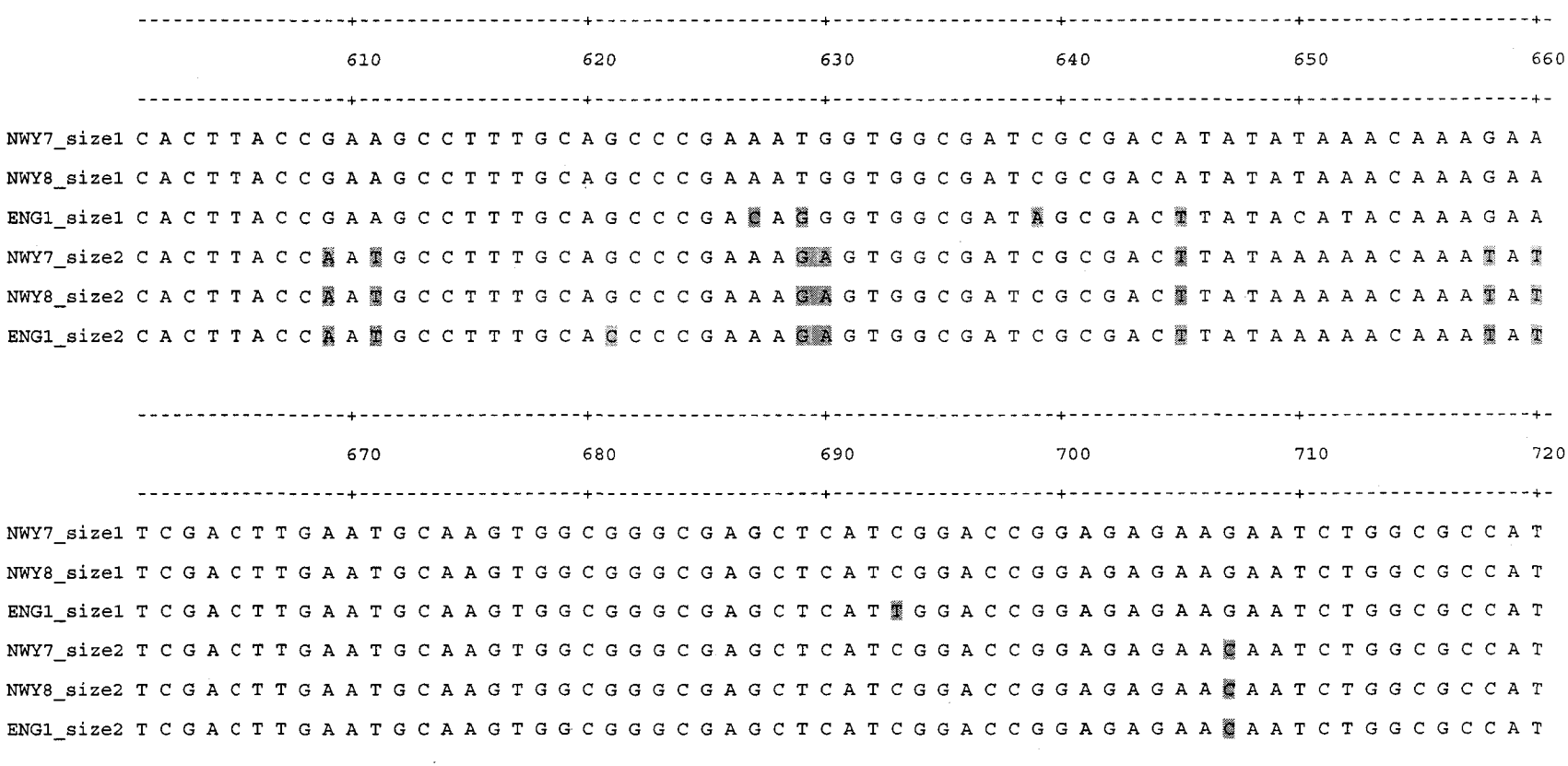


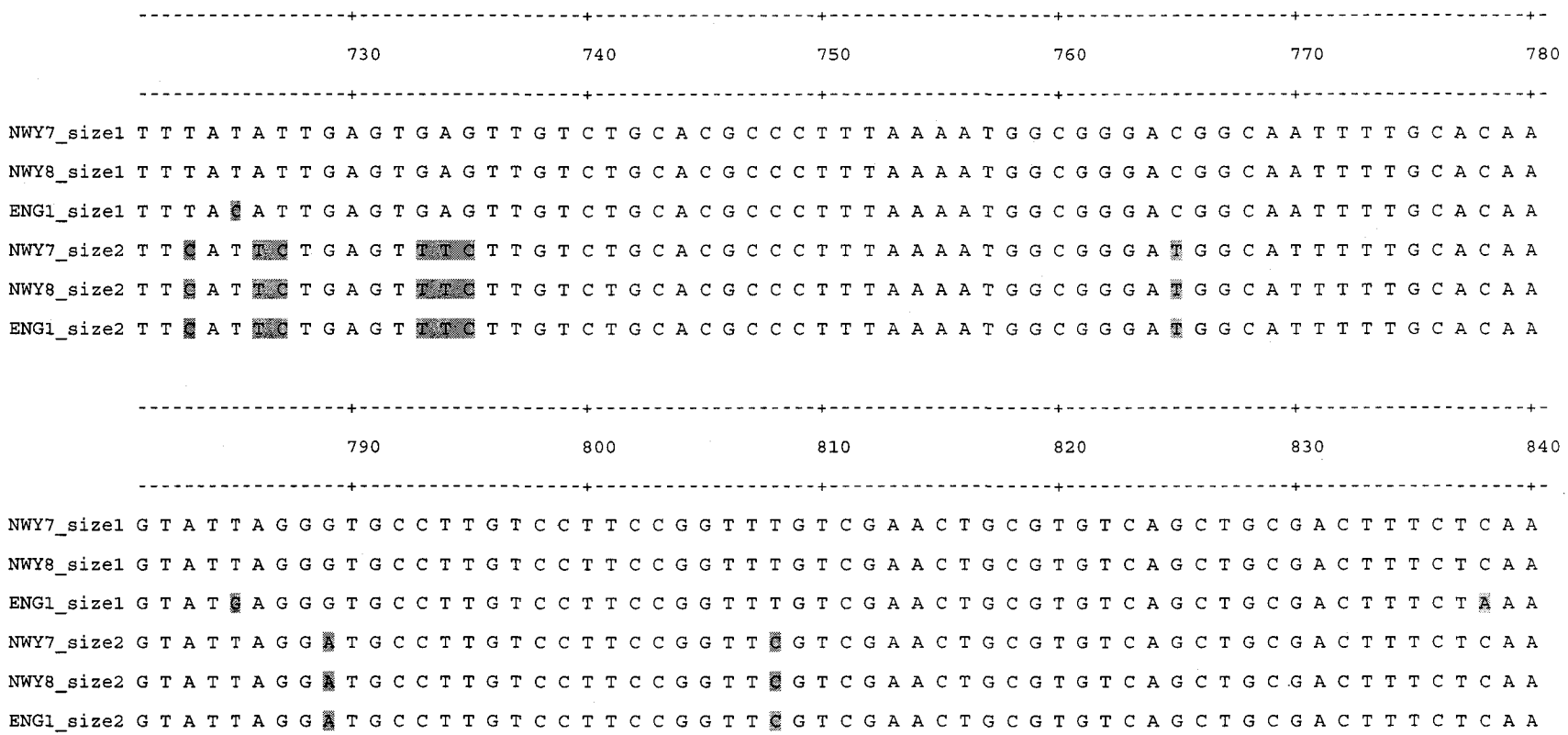


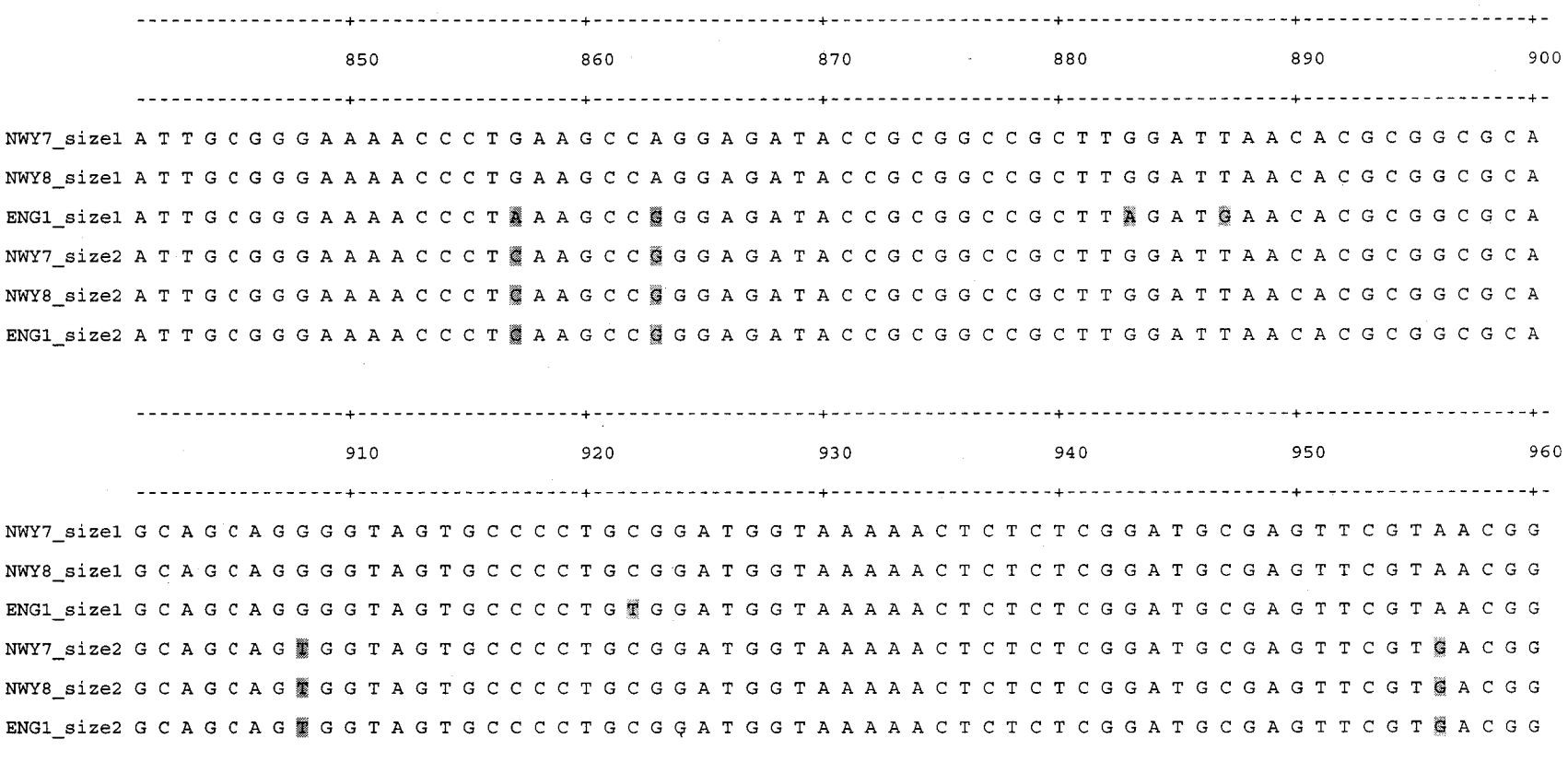
```
               ----------------+-----------------+-----------------+-----------------+-----------------+-----------------+-
                              370               380               390               400               410               420
               ----------------+-----------------+-----------------+-----------------+-----------------+-----------------+-
NWY7_size1     ------------------------------------------------------------
NWY8_size1     ------------------------------------------------------------
ENG1_size1     ------------------------------------------------------------
NWY7_size2     AGATGCAGGCGACGACGTGACCGTAGTAAATACGGCCATGCATGCGCAGCTGCACGGAAC
NWY8_size2     AGATGCAGGCGACGACGTGACCGTAGTAAATACGGCCATGCATGCGCAGCTGCACGGAAC
ENG1_size2     AGATGCAGGCGACTACGTGACCGTAGTAAATACGGCCATGCATGCGCAGCTGCACGGAAC

               ----------------+-----------------+-----------------+-----------------+-----------------+-----------------+-
                              430               440               450               460               470               480
               ----------------+-----------------+-----------------+-----------------+-----------------+-----------------+-
NWY7_size1     ------------------------------------------------------------
NWY8_size1     ------------------------------------------------------------
ENG1_size1     ------------------------------------------------------------
NWY7_size2     CAGCAGGGTTTACCACAGCGTAGTTAGTTCTGTAGCAGCTAAACGTACCAATGCGAAATG
NWY8_size2     CAGCAGGGTTTACCACAGCGTAGTTAGTTCTGTAGCAGCTAAACGTACCAATGCGAAATG
ENG1_size2     CAGCAGGGTTTACCACAGCGTAGTTAGTTCTGTAGCAGCTAAACGTACCAATGCGAAATG
```









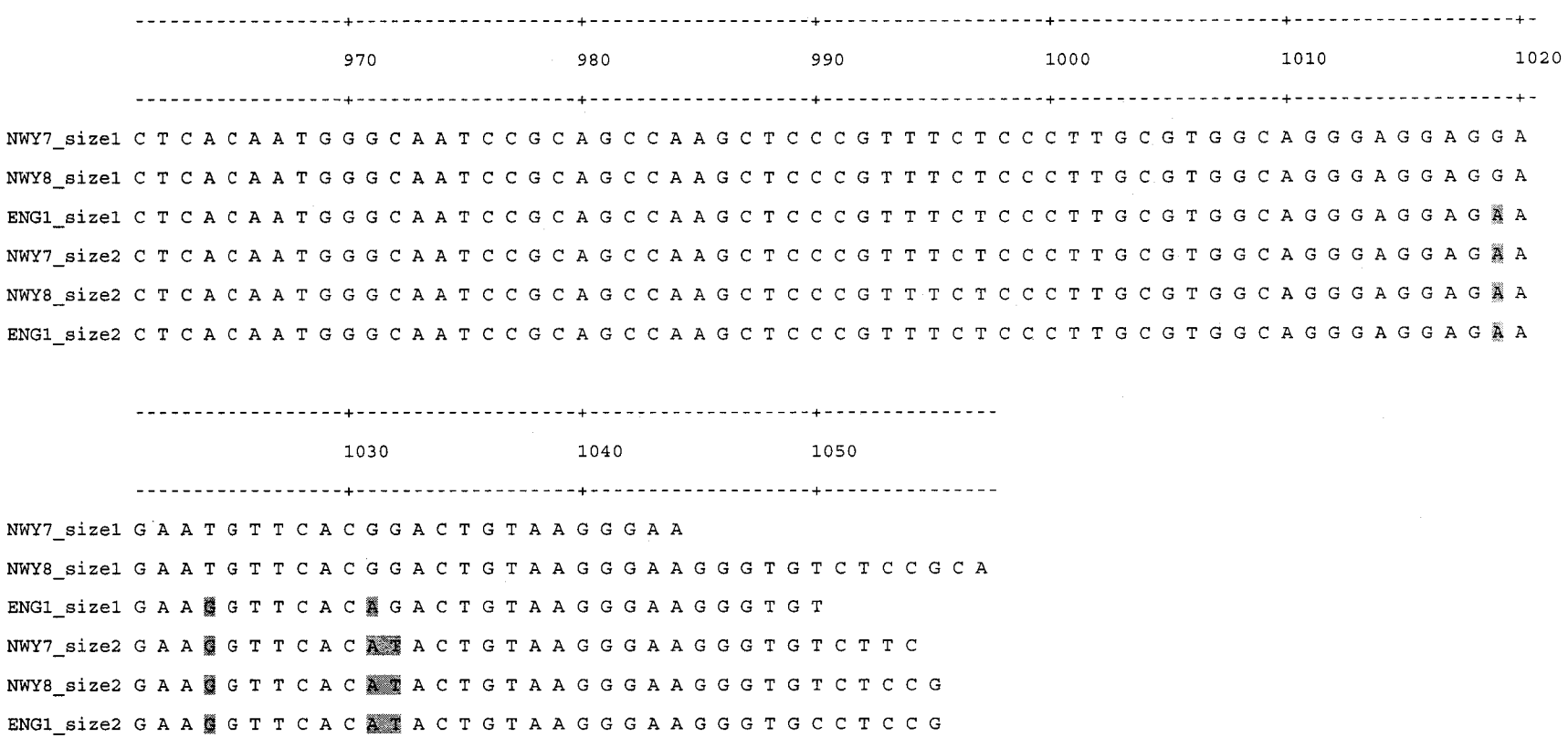


Table 6. Percent sequence similarity between helix 50 introns without a His-Cys ORF (Intron 1) and introns with the insertion (Intron 2) in three individuals of *Porphyra umbilicalis* that contain both types. The ORF was not included in the percent identity calculation.

Porphyra umbilicalis isolate	NWY7(1)	NWY8(1)	ENG1(1)	NWY7(2)	NWY8(2)	ENG1(2)
NWY7-Intron 1						
NWY8-Intron 1	100					
ENG1-Intron 1	96.2	96.2				
NWY7-Intron 2	94.2	94.2	93.3			
NWY8-Intron 2	94.2	94.2	93.3	100		
ENG1- Intron 2	93.9	93.9	93.1	99.8	99.8	

Figure 9. The proposed secondary structure of Helix 50 group-I intron in *P. umbilicalis* with secondary structure annotations (Burke *et al*, 1987; Michel and Westhof, 1990) as follows. P, Q, R, and S represent regions of highly conserved sequence motifs. P1-P9 annotations represent sequential base pairing regions. *lowercase* letters= 18S rRNA; *uppercase*= intron RNA

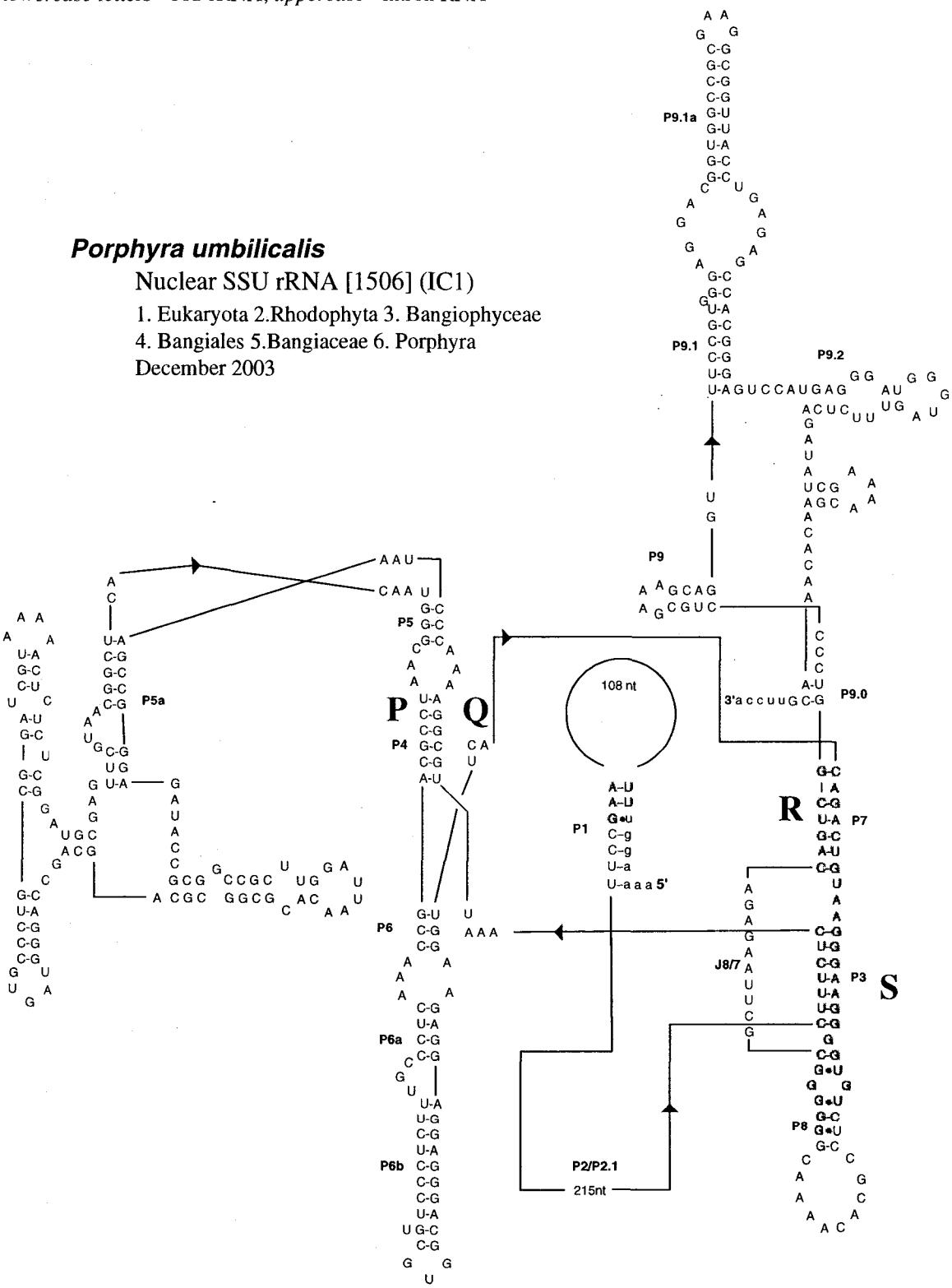


Figure 10. The 453 bp P1 extension sequence from *Porphyra umbilicalis* isolate (GenBank #). The ORF sequence is shown in *uppercase*, flanking sequence in *lowercase* letters, with the corresponding amino-acid sequences given above the ORF. Nucleotides corresponding to the conserved U:G pair in P1, which define the 5'-splice site, are in *bold*. Note that the ORF is located on the complementary strand.

```
tttgctaaggcactaaaacagactatcatggacatgcaaccaatcgaccacccatgtcgtccaccttcaagcgaatccct

                          *  Q  P  Y  G  P  E  G  E  L  P  T  D  I  N  G  H  V  R
cgtttactttctatcgttcataCTACTGCGGGTAACCAGGCTCACCCTCCAGGGGGGTGTCGATGTTACCATGCACGCGA

 I  C  Q  P  Q  H  L  C  N  H  D  P  H  D  K  F  H  D  C  Y  Q  R  T  K  N  V  R
ATGCACTGCGGCTGATGAAGACAGTTGTGGTCCGGGTGGTCTTTGAAATGGTCGCAGTATTGTCGCGTCTTGTTGACCCT

  E  T  E  F  V  L  H  Q  P  N  V  C  A  G  R  H  C  R  H  S  A  Q  E  G  E  V
CTCGGTTTCAAAGACCAAATGCTGCGGGTTCACACAGGCACCCCGATGACAACGGTGAGACGCTTGTTCCCCTTCGACAG

P  V  R  G  A  N  F  I  C  A  V  V  H  G  Y  Y  K  R  G  H  L  R  L  Q  V  Y  G
GGACACGACCAGCGTTGAAGATGCAGGCGACGACGTGACCGTAGTACTTACGGCCATGCAAGCGCAGCTGCACATAACCA

 A  P  K  V  V  A  Y  N  T  R  Y  C  S  F  T  G  I  R  F  A  M  P  T  H  A  D  L
GCAGGTTTCACCACAGCGTAGTTAGTTCTGTAGCAGCTAAACGTACCAATGCGAAATGCCATGGGAGTATGCGCGTCAAG

  A  A  M  A  R  A  L  L  A  P  N  N  Y  A  A  Y  N  N  V  D  I  A  N  D  M
AGCGGCCATAGCCCTGGCGAGCAAGGCGGGATTGTTGTAGGCGGCATAATTGTTGACGTCGATAGCGTTGTCCATgaaga

atgaccttctgtgggaaacacttaccgaag
```

Figure 11. Similar putative endonuclease sequences from the Bangiophyceae in the Helix 50 ribosomal group-I intron. The amino acid sequence of the *Porphyra umbilicalis* intron ORF found in four individuals (Pumb) is aligned to the corresponding sequences from *Porphyra spiralis* (Psp 1), and *Bangia atropurpurea* (Bat 1) as given in Haugen *et al.* (1999). Identical positions are indicated by *dots*, deletions by *dashes*. The His-Cys box motif (***bold line***) is compared to the homing endonuclease I-*Ppo*I. Conserved residues proposed to be directly involved in zinc binding (C100, C105, H110, C125, C132, H134, C138) and the active site (H98, N119) of the I-P*po*I endonuclease (Flick *et al.*, 1998) are indicated by shading.

```
PUMB_IRE2  M D N A I D V N N Y A A Y N N P A L L A R A M A A L D A H T
PUMB_NWY7  . . . . . . . D T . . . F . . . . . . . . V . . . . . . . .
PUMB_NWY8  . . . . . . . D T . . . F . . . . . . . . V . . . . . . . .
PUMB_ENG1  . . . . . . . D T . . . F . . . . . . . . V . . . . . . . .

PUMB_IRE2  P M A F R I G T F S C Y R T N Y A V V K P A G Y V Q L R L H
Psp 1                                                 . . . . . . Y Q
BAT 1                                                 . . . . M . I D
PUMB_NWY7  . . . . . . . . . . . . . . . . . . . . N . . . S . . . . M .
PUMB_NWY8  . . . . . . . . . . . . . . . . . . . . N . . . S . . . . M .
PUMB_ENG1  . . . . . . . . . . . . . . . . . . . . N . . . S . . . . M .

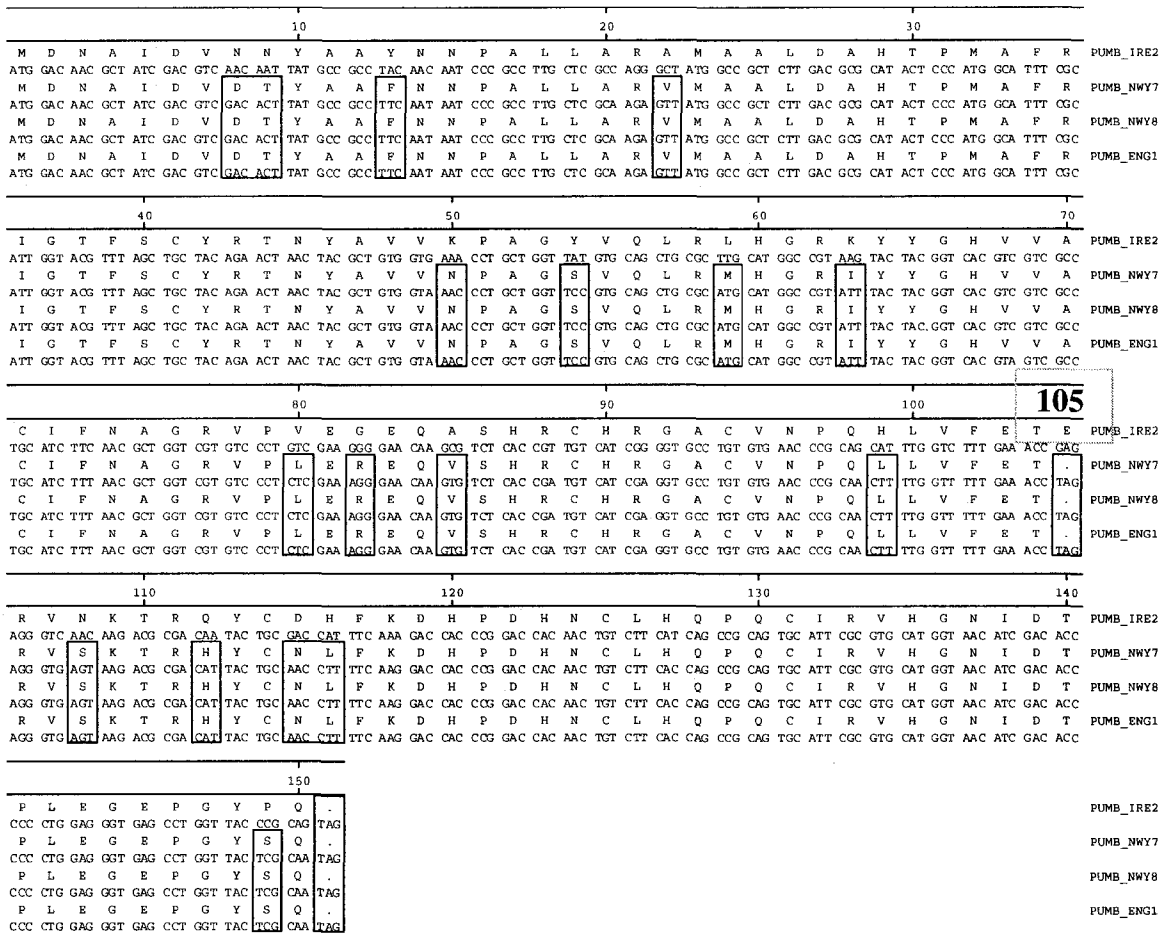
PUMB_IRE2  G R K Y Y G H V V A C I F N A G R V P V E G E Q A S H R C H
Psp 1      . T . . . C . I I V A V A A T E . L . L P . . E . . . T . .
BAT 1      . . . . . C . L I S T . A A F N . L . T . . . E . . . . . .
I-Ppo I                                                   . . . L . .
PUMB_NWY7  . . I . . . . . . . . . . . . . . . . . L . R . . V . . . . .
PUMB_NWY8  . . I . . . . . . . . . . . . . . . . . L . R . . V . . . . .
PUMB_ENG1  . . I . . . . . . . . . . . . . . . . . L . R . . V . . . . .
                                                          H98   C100

PUMB_IRE2  R G A C V N P Q H L V F E T E R V N K T R Q Y C D H F K D H
Psp 1      N A K . . . K A . . T L . S G D L . . S . I . . R L M R N .
BAT 1      N A K . . . L . M A . . S G D . . . S . L . . R L . . N .
I-Ppo I    N T R . H . . L . . C W . S L D D . . G . N W . - - - - P G
PUMB_NWY7  . . . . . . . L . . . . . . . . . S . . . H . . N L . . . .
PUMB_NWY8  . . . . . . . L . . . . . . . . . S . . . H . . N L . . . .
PUMB_ENG1  . . . . . . . L . . . . . . . . . S . . . H . . N L . . . .
                C105          H110                N119          C125

PUMB_IRE2  P D H N C L H Q P Q C I R V H G N I D T P L E G E P G Y P Q
Psp 1      . E f k . . . T . . V *
BAT 1      . E F K . . . V . E . H T Q T *
I-Ppo I    . N G G . V . A V V L . - Q . P L Y G . G A T V A . P Q .
PUMB_NWY7  . . . . . . . . . . . . . . . . . . . . . . . . . . . . S .
PUMB_NWY8  . . . . . . . . . . . . . . . . . . . . . . . . . . . . S .
PUMB_ENG1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . S .
              C132  H134      C138

PUMB_IRE2  . *
Psp 1
BAT 1
I-Ppo I    R G S H F V V *
PUMB_NWY7  . *
PUMB_NWY8  . *
PUMB_ENG1  . *
```

Figure 12. The amino acid and nucleotide alignment of ORFs in four individuals of *Porphyra umbilicalis*. Amino acid changes differing from isolate IRE-2 are *boxed*. A change in amino acid at position 105 (***bold***) resulted in a stop codon in NWY7, NWY8, and ENG1.



CHAPTER III

GENETIC VARIATION AND BIOGEOGRAPHICAL BOUNDARIES WITHIN *PORPHYRA UMBILICALIS* (BANGIOPHYCIDAE, RHODOPHYTA).

Abstract

Information about historical periods of climate change can provide a framework for investigating how marine communities may have adapted to changes both geographically and ecologically. The spatial distribution of variable haplotypes from the nuclear ribosomal DNA internal transcribed spacer region (ITS1-5.8S-ITS2) and a ribosomal DNA group-I intron from the obligate, rocky intertidal red alga, *Porphyra umbilicalis* Kützing were used to reconstruct its biogeographic history since the last glacial maximum in the North Atlantic. Haplotype distributions from European and North American samples representing the range of *P. umbilicalis* are consistent with the hypothesis that North American populations were extirpated during the last glacial maximum and subsequently recolonized from European donor populations. A non-coding intergenic region between the mitochondrial cytochrome oxidase (*cox*)2 and *cox*3 genes was also investigated but because it exhibited extremely low levels of intraspecific variation, the spacer was not useful for testing phylogeographic hypotheses.

Introduction

The North Atlantic is primarily a cold-temperate biogeographical region (Briggs, 1974; Hoek, 1975; Lüning, 1990) with the southern bi-continental boundaries of Cape Hatteras, (North Carolina, USA) and the English Channel (Europe) and corresponding northern boundaries at the Strait of Belle Isle (Newfoundland, Canada) and the Barents Sea at Kola fjord (Finland) (Hooper *et al.*, 2002; Lüning, 1990). The North Atlantic coastlines contain diverse assemblages of intertidal marine organisms including macroalgae, many of which are endemic to the cold-temperate habitats of the North Atlantic. Of these seaweeds, many are common to both the eastern and western coasts of the North Atlantic. However, the degree of endemism between the coasts contrasts significantly with Europe exhibiting both higher species diversity and number of endemics versus the Northwest Atlantic. Hoek (1975) attributed most of the discontinuities and differences between the two coasts to the degree of the Pleistocene glaciation; that is, although these coastlines share many similarities (*i.e.*, substrata, surface seawater isotherms), the historical events that influenced current seaweed distribution contrast significantly. For North Atlantic intertidal marine communities, the last glacial maximum of ~20,000 years ago (McIntyre *et al.*, 1976; Holder *et al.*, 1999) is thought to have been especially harsh for obligate rocky intertidal species on the North American coast (Ingolfsson, 1992; Riggs *et al.*, 1996; Wares and Cunningham, 2001). The last Pleistocene glaciation would have displaced many species from their original (pre-glaciation) habitat since the availability of hard substrata was limited beyond the southernmost extent of the glaciers at Long Island Sound, except for a few off-shore reefs

(Ingolfsson, 1992; Riggs *et al.*, 1996). For many cold-temperate seaweeds, attachment to hard substrata is a requirement for survival and this change in available habitat probably resulted in localized extirpation. Additionally, it is probable that a steep temperature gradient existed below the glaciation that would inhibit the survival of arctic and cold-temperate species (van den Hoek and Breeman, 1990). If extirpation occurred, then most of the current Northwest Atlantic rocky intertidal species were probably recruited from European "donor" populations where the Pleistocene glaciation was less severe and hard-substratum was more available (Vermeij, 1991; van Oppen *et al.*, 1995).

Ingolfsson (1992) was the first to look at marine organisms that required rocky substrata for attachment and recruitment in the North Atlantic. His comparison of species composition in Northern Norway, Iceland, and the Canadian Maritimes suggested that the last two communities are impoverished subsets of the rocky shore fauna of Northern Norway; this observation is in agreement with a hypothesis of post-glacial dispersal of marine organisms from Europe. Based on van den Hoek and Breeman's (1990) comparison of seaweeds from the cold temperate Northeast Atlantic with the cold temperate Northwest Atlantic, Ingolfsson (1992) speculated that rocky shore algae in the North Atlantic should present a similar picture to his study of marine animals.

Wares and Cunningham (2001) revisited the post-glacial recolonization hypothesis for rocky, intertidal species using mitochondrial DNA sequences from three cold-temperate obligate rocky intertidal species (*Semibalanus balanoides* L., barnacle; *Nucella lapillus* L. and *Littorina obtusata* L., gastropods), as well as three generalist species (*Asterias rubens* L., sea star; *Mytilus edulis* L., mussel; and *Idotea balthica* Pallas, isopod). Using allelic diversity measurements and estimated lineage-specific

mutation rates, they provided evidence supporting the hypothesis that some obligate rocky intertidal taxa had colonized New England from European populations. Their work also suggested that in some of these organisms, life history traits, including mechanisms of dispersal, might have played an important role in organisms surviving the drastic changes associated with the last glacial maximum. The study was followed by a more detailed investigation of *Asterias* (Wares, 2001a), using both the cytochrome oxidase I mitochondrial gene and a fragment of the nuclear ribosomal internal transcribed spacer. The results of this analysis suggested an initial vicariance of *Asterias* populations during the formation of the Labrador Current 3.0 million years ago (Ma) followed by recent recolonization of the Northwest Atlantic populations from a European source population.

In contrast, an alternative hypothesis of post-Pleistocene recolonization by Wilce (unpubl., in South, 1983) attributed the low endemism of shore algae on the western coasts of the Atlantic to a one-way post-glacial exchange of species by the Paleo-Gulf Stream (Cronin, 1980) from America to Europe. Wilce emphasized that the Gulf Stream is a more effective vector for transporting cold-temperate species than for species migrating in the east-west direction. His hypothesis adds one more factor besides glaciation and substratum barriers to the differences in species richness between the two continents.

The large-scale biogeographic patterns observed today can be linked to vicariant events of the past such as fragmentation of continents, emergence of land barriers, and extreme shifts in global temperature (*i.e.,* glaciation events) through phylogeographic

studies (Avise, 2000). *Porphyra umbilicalis* Kützing., an obligate[12], rocky-intertidal red alga (Bird and McLachlan,1992) is a suitable species to test these models of historical migration following glaciation. The species has a cold-temperate distribution in the North Atlantic (North America, Greenland, Iceland and Europe), it requires a hard substratum for attachment, and resides in an intertidal environment that would have been severely impacted by ice sheets. Additionally, the species' North Atlantic boundaries are unclear because it is frequently confused with similar taxa such as *P. purpurea* (Roth) C. Agardh, *P. birdiae* Neefus *et* Mathieson, and *P. insolita* P. Kornmann *et* P.-H. Sahling (Bird and McLachlan, 1992; Mitman and van der Meer, 1994; Klein *et al.*, 2003).

Based upon the hypothesis that substratum requirements played a major role in the species' historical biogeography, genetic differences were examined among *Porphyra umbilicalis* isolates to describe the post-glacial recolonization of the species in New England and the Canadian Maritimes. The DNA sequences from both the mitochondrial (*cox*2-*cox*3 spacer) and nuclear genomes (ITS1 and 2 spacer region; SSU rDNA Helix 50 group-I intron) were used to examine the patterns of intraspecific variation within *P. umbilicalis*.

The mitochondrial encoded *cox2-cox3* (COX) intergenic spacer region separates the cytochrome oxidase subunits 2 and 3 (*cox2/cox3*) genes. The spacer has been used to examine intraspecific variation in animals and has proven to be a useful, highly variable molecular marker. The variation found in COX spacer region is not as great in plant mitochondrial DNA, which may explain its scarcity of use in studies of other

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[12] Records of an epiphytic form, *Porphyra umbilicalis* forma *epiphytica* F.S.Collins (1903), are here treated as *Porphyra leucosticta,* based on personal observations and a review of the literature.

photosynthetic organisms such as marine algae. However, the large evolutionary divergence between marine red algae and land plants (Baldauf *et al.*, 2000) should encourage examination of the mitochondria as a candidate organelle for variable markers. Recent work in the Rhodophyta has shown that the COX region is variable within populations of the red algae *Caloglossa leprieurii* (Montagne) G. Martens and *Caulacanthus ustulatus* (Mertens ex Turner) Kützing (Zucarello *et al.*, 1999, 2002).

The nuclear ribosomal DNA (rDNA) internal transcribed spacer regions (ITS1 and 2) separate three ribosomal genes (18S-5.8S-26S) in the rDNA cistron of eukaryotes. Though the biological function of the internal transcribed spacers is not fully understood, there is evidence that they may play a role in rRNA processing (Sande *et al.*, 1992). Internal transcribed spacer regions have been shown to evolve at a rapid rate (Bakker *et al.*, 1995) and are therefore useful in resolving differences between closely related species or different populations within species (Wares, 2001b). In marine algae, ITS regions have been utilized in biogeographic studies to support theories of dispersal direction and postglacial recolonization in the Rhodophyta (van Oppen *et al.*, 1995; Rueness and Rueness, 2000; Marston and Villard-Bohnsack, 2002), Phaeophyta (van Oppen *et al.*, 1993), and the Chlorophyta (van Oppen *et al.*, 1993; Bakker *et al.*, 1995; Olsen *et al.*, 1998; and Kooistra *et al.*, 2002). One caveat to using ITS regions is the occurrence of high levels of intra-individual polymorphism in a number of plant and algal species (Bobola *et al.*, 1992; Serrão *et al.*, 1999; Fama *et al.*, 2000) due to incomplete homogenization of ribosomal arrays under concerted evolution (Dover *et al.*, 1982). Recent studies examining ITS variation in *Porphyra* suggest its usefulness for

intraspecific studies of cultured strains (Kunimoto *et al.*, 1999a) and for phylogenetic evaluations (Broom *et al.*, 2002).

Group-I introns are found throughout many distantly related organisms, including green algae, ciliates, myxomycetes, fungi, filose amoeba, euglenids and rhodophytes (Bhattacharya *et al.*, 1994, 1996a,b; Saldanha *et al.*, 1993; Damberger and Gutell, 1994, Müller *et al.*, 2001; Busse and Preisfeld, 2003). Several studies on group-I introns in the small subunit of the ribosomal repeat within Bangiales (Oliveira *et al.*, 1995; Kunimoto *et al.*, 1999) have suggested the possibility of using group-I introns for intraspecific investigations such as strain identification, biogeographical boundaries and phylogeography. The present study represents the first investigation of ribosomal group-I intron variation within *Porphyra* to test phylogeographic hypotheses.

Sequence variation for the mitochondrial COX, nuclear ribosomal ITS1 and ITS2, and a ribosomal group-I intron found within Helix 50 of the 18S gene (see Chapter 2; also known as Intron 1506 in Müller *et al.*, 1998, 2001) were surveyed across the geographical range of *Porphyra umbilicalis* populations from the Northwest and Northeast Atlantic, plus the North Sea. The corresponding sequences were also obtained from the closely related *P. mumfordii* Lindstrom *et* Cole, *P. linearis* Grev. and *P. dioica* J. Brodie *et* L.M. Irvine (Klein *et al.,* 2003; Lindstrom and Fredericq, 2003). The data were used in tandem with spatial and historical biogeography information to test phylogeographic hypotheses on the post-glacial recolonization of obligate, rocky intertidal organisms such as the cosmopolitan red alga *P. umbilicalis.* The present study was also intended to re-examine the current range and species designation of this alga in the North Atlantic.

Materials and Methods

Sample collections.

The specimens used in this study were freshly collected, obtained as herbarium samples, or from silica-gel dried specimens. Table 1 lists the collection, voucher, and GenBank information for each sample. Silica-gel specimens were usually not adequate in size for herbarium vouchers and were used only for DNA extraction. Thirty-four samples of *Porphyra umbilicalis* were collected (18 European; 16 North American) from twenty nine geographical locations (Table 1, Figure 1).

In addition, samples of *Porphyra mumfordii*, *P. linearis*, and *P. dioica* were included in this study. All samples were verified for identification by a species-specific RFLP assay (Teasdale *et al.*, 2002; Chapter 1). The following DNA sequences from other studies were included in various analyses: sequences for the ITS region (*Porphyra umbilicalis*: AJ318959; AB017088, Kunimoto *et al.*, 1999), 18S 1506 intron ribosomal intron (*Porphyra purpurea*: AF172588, Müller *et al.*, 2001) and 18S ribosomal sequence (*P. umbilicalis*: L36049, L26202, Oliveira *et al.*, 1995; AB013179, Kunimoto *et al.*, 1999). The mitochondrial genome of *P. purpurea* (GenBank Accession NC002007; Burger *et al.*, 1999) was used in the alignment and characterization of the COX region for this study.

DNA extraction.

Tissue samples (0.03-0.25 g) were ground in a 1.5 ml microcentrifuge tube using liquid nitrogen and a micropestle. Genomic DNA was extracted either by a standard

CTAB method modified by Stiller and Waaland (1993) or by using the Puregene® DNA Isolation Kit for plant tissue (#D5500A; Gentra Systems, Minneapolis, Minnesota); the samples were either stored at 4° C (short-term) or -80° C (long-term).

Amplification and sequencing.

The ITS1-5.8S-ITS2 region was PCR amplified using a forward primer JBITS7 (Table 2) as prescribed in Broom *et al.* (2002) and a universal reverse primer AB28 (Table 2; Steane *et al.*, 1991). Polymerase chain reactions (PCR) were performed in 50 µL volumes that contained 1-2 µL (25-75 ng) genomic DNA, 0.2 mM of each dNTP, 2.0 mM Mg^{2+}, 1X Magnesium Free Reaction Buffer B (Promega, Madison, WI), 0.4 µM of each primer, and 0.4 µL Taq DNA Polymerase (5 U μL^{-1}, Promega). The amplification profile was as follows: 1 cycle of 3 min at 94; 34 cycles of 1 min at 46°C, 1.5 min at 72° C, 30 s at 94° C; 1 cycle of 1 min at 45° C; 1 cycle of 10 min at 72° C; ending with a hold cycle at 10 °C. Two additional sequencing primers (ITS1-R and ITS2-F) were used for sequence determination (Table 2).

For the ribosomal group-I intron analyses, two sets of primers (H50-1 and H50-2) and INT-5FL and ITS1-R (Table 2; Figure 1 in Chapter II) were designed based on *Porphyra* SSU sequences (Klein *et al.*, 2003) and flanking ITS1 sequence (see above). PCR reactions were performed in 50µL volumes under identical reagent conditions of the ITS (see above). The amplification profile for the H50 primers were as follows: 1 cycle of 3 min at 94° C; 35 cycles of 1 min at 55° C, 1 min at 72° C, 30 s at 94° C; 1 cycle of 1 min at 55° C; 1 cycle of 10 min at 72° C; ending with a hold cycle at 10° C. The INT-5FL/ITS1-R primers were used with the H50 profile, with the exception of a longer

extension time at 72 °C at 1 min 30 s. The amplification product from the H50-1 and H50-2 primers were used for intron sequence comparison, whereas the INT-5FL and ITS1-R primers were specific for *P. umbilicalis* and used to determine intron size and insertion sites by amplifying the full size intron and flanking areas of the rDNA and adjoining ITS1 spacer.

The *cox*2-*cox*3 spacer regions were amplified using the degenerate primer pair cox2-for and cox3-rev (Table 2; Zuccarello *et al.*, 1999). Polymerase chain reaction (PCR) amplifications were done in 50 μL volumes using the following final concentrations: 1X Taq reaction buffer (Promega), 100 μM of each dNTP, 1.8 mM $MgCl_2$, 0.5 μM of each primer, 0.4 units of Taq DNA polymerase (Promega), and 25-75 ng of template DNA. The following reaction profile was used: 1 cycle of 4 min at 94° C; 5 cycles of 1 min 93° C, 1 min 45° C, 1 min 72° C; 30 cycles of 30 s 93° C, 30 s 55° C, 30 s 72° C; 1 cycle for 15 min 72° C, and a hold cycle at 10° C (Zuccarello et. al, 1999).

All PCR amplifications were performed using a PTC-100 MJ Research DNA Thermalcycler (Watertown, MA). For each set of reactions, a control sample containing all reagents but template DNA was included as a test for contamination.

The PCR-product from each individual was separated by size using electrophoresis in a 0.9% low melting point agarose gel and bands were visualized by Ethidium bromide staining. Sizes were estimated using a ΦX/*Hae*III (Promega) DNA ladder under UV light. The amplified bands were excised from the gel and treated with 5 U agarase (Sigma®, St. Louis, Mo.) per 100 μL gel with a 1 h incubation at 37°C before use in direct sequencing reactions.

Cycle sequencing was done using a Perkin Elmer ABI Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA), while sequencing reactions were performed on a thermocycler using the profiles recommended by the manufacturer. Cycle sequencing products were ethanol precipitated and separated on a Perkin Elmer ABI377 Automated Sequencer at the Hubbard Genome Center Sequencing Facility (University of New Hampshire, Durham, U.S.A). All sequences were assembled using the program Seqman. The sequences were then aligned for phylogenetic analyses using Megalign, both of which are part of the Lasergene suite of programs (DNASTAR Inc. Madison, WI).

Cloning of ITS

Purified PCR fragments were used to test for intra-individual variation in ITS. The ITS fragments were cloned using the pGEM®-T Easy Vector System (Promega) following manufacturer's instructions. Plasmid DNA was isolated using the Qiagen mini-prep kit (Qiagen Inc., Valencia, CA, U.S.A).

Haplotype diversity

Haplotype diversity (*h*, Nei, 1987) and its sampling variance were calculated for each continental population for both ITS and intron sequences using Arlequin vers. 2.001.

Phylogenetic analysis

Intraspecific genealogies were inferred in *Porphyra umbilicalis* for both ITS and Helix 50 group-I intron using the phylogenetic criteria of Maximum Parsimony (MP), Maximum-Likelihood (ML), and Statistical Parsimony (SP). The MP algorithm looks for trees that require the fewest changes to explain the differences observed in the taxa under study (Nei, 1987). The MP method is generally used when sequence heterogeneity is low and when mutational rates are different (*i.e.* transitions vs. tranversions). A disadvantage of using the MP method is it can generate numerous trees with the same score. Whereas the ML algorithm, which identifies neighbor pairs that minimize the total length of a tree and evaluates the probability that the chosen evolutionary model (tree) has generated the observed data, results in only one "best" tree (Nei, 1987; Felsenstein, 1988). Finally, the SP method was specifically designed for estimating intraspecific haplotype trees (Templeton *et al.* 1992). Since the MP model minimizes the total number of mutational steps in the tree under a neutral model of intraspecific evolution, it is unlikely for haplotypes that are separated by only one or very few nucleotide differences to have multiple mutational hits at the few sites by which they differ. The SP algorithm thereby makes a correction in the MP assumptions and gives precedence to connections between haplotypes (Clement *et al.*, 2000).

Using the software TCS (Clement *et al.*, 2000), Statistical Parsimony initially defines the uncorrected distance above which the parsimony criterion is violated with more than 5% probability. Subsequently, all connections are established among haplotypes starting with the smallest distances and ending either when all haplotypes are connected or the distance corresponding to the parsimony limit has been reached.

Intraspecific unrooted cladograms (parsimony networks) based on the SP procedure have been shown to have greater statistical power and accuracy when there are limited numbers of variable sites (Templeton *et al.*, 1992; Crandall, 1994; Clement *et al.*, 2000), as is commonly found in biogeographic studies. The network for each locus was compared with the topology of the ML and MP phylogenies (estimated in PAUP* 4.0; Swofford, 1998) to ensure concordance; the best-fit ML model for each dataset (Cunningham *et al.*, 1998) was determined using ModelTest (Posada and Crandall, 1998). Searches were performed with stepwise addition (simple addition sequence) and TBR branch swapping with zero-length branches collapsed.

Rooting techniques

Based on sequence similarity, the Pacific taxa *Porphyra mumfordii* was used to create a rooted phylogram employing both MP and ML for determining a basal North Atlantic clade within *Porphyra umbilicalis*. Alternatively, each haplotype in the statistical parsimony network was assigned a so-called "outgroup probability" (Donnely and Tavare, 1986; Castelloe and Templeton, 1994), TCS version 1.13 (Clement *et al.*, 2000). The likelihood value is calculated as a function of the position of the haplotypes in the network, its frequency, and its number of connections with neighbor haplotypes.

Lineage-Specific Estimates of Mutation Rate

Estimates of clade divergence for *Porphyra umbilicalis* within the North Atlantic require an estimate of the mutation rate (μ). Because phylogenetic and paleontological evidence suggests that *P. umbilicalis* diverged from its closest ancestral sister-taxon *P.*

mumfordii during the trans-Arctic interchange about 3.5 Ma (Teasdale *et. al.*, 2000; Lindstrom and Fredericq, 2003; Lindstrom, 2001; Vermeij, 1991), this date was used to calibrate the divergence between the two closely related North Pacific and North Atlantic species. Therefore, the ML estimates of the internal branch length separating the sister taxa/populations from the Pacific and Atlantic were used to estimate the appropriate amount of divergence per site (Edwards and Beerli, 2000). The measure represents the net nucleotide divergence, *d* (Nei and Li, 1979), and it allows a calculation of the mutation rate as $\mu = (1/2)\, d / (3.5 \times 10^6$ years) using the trans-Arctic divergence estimate discussed above. Because prior investigations on intron mutation rates are limited, only ITS sequences were used for estimating the time of clade divergence.

Results

The location for samples used in this study and GenBank accession numbers for sequence data for COX, ITS, and group-I intron sequences are given in Table 1. The distributions of these collection sites in North America and Europe are shown in Figure 1. In some cases, herbarium vouchers were not available or could not be made from the material provided by other collectors.

Several additional reference sequences of *Porphyra umbilicalis* in GenBank (both the ITS and Helix 50 intron) were not used in my analyses as they showed limited sequence similarity with the sequences documented here. Overall the "*P. umbilicalis*" ITS accession AJ318959 was approximately 57-62.5% similar to my samples, which was below the ~77% similarity found in the interspecific comparison between *P. mumfordii* and *P. umbilicalis*. Similarly, the *P. umbilicalis* Helix 50 intron accessions AF172573

and AF172602 showed sequence similarities of 18.5-37.4% when compared to this study's sequences. The relative comparisons of the intron sequences again show very low sequence identity in contrast to a comparison of *P. umbilicalis* samples and its sister species *P. mumfordii* (65-70% sequence similarity). The large separation between the reference sequences and my data suggest that AF172573, AF172602, and AJ318959 probably represent other taxa than *P. umbilicalis*.

COX sequences

Complete sequences for the COX region were obtained from five Northwest Atlantic and five Northeast Atlantic *Porphyra umbilicalis* accessions (also used in the ITS and intron analyses). The COX sequences from two additional taxa were sequenced for interspecific comparisons (*P. dioica* and *P. mumfordii*). The length of the amplified region after editing was approximately 291 base pairs (bp) among the taxa and included the 3' end of the *cox*2 gene (118 bp), the intergenic spacer region (166-167 bp; Appendix B), and the 5' end of the *cox*3 gene (6-7 bp). The sequences of all 12 taxa were aligned easily and were found to be identical in length for the intergenic spacer, except for a 1 bp size difference in *P. dioica*.

Sequence variation for COX in ten *Porphyra umbilicalis* individuals used in this sequence comparison was limited to single nucleotide differences in two individuals (ENG1, NWY4). The North Pacific taxon *P. mumfordii* shared identical spacer sequence with eight *P. umbilicalis* specimens (see Haplotype A, Table 3). The two other *Porphyra* species used in the alignment, *P. dioica* and *P. purpurea* (GenBank # NC002007; Burger

et al., 1999) diverged from the *P. umbilicalis/P. mumfordii* consensus sequence by approximately 8-9% of the total base pairs (Table 3).

ITS 1 and 2 sequences

The universal primers JBITS and AB28 (Table 2) were used to amplify across the entire ITS1-5.8S- ITS2 region of the ribosomal repeat. In many samples, this amplification produced several DNA fragments of different sizes, including the band of expected size. The extraneous bands presumably resulted from the amplification of other DNA templates contaminating *Porphyra umbilicalis*. A number of recurring bands (two main size classes) that did not correspond to the predicted size of the *Porphyra* ITS were sequenced and submitted to a BLASTn search [BLASTN 2.2.4; (October 17, 2002); Altschul *et al.*, 1997]. The results of this search showed high sequence similarity (size class 1: ~420 bp) to the ITS1 region from the copepod, *Lepeophtheirus salmonis* Krøyer, (BLASTN E Value = $2e^{-38}$; GenBank Accession AF043980; Shinn *et al.*, 1999) and the endophytic sporophytic stage of the green alga *Acrosiphonia coalita* (Ruprecht) Scagel, Garbary, Golden and Hawkes (Size Class 2:490 bp;BLASTN E Value = 0.0; GenBank Accession AF047682.1; Sussmann *et al.*, 1999).

The degree of intra-individual ITS variation was determined from five clones of *Porphyra umbilicalis* isolate NHP-2 (New Hampshire) and another eight clones from the geographically distinct *P. umbilicalis* isolate NWY-3b (Norway). The intra-individual sequence variation from an alignment of 927 bp had ranges of 0.0-0.3 % in the NHP-2 specimen and 0.0-0.2% in the NWY-3b specimen. The single nucleotide polymorphisms

were randomly distributed among the clones and can probably be ascribed to artifactual point mutations from *Taq* amplification.

The entire sequence of the ITS1-5.8S-ITS2 region was determined from 34 individuals of *Porphyra umbilicalis* collected at 29 locations within the North Atlantic (including the North Sea) to evaluate the amount of intraspecific variation across its wide geographical range. The length of ITS1 is between 274 and 275 bp depending upon the presence of an indel (insertion or deletion) at alignment position 91 (Appendix C). Additionally, the ITS1-5.8S-ITS2 region was amplified and sequenced for *P. purpurea*, *P. dioica, P. birdiae*, and *P. mumfordii* to provide outgroups for phylogenetic analysis. However, high levels of sequence divergence between the outgroup taxa prevented confident alignment of the first three taxa with *P. umbilicalis*. Only ITS sequence from *P. mumfordii* exhibited a high degree of sequence similarity, allowing successful alignment with *P. umbilicalis*.

The length of the ITS1 region in *Porphyra umbilicalis* ranged from 274 to 275 bp due to the presence of an indel at bp position 91. The 5.8S rDNA coding region was 160bp long and identical for all *P. umbilicalis* accessions, except for a single point mutation found in NBK-1. The length of ITS2 showed more variation, ranging from 507 and 515 bp, primarily due to a single 5 bp deletion in IRE-2 (Ireland) and two indels common in two distinct haplotypes. Other sequence variation over the ~950 bp ITS1-5.8S-ITS2 region was limited to twenty-two single nucleotide substitutions, eleven of which were unique to a single individual; thus, they were not parsimony informative.

In the phylogenetic analyses of ITS sequences, the unrooted cladograms for both MP (Figure 2) and ML (data not shown) showed similar haplotype relationships with

significant bootstrap resampling, which supported four primary *Porphyra umbilicalis* haplotypes. When the same populations are rooted with an outgroup in ML (Figure 3) or by coalescence in the Statistical Parsimony Network (Figure 4), the relationship between North American samples either remains close or is lacking expected intermediate haplotypes, respectively. Figure 3 shows that the North American haplotypes have limited divergence from six European haplotypes within a well supported clade (Clade I). Besides the mixed composition of haplotypes (*e.g.,* both North American and European) in this clade, the divergence in this mixed clade showed a reduced level of missing haplotypes when compared to the other exclusively European clade (Clade II, Figure 3).

Intron Sequences

Appendix D shows an alignment of 28 geographic isolates of *Porphyra umbilicalis* (18 European, 10 North American) for the ribosomal group-I intron at Helix 50 in the nuclear SSU, with these representing a subset of the samples analyzed for their ITS sequence. The full-length intron sequence of IRE-1a and gel-band sizing was used in tandem with other *Porphyra* (*P. umbilicalis; P. purpurea*; as referenced previously) group-I Helix 50 introns and 18S ribosomal sequences for accurate size and positioning. The full size of the intron was ~710 bp. For the phylogenetic analysis, a smaller sized PCR amplification of the intron using H50-1 and H50-2 primers resulted in a ~548 bp fragment with the only size variation occurring with the presence of an indel at alignment position 510 (Appendix D). Other sequence variation over the 548 bp intron fragment included 53 nucleotide substitutions, 18 of which were unique to a single individual and not parsimony informative. An additional group-I intron size variant was weakly

amplified at the same insertion point in several samples (ENG1, IRE2, NWY7, NWY8). The size variants contained a ~478 bp insertion (position 64; Appendix D) and were not included in the phylogenetic analyses (see Chapter II).

The intron analyses were consistent with many observations found with the ITS results. It is evident based on the number of polymorphisms, that the Helix 50 intron is diverging at a considerably higher rate (~2.5 fold higher) than the ITS spacer regions. The unrooted Maximum Parsimony cladogram of the group-I intron exhibits more divergence of both European and North American haplotypes when compared to the unrooted MP cladogram for ITS (Figure 5). The SP network (Figure 6) confirms the clade separation seen in ITS but also exhibits larger divergence between the North American haplotypes and the mixed-European haplotypes than seen in ITS (Clade I, Figure 3) based on missing haplotypes assumed by coalescence theory.

Phylogeographic Analysis

Haplotype networks representing the complete datasets for ITS and the Helix 50 group-I introns in *Porphyra umbilicalis* are shown in Figures 4 and 6. Because outgroup rooting is not reliable for intraspecific genealogies (Castelloe and Templeton, 1994), the most likely root haplotypes are indicated on each network (asterisk see Figures 4,6). The root haplotype in ITS was the largest common haplotype in the SP network, being exclusively European in origin. The ancestral haplotype contained samples from Norway (NWY1,2,4,5,6), England (ENG2, ENG4) and the Republic of Ireland (IRE1a). Consistent with the ITS root haplotype, the Helix 50 group-I intron SP analysis also calculated a root haplotype containing NWY1 and NWY4-6. Because of increased

sequence variation in the group-I intron, ENG2, ENG4, and IRE-1a did not fall into the dominant, root haplotype.

Haplotypes Diversity and Mutation Rate Estimates

Haplotype diversities (*h*) of North American and European populations are shown in Table 4. North American populations showed lower haplotype diversity than European populations for both ITS and group-I intron sequences.

The internal branch length (based on best-fit maximum-likelihood model F81, no rate variaton) separating *Porphyra umbilicalis* from *P. mumfordii* for the ITS data was 0.03274, with a standard error of ± .0001 (calculated from Figure 3). The ITS data under the F81 ML model did not reject a molecular clock model, which implies that the different haplotypes are diverging at a constant rate. Using the calibration date of 3.5 Ma (the estimated time the trans-arctic interchange was closed), the ITS region has a theoretical mutation rate of $\mu=4.67 \times 10^{-9}$ (substitutions per site per generation). Analysis of the branch length showing the first split of North American haplotypes from European haplotypes indicates that the event may be quite recent with an estimated time to most recent common ancestor [TMRCA] = 1.28×10^{5} years or an estimated divergence between European and North American haplotypes within the last 128,000 years. The "older" root clade appears to predate the last glacial maximum.

Discussion

Large-scale biogeographic studies in some seaweeds have been problematic due to the lack of morphological characters that are needed for taxonomic identification and

species delimitation. While the advent of DNA-molecular methods has allowed considerable progress in macro-geographic studies of marine animals, comparable genetic loci (*i.e.*, hypervariable regions in animal mitochondria) in seaweeds have not shown the same levels of resolution and thus have limited the number of biogeographic studies.

In this study, the genetic diversity of North Atlantic populations of *Porphyra umbilicalis* was assessed using ITS, ribosomal intron and COX spacer sequences. Both ITS and COX have been successfully used to estimate intra-specific levels of genetic diversity in red algae (Patwary 1993; van Oppen *et al.*, 1995; Vis and Sheath 1997; Zuccarello *et al.*, 1999; Marston and Villalard-Bohnsack, 2002). Analysis of ITS and group-I intron sequences throughout the species range of *P. umbilicalis* revealed regional differences. By contrast, the mitochondrial COX region showed no significant intraspecific variation and only low levels of interspecific variation among the four *Porphyra* taxa used for comparison. The limited variation in the mitochondrial spacer suggests that the mitochondrial genome in the Bangiaceae may be under very different evolutionary constraints than the members of the Florideophyceae that have been surveyed to date (Zuccarello *et al.*, 2000). Although not useful for biogeographic studies, the 8-9% variation found between *P. umbilicalis* and *P. dioica* and between *P. umbilicalis* and *P. purpurea* in the COX region suggest that this locus is useful for confirming topologies for a genus-wide phylogeny. The comparatively high sequence similarity of this region between *P. umbilicalis* and *P. mumfordii* (see Table 2) supports a close relationship between these two taxa as previously calculated using *rbc*L sequence

data (Lindstrom and Fredericq, 2003); it also justifies using *P. mumfordii* as the closest known relative to *P. umbilicalis*.

The ITS region and group-I intron sequence data have previously been investigated in other *Porphyra* species, and they can be used as a baseline comparison of sequence divergence in *P. umbilicalis* and *P. mumfordii*. Studies by Kunimoto *et al.* (1999) and Broom *et al.* (2002) on *P. yezoensis* and *P. suborbiculata*, respectively, used regions of the ITS spacer and group-I introns to investigate the molecular divergence and taxonomic status of their representative taxa. Both studies showed comparable levels of intraspecific sequence similarities in ITS1 (*P. yezoensis*, 96-100%; *P. suborbiculata*, 94.6-100%). Kunimoto *et al.* (1999) also looked at the interspecific differences at ITS1 between *P. yezoensis* and *P. tenera* Kjellman, two closely related species, and found 88-90% sequence similarity. Furthermore, the level of sequence similarity between *P. yezoensis* and a specimen of *P. umbilicalis* (Nahant, MA; AB013179) was much lower (38-44% sequence similarity). The ITS variation in my samples is similar to their results with ITS1 sequence similarity between all *P. umbilicalis* isolates of 96.3-100% and interspecific similarity between *P. umbilicalis* and *P. mumfordii* of 77.7-79.2%. Together these analyses suggest that an individual species in the genus *Porphyra* has roughly 4-5% base pair variation in the ITS region. Interestingly, there is slightly higher variation in the ITS2 (*P. umbilicalis*; 95.9-100%) when compared to ITS1. The ITS2 was not examined in earlier studies and I feel that because of the small size of the entire ITS region, both the ITS1 and ITS2 regions should be analyzed together to enhance the statistical significance of phylogenetic analyses. The high level of sequence similarity between geographically distinct *Porphyra umbilicalis* samples from this study provide

additional information for ascertaining the current distributional range of *P. umbilicalis* and its phylogenetic relationship to *P. mumfordii* as well as other closely related species.

Impact of Glacial Events

The primary goal of this study was to test the hypothesis that glaciation effects upon rocky, intertidal marine seaweeds in the Northwest Atlantic were severe and resulted in the extirpation of organisms that required rocky substrata for attachment and recruitment. Since *Porphyra umbilicalis* is considered to be an obligate, rocky intertidal species (Bird and McLachlan, 1992), historical phylogeography may help to explain the current distribution of populations of this alga and its possible recolonization routes in the North Atlantic. An analysis of genealogical patterns within *P. umbilicalis* fits the following models: 1) a recent range expansion and; 2) that North American populations are descendants of a recent colonization from Europe that probably followed the last glacial maximum (about 20,000 BP).

Using data from the ITS region it would appear that all North American haplotypes are closely related to many European haplotypes, depicted as Clade I in Figure 3. Phylogenetic analysis using statistical parsimony further resolves these relationships by suggesting that the haplotype consisting of samples NBK2, MAE1, MAS1, and NY1 should also be found in European populations with increased sampling. The assumption that this haplotype exists on both sides of the North Atlantic would explain the divergence of the three European samples from this haplotype (Figure 4). Based on the bootstrap support of MP and ML analysis, the two Norwegian samples NWY 3a,b were not included in this mixed North American and European clade (Figure

3). This suggests NWY 3 a,b are an intermediate haplotype and possible source population for postglacial recolonization to North America. The addition of a second set of exclusively European haplotypes (Clade II, Figure 3) confirms the calculations of diversity (*h*) that show an increased number of European haplotypes compared to North America (Table 4). The lower allelic diversity found within North American *Porphyra umbilicalis* compared to the diversity calculated for European samples provides another indicator of recent range expansion (Hewitt, 1996; Austerlitz *et al.*, 1997, Wares and Cunningham, 2001). The observation becomes significant when evaluating the larger divergence between North American and European haplotypes for intron data (see below), where more genetic variation exists between members of the geographically mixed clade (Clade I), presumably due to a relaxed selection rate. However, a reduced degree of allelic diversity for the intron sequence is again seen among the North American populations.

Coalescent theory was used to predict that NWY1 was the most ancestral ITS haplotype. The result is consistent with the hypothesis that European populations gave rise to extant Western North Atlantic populations of *Porphyra umbilicalis* after Pleistocene glaciation events. The ITS analyses suggest that the TMRCA between European and North American isolates of *P. umbilicalis* was within the last 125,000 years, probably as a result of isolation during the glacial periods of the Pleistocene. Clade II (Figure 3), which is made up of only European haplotypes, represents a much older separation and probably does not represent recolonization haplotypes. However, haplotypes NWY3a and NWY3b provide intermediate haplotypes that appear to link Clade I and Clade II. Based on the SP network and ML tree, a Norwegian donor

haplotype related to the NWY3a,b haplotype supports bi-directional dispersal southward to other parts of Europe and westward as the seed population for North America. Norway has previously been supported as a likely donor refugium of marine organisms for post-Pleistocene dispersal in the North Atlantic (Ingolfsson, 1992).

Although the Helix 50 group-I intron sequences were not used in estimating times of divergence, they provided valuable information in understanding and confirming ITS haplotype relationships and providing an alternate set of data (perhaps not independently) from the same individuals for analysis and comparison. The most obvious result of the intron analysis is the separation of European from North American haplotypes (Figures 5 and 6). The degree of divergence between European and North American populations suggests that geographical isolation is the primary factor influencing the Helix 50 intron haplotype relationships and that gene flow is occurring at a higher rate within than between continental populations. A hypothesis explaining this divergence is that the higher rate of mutations occurring within the group-I intron has decreased the historical resolution of the haplotype relationships found in the slower evolving ITS. However, like ITS the North American intron haplotypes show lower overall diversity when compared to European accessions. The lower haplotype diversity is consistent with the hypothesis that North American *Porphyra umbilicalis* populations have been recently colonized (within the last 125,000 years) versus Europe.

When the results of statistical parsimony analyses of the ITS region and the Helix 50 intron are compared to some of the criteria set forth by Wares and Cunningham (2001) in substantiating a recolonization event, the analyses generally support the theory that Northern Europe was a donor population for *Porphyra umbilicalis* in the Northwest

Atlantic for the following reasons: 1) both the ITS and Helix 50 intron showed lower haplotype diversity in North America; 2) the ancestral *P. umbilicalis* haplotype was related to members of the exclusive European Clade II; 3) the divergence between many European and North American haplotypes in Clade I is small and gives support for shared haplotypes between the two distant geographic regions; and 4) haplotypes NWY3a and NWY3b appear to be intermediate between European and North American populations and are perhaps closely related to the source haplotype of a North American recolonization event.

Although the results of this study can be used to support postglacial expansion events, an alternative hypothesis is also consistent with some but not all of my observations. If North American populations were not extirpated but instead existed in glacial refugia, extant North American *Porphyra umbilicalis* would still be expected to exhibit significantly lower haplotype diversity than the less severely impacted European populations. So although the results of the ITS and group-I intron statistical parsimony analyses show distinct haplotype clustering by geographical location, the lack of shared haplotypes along with the estimate of divergence time for the most recent common ancestor between the European and North American alleles do not rule out the alternative hypothesis of recolonization of New England and the Canadian Maritimes from a North American glacial refugia. Additionally, the assumption that available rocky intertidal habitat was not available at the last glacial maximum in North America for *P. umbilicalis* could be disputed; for instance, lower sea levels (~150m) may have exposed consolidated sediments on the continental shelf (Riggs *et al.*, 1996), which could act as habitat for *P. umbilicalis*.

Since no shared haplotypes were found in either the ITS or intron datasets, interpretation of the root haplotypes and the assumptions and implications of coalescent theory that was used to support the root haplotypes becomes important. Coalescent theory describes the genealogical branching process backwards in time until a single common ancestral gene sequence is estimated using parameters such as likelihood estimation of divergence rates and migration rates (Knowles and Maddison, 2002; Li, 1997). The TCS program used in this study to estimate the root haplotype is based on coalescent assumptions of allele genealogy. An important assumption in coalescent models for historical inference is that a simple population history is assumed in contrast to other models that try to distinguish an array of historical processes..

Given the length of time that hypothetical North American refugia and European refugia would have been separated due to unfavorable climatic conditions during glacial maxima, simulations by Johnson *et al.* (2000) indicate that the likelihood of observing shared alleles between these populations today is small if gene flow was not maintained., and in fact no share haplotypes were detected in this study. Furthermore if separate refugia for *P. umbilicalis* had existed in North America and Europe, this would generate greater divergence among alleles from each population. In my study, the statistical parsimony networks of the intron and ITS show that the haplotypes are less diverged in North America than in Europe. In summary, the results show that the root haplotypes for both ITS and the helix 50 intron were European, and that the haplotype divergences for both loci were greater in Europe than in North America, which are consistent with the hypothesis that the obligate rocky intertidal species *P. umbilicalis* recolonized North America from an European refugium after the last glacial maximum.

Species Range

The rDNA ITS, COX, and Helix 50 intron data demonstrate that all of the *Porphyra umbilicalis* populations examined in this study represent a distinct species that exhibits a unique molecular profile. The variable ribosomal ITS and intron sequence data have led to the discrimination of two well supported clades and have provided a molecular baseline for the species range of *P. umbilicalis* that may be used to confirm whether new accessions fall within this taxon. An interesting question arises as to the differences between the two most divergent clades (Clade I, III; Figure 3) and whether they may be precursors to a speciation event based on geographic isolation.

The inherent high levels of sequence variation within these accessions of *Porphyra umbilicalis* for both ITS and the ribosomal intron are consistent with the species-specific RFLP assay based on *rbc*L (Chapter One). The results also bring up an important observation that may affect previous phylogenetic analyses of these loci. During comparative sequence analysis of *P. umbilicalis* ITS and the Helix 50 intron, three GenBank accessions were found that were incompatible with the species designation in this study based on sequence similarity: GenBank Accession AJ318959 for the ITS region (Antoine and Fleurence, 2003) and 1506 intron accessions AF172573 and AF172602 (Müller *et al.*, 2001). Because previous studies did not utilize a species-specific molecular assay for sample identification and samples included in this study were obtained by multiple collectors and showed high degrees of sequence similarity, I conclude that GenBank accessions AJ318959, AF172573, and AF172602 are not *P. umbilicalis,* but other mis-identified *Porphyra* taxa that have been mis-identified.

Issues of Contamination

The amplification of extraneous fragments from mixed DNA templates (total genomic DNA extractions from *Porphyra umbilicalis*) and identification of the corresponding contaminating organisms resulted in two potentially important observations. Foremost, the appearance of multiple bands during ITS amplification was not unexpected and is considered a common phenomenon and problem when using universal or non-species specific PCR primers. There are also inherent problems of epiphytic and endophytic organisms associated with field collections of marine macroalgae (and other biological samples for ecological studies). Together these two factors can produce false positive banding patterns for the target taxon when there are mixed DNA templates. Such a problem is of particular concern when analyzing markers produced from PCR using short, non-specific oligonucleotide primers such as randomly amplified polymorphic DNAs (RAPDs) or inter-simple sequence repeats (ISSRs) because of the non-specificity of the universal PCR primers and the inherent problems of epiphytic and endophytic organisms associated with field collections of marine macroalgae.

The contamination problems may have been alleviated or reduced by culturing each sample to reduce epiphytic and endophytic organisms that may have been attached to the isolate, but for this study culturing was both time and cost prohibitive.
The other observation involving contaminants occurred when examining the sequences of the non-specific bands that consistently appeared in the ITS amplifications. Surprisingly, one of these sequences was found to have high sequence similarity with the green alga

Acrosiphonia coalita. It has been reported that *Porphyra* may be a suitable host for the endophytic sporophyte phase of *Acrosiphonia*, previously identified as *Chlorochytrium inclusum* and *Codiolum petrocelidis* (UBC Herbarium specimen A39186 in Sussmann and Dewreede, 2002). However a recent study looking for *Acrosiphonia* endophytes in 28 *Porphyra* samples based on ITS sequence data failed to detect the endophyte in all of their samples and *Porphyra* was subsequently ruled out as a possible host (Sussmann *et al.*, 1999; Sussmann and DeWreede, 2002). An alternative green algal endophyte species is a possible explanation to this result. For example, West *et al.* (1988) described a unicellular green endophyte, *Chlorochytrium porphyrae,* specifically found in *Porphyra*. Although the conspecificity of these organisms remains speculative, the results suggest that vegetative thalli of *Porphyra umbilicalis* are frequently contaminated by green algal endophytes and thus should not be excluded as a possible host for either of the previously mentioned *Chlorochytrium* species. The only other recurring band resulting from a contaminating organism was from the sea louse *Lepeophtheirus salmonis*, with all of the molecular work and sequence information derived from European populations. Interestingly, only European *P. umbilicalis* isolates exhibited these contaminating bands.

Assumptions and Limitations

When trying to interpret the phylogeography of an organism, it is important to outline the main assumptions being made and how they limit the interpretation of results. One of the primary concerns in this study is the use of only one locus for estimating divergence times and substitution rates. Although ITS has been extensively studied, additional loci may provide more reliable or advantageous data for historical

biogeographic studies of marine algae. Organelle DNA such as mitochondrial or chloroplast DNA have advantages of not undergoing recombination and having a smaller effective population size (Avise *et al.*, 1987). Unfortunately, these organelles have not shown the same level of variability in protists (*i.e.,* mitochondrial *cox* spacer; this study) as they have in animals (mitochondria) or plants (chloroplast).

My analyses further assumed that the trans-arctic interchange was the most likely time of dispersal between the Pacific and Atlantic Ocean with subsequent vicariant speciation occurring as the Arctic Ocean froze and the Bering Strait closed during the glacial periods. Under this assumption, the estimation of the mutation rates in ITS was based on the further assumption that North Atlantic and North Pacific taxa diverged 3.5 million years ago, the estimated time since the submergence of the Bering land bridge. Although this date was based on fossil evidence, dates of 6.4 million years ago have also been proposed for this vicariant event (Vermeij, 1991; Marincovich and Gladenkov, 1999). My data set is limited in it is not possible to resolve which ocean (Pacific or Atlantic) was the origin of the most common ancestor (TMCA) for *P. umbilicalis* and *P. mumfordii.* However, a deeper phylogenetic reconstruction of *Porphyra* from both the Atlantic and Pacific Oceans may provide useful information on invasion direction (Lindstrom, 2001).

Finally, the use of the coalescent model in the statistical parsimony network to estimate a root haplotype assumes that the ancestral haplotype still remains in the population and is often the most common haplotype. The assumptions are just such and if incorrectly interpreted they could alter some of the observations and conclusions from this study.

Conclusions

In conclusion, the ITS and ribosomal group-I introns show utility for phylogeographic and population-level studies in *Porphyra*. The apparently higher rate of mutation within the intron and presence of multiple intron sizes within a single individual (see Chapter 2) decreases the utility of this locus for the goal of understanding the historical biogeography of *Porphyra umbilicalis* in the North Atlantic. However, the intron data does confirm much of the clade formation and root probabilities seen in the ITS data. As more information becomes available on intron evolution and mutation rates it may become possible to utilize rDNA intron sequence comparisons more effectively in biogeographic analyses.

Table 1. Collection locations of *Porphyra* specimens sequenced for this study. Map # corresponds to geographical location (see Figure 1). * = not available

Species	Map #	Code	Location	Coordinates	Date	Collector	Herb#	Cox Spacer Genbank	ITS1-5.8S-ITS2 Genbank	Group-I Intron Genbank
P.umbilicalis	1	ENG-1	North Island, Cook St. Pier		8/2/95	D. Birkett/JB170		AY316138	AY322113	AY347883
	2	ENG-2	Sidmouth, Devon, England	50°41',3°15'W	3/24/00	J. Brodie/JB222	76784	-	AY322114	AY347884
	3	ENG-3	Overstrand, England	52°56, 1°20E	11/8/00	J. Brodie/JB247	76783	-	AY322115	AY347885
	2	ENG-4	Sidmouth, Devon, England	50°41',3°15'W	1/21/00	J. Brodie/JB218	76578	AY316137	AY322116	AY347909
	4	IRE-1a	Clare Island, County Mayo	53°46'/9°51'W	8/28/00	F. Rindisi	76580	-	AY322118	AY347910
	4	IRE-1b	Clare Island, County Mayo	53°46'/9°51'W	8/28/00	F. Rindisi	76580	-	AY322119	AY347887
	4	IRE-2	Clare Island, County Mayo	53°46'/9°51'W	8/28/00	F. Rindisi	76580	-	AY322120	AY347888
	5	NWY-1	Finnoy, NW of Alesund	62°47'/6°30'E	10/8/02	J. Rueness	76577	-	AY322123	AY347897
	6	NWY-2	Ona, NW of Alesund	62°52'/6°34'E	10/9/02	J. Rueness	76577	-	AY322124	AY347898
	7	NWY-3a	Golten, Island of Sotra	60°18'/5°4'58E	9/21/02	K. Sjotun	*	AY316140	AY322125	AY347904
	7	NWY-3b	Golten, Island of Sotra	60°18'/5°4'58E	9/21/02	K. Sjotun	*		AY322126	AY347905
	8	NWY-4	Southern Norway	59°02'538",10°17'77"	3/6/01	A. Pedersen	76579	AY316139	AY322127	AY347906
	9	NWY-5	Southern Norway	58°16'40",08°32'24"	3/6/01	A. Pedersen	76579	-	AY322128	AY347901
	10	NWY-6	Southern Norway	58°05'68",08°12'65"	3/6/01	A. Pedersen	76579	-	AY322129	AY347908
	11	NWY-7	Southern Norway	58°02'88",06°47'750"	3/6/01	A. Pedersen	76579	-	AY322130	AY347899
	12	NWY-8	Southern Norway	58°28'72",05°49'60"	3/6/01	A. Pedersen	76579	-	AY322131	AY347900
	13	GNY-1a	Helgoland, North Sea	54°12,7°53E	10/15/02	A. Wagner	*	-	AY322121	AY347886
	13	GNY-1b	Helgoland, North Sea	54°12,7°53E	10/15/02	A. Wagner	*	-	AY322122	-
	14	PRT-1	Praia da Luz, Porto	37°06,8°40W	9/4/00	R. Pereira	74036	AY316136	AY322117	AY347907
	15	NBK-1	St. Martins, New Brunswick	45°21,65°32	7/10/02	T. Bray	76582	AY316144	AY322133	AY347894
	16	NBK-2	Dipper Harbor, New Brunswick	45° 05'50",66°24'80'	7/11/02	T. Bray	*	-	AY322134	AY347895
	17	NVS-1	Port George, Nova Scotia	44°57,65w04	7/22/02	T. Bray	76581	AY316145	AY322132	-
	18	MAE-1	Campobello Bridge, Maine	44°52,66w69	10/7/96	A. Mathieson	*	AY316142	AY322135	AY347889
	19	MAE-2	Parsons Beach, Maine	44°51,68w69	2/4/96	A. Mathieson	72329	-	AY322136	AY347890
	20	MAE-3	Peaks Island, Casco Bay, Maine	43°39,70°12	7/8/95	A. Mathieson	*		AY322137	AY347891
	21	MAE-4	Two Lights State Park, Maine	43°38,70°16	1/28/96	A. Mathieson	*	AY316143	AY322138	AY347892
	22	MAE-5	Diamond Island, Casco Bay, ME	43°39,70°12	6/3/00	A. Mathieson, E. Hehre	71678	-	AY322139	AY347893
	23	NHP-1	North Wallis Sands, NH	43°01,70°44	7/14/02	B. Teasdale	*	AY316141	AY322140	AY347896
	24	NHP-2	Jaffrey Point, NH	43° 03' 70° 43'	7/15/02	B. Teasdale	*	-	AY322141	AY347902
	25	NHP-3	Hampton Beach, NH	42°54,70°49	7/15/02	B. Teasdale	*	-	AY322142	AY347903
	26	NHP-4	Rye Harbor, NH	42°59,70°46	7/15/02	B. Teasdale	*	-	AY322143	-
	27	NHP-5	Dover Point, NH	43n12, 70w53	8/25/98	D. West	71783	-	AY322144	-
P.umbilicalis	28	MA-1	Gloucester, MA	42.63179 N,70.68342 W	9/22/97	C. Neefus	68821	-	AY322145	-
P. umbilicalis	29	NY-1	Shinnecock Inlet, Long Island, NY	40 50 52, 72 28 70	6/15/99	E.J. Hehre	69771	-	AY322116	-

Table 1 continued. Collection locations of *Porphyra* specimens sequenced for this study. Map # corresponds to geographical location (see Figure 1). * = not available

P. linearis	n.a	n.a	Millstone Point, CT	41° 18'	72° 10'	8/14/96	C. Yarish	CT 7-1	AY316148	-	-
P. mumfordii	n.a	n.a	Orlebar Point, British Columbia	49 12.20	123 49.20	*	S. Lindstrom	-	AY316146	-	-
P. dioica	n.a.	n.a.	Sidmouth, Devon	50°41',3°15'W		7/29/99	J. Brodie	-	AY316147	-	-
P.birdiiae	n.a.	n.a	Herring Cove, Nova Scotia	n.a.		9/28/96	A. Mathieson	-	pending	-	-
P.purpurea	n.a.	n.a.	Ross Island, New Brunswick	n.a.		11/1/96	A. Mathieson	65186	pending	-	-

Figure 1. Geographical sampling locations of *Porphyra umbilicalis*. Species identities were confirmed by RFLP assay of the *rbc*L and *rbc*L-*rbc*S spacer region. A= North American sites, B= European sites. *Note: Number groupings represent sampling regions and not identical sample locations; see Table 1 for specific locations.*

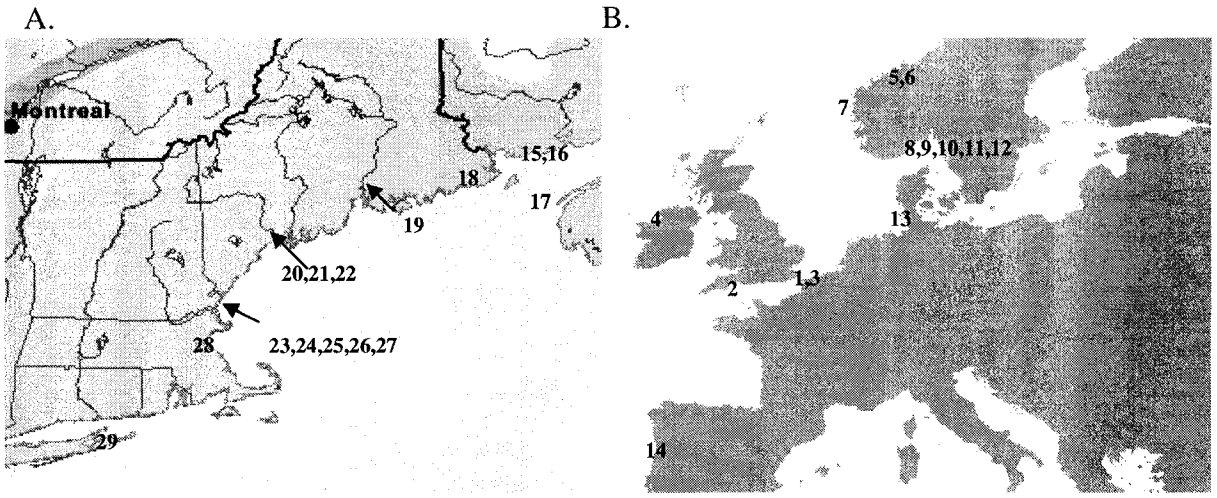


Table 2. A list of amplification and sequencing primers for this study.

Primer Label	5′-Primer sequence-3′	Reference
ITS		
JBITS	GTAGGTGAACCTGCGGAAGG	Broom *et al.*, 2002
AB28	CCCCGGGATCCATATGCTTAAGTTCAGCGGGT	Steane *et al.*, 1991
ITS-R	GAAACTGCGGTATCCTGTCGT	This study
ITS-F	TATCCACCGTTAAGAGTTGTAT	"
INTRON		
H50-1	GAAGGAGAAGTCGTAACAAGGTTT	This study
H50-2	CAGGGGACCGACTGTCTCTTA	"
H50-FL	GAGGAAGGAGAAGTCGTAACAA	"
H50-4	CTGATCCTTCTGCAGGTTCACCTAC	"
PU-INT-3	TCCCTTACAGTCTGTGAACCTT	"
PU-INT-5	CATTTAGAGGAAGGAGAAGTCGT	"
COX		
cox2-for	GTACCWTCTTTDRGRRKDAAATGTGATGC	Zuccarello *et al.*, 1999
cox3-rev	GGATCTACWAGATGRAAAWGGATGTC	Zuccarello *et al.*, 1999

Table 3. Sequence divergence between the mtDNA cox2-cox3 spacer and three outgroup species as Kimura 2-parameter distances (upper diagonal) and absolute differences (lower diagonal).

Species	No. of ind.	COX Haplotype	A	B	C	D	E
P. umbilicalis, *P. mumfordii*	8/1	A	-	.00606	.00606	.08378	.09684
P. umbilicalis (ENG1)	1	B	1	-	.01220	.09082	.10393
P. umbilicalis (NWY4)	1	C	1	2	-	.07683	.10393
Porphyra dioica	1	D	13	14	12	-	.15478
Porphyra purpurea	1	E	15	16	16	23	-

Figure 2. Single most parsimonious tree of the 947 bp region of ITS from *Porphyra umbilicalis* isolates using the evolutionary criterion of Maximum Parsimony. Tree length= 27 steps, CI= 1.00, RI= 1.00, RC= 1.00. Numbers represent bootstrap support values (1000 bootstrap resamplings)

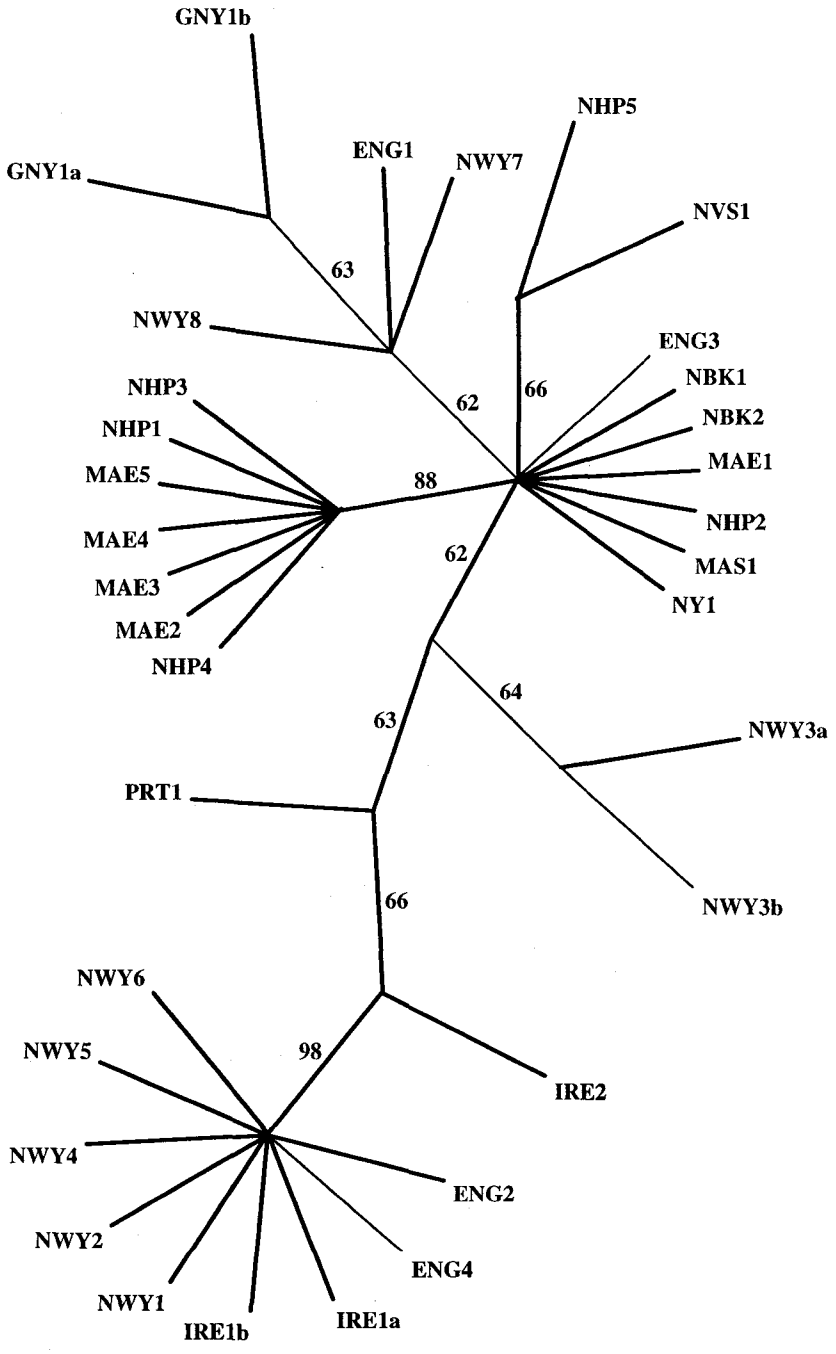


Figure 3. Phylogram of ITS sequence from *Porphyra umbilicalis* isolates using the Maximum-Likelihood (ML) algorithmn with *Porphyra mumfordii* as the outgroup. ML settings followed the best-fit model corresponding to the K80 (K2P) model. Bootstrap values (numbers in bold) represent 100 replicates with 884 characters resampled in each replicate. ML branch lengths are given in parentheses and represent the degree of divergence per site.

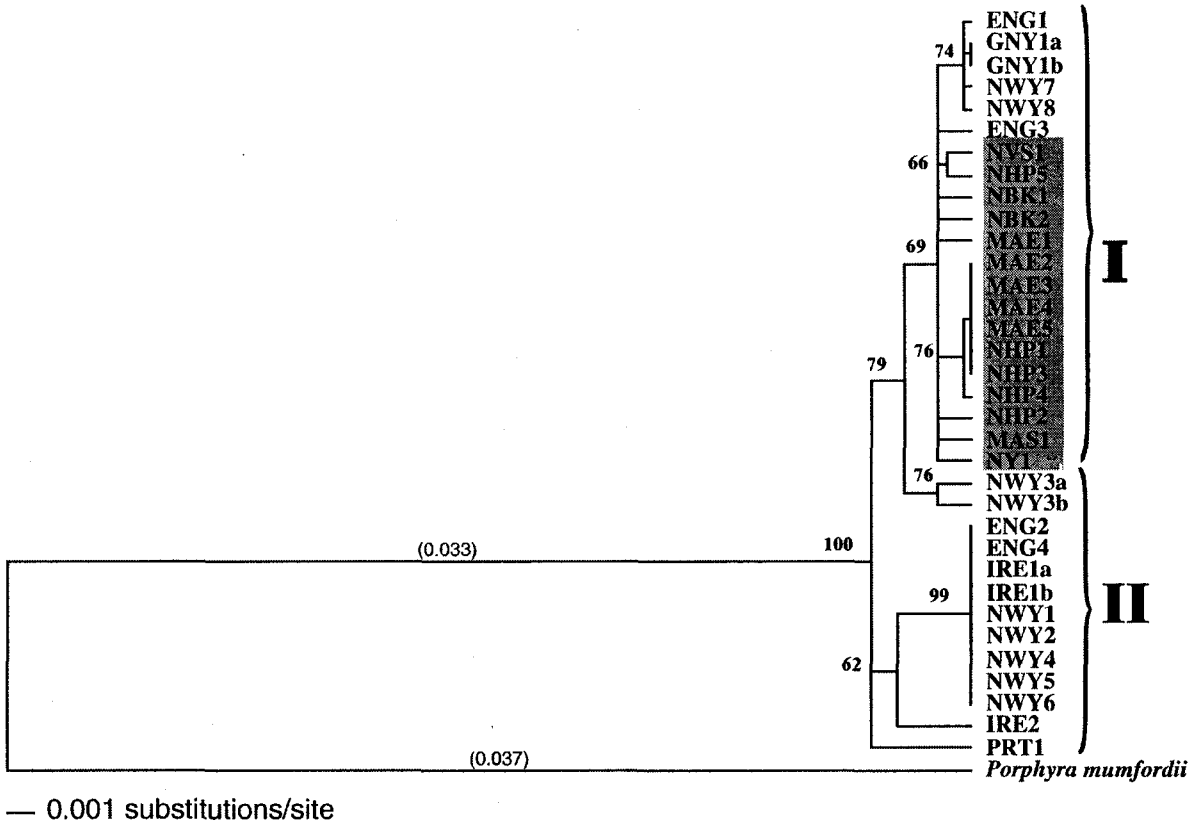


Figure 4. Statistical Parsimony Network depicting the phylogenetic relationships among, and geographical assignment of, all *P. umbilicalis* ITS haplotypes found throughout its range in the North Atlantic: white, European; grey, North American. Missing intermediate haplotypes are designated by darkened circles. The size of each circle is proportional to the corresponding heplotype frequency. Asterick (*) corresponds to haplotype with highest rooting probability based on coalescence/TCS analysis.

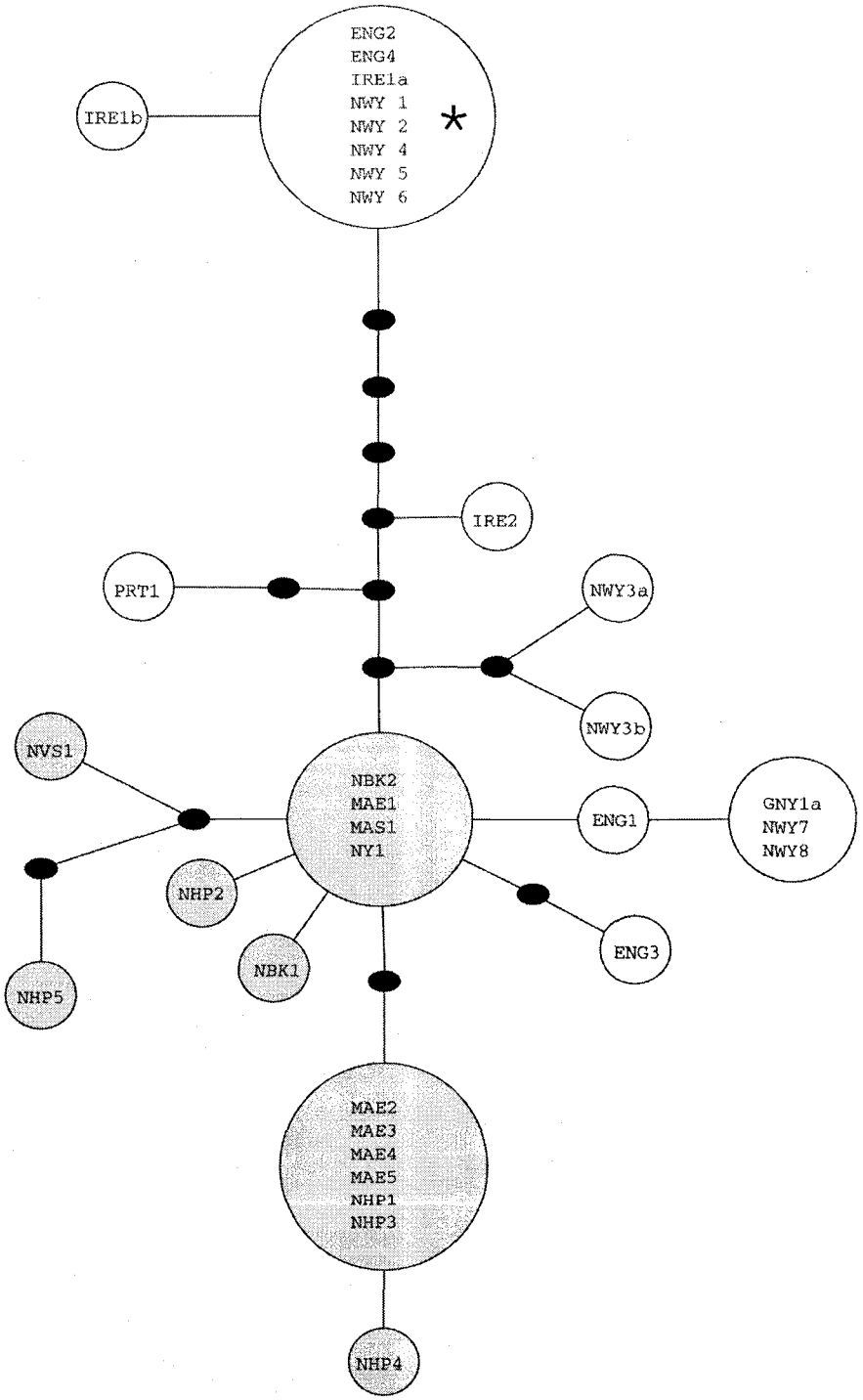


Figure 5. Unrooted cladogram of Helix 50 group-I intron from *Porphyra umbilicalis* isolates determined using the evolutionary criterion of Maximum Parsimony.

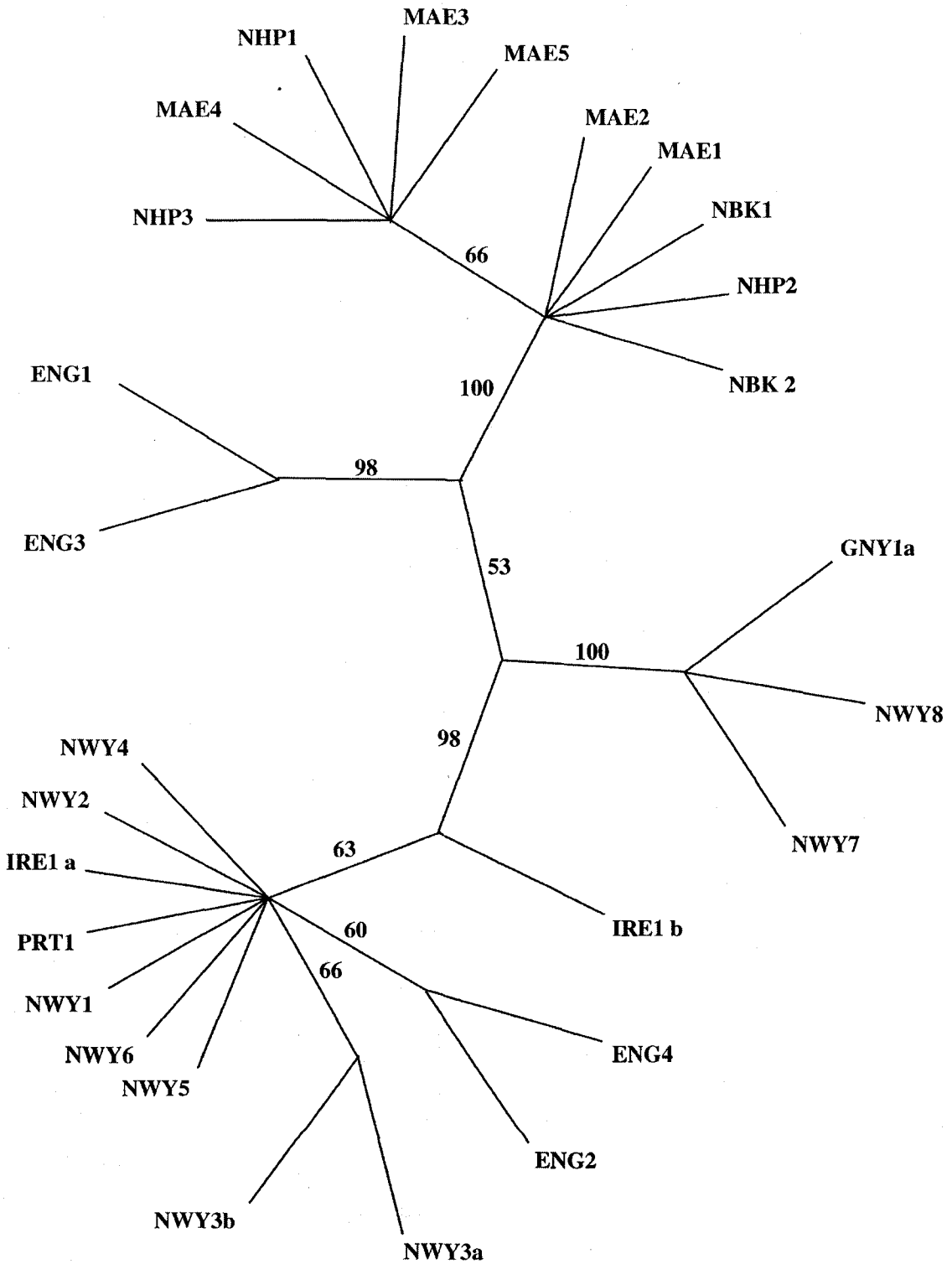


Figure 6. Statistical Parsimony Network depicting the phylogenetic relationships among, and geographical assignment of, all *P. umbilicalis* group-I intron haplotypes found throughout its range in the North Atlantic: white, European; grey, North American. Missing intermediate haplotypes are designated by darkened circles. The size of each circle is proportional to the corresponding haplotype frequency. Asterisk (*) corresponds to haplotype with highest rooting probability based on coalescence/TCS analysis.

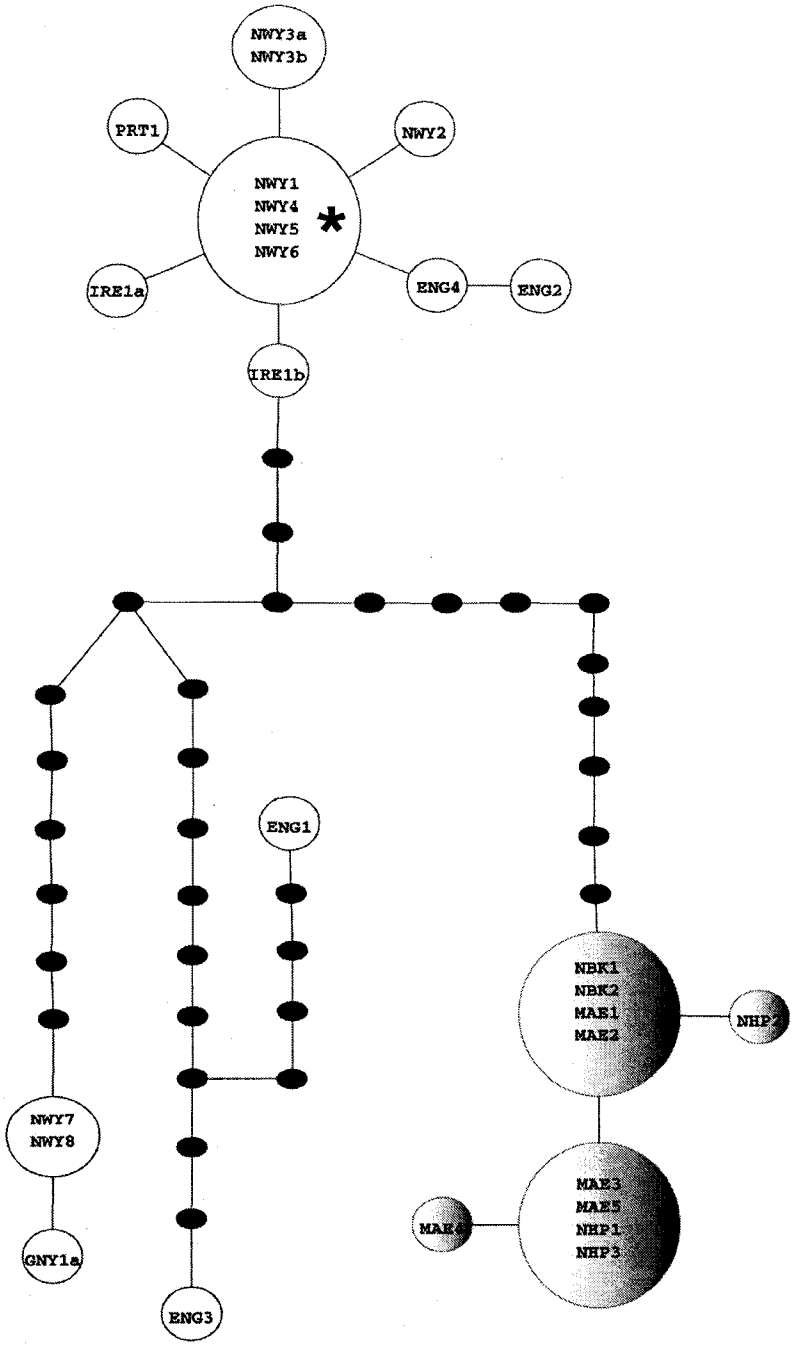


Table 4. Comparisons of haplotype diversity (*h*, Nei 1987) for the ITS and ribosomal Helix 50 group-I intron in different populations of *Porphyra umbilicalis*.

Locus/Population	Haplotype diversity (*h*)	σ^2
ITS		
European	0.8947	0.0437
North American	0.5556	0.0745
Helix 50 group-I Intron		
European	0.7524	0.0918
North American	0.5556	0.0902

CHAPTER IV

ISOLATION OF MICROSATELLITES FROM *PORPHYRA UMBILICALIS*.

Abstract

A hybrid capture method was used to isolate sequences containing dinucleotide repeats in a search for microsatellite markers in the red alga *Porphyra umbilicalis*. The resulting genomic libraries were moderately enriched with 22.4% of clones containing CA, GA, or TA microsatellite repeats, respectively. The number of repeats ranged from 4 -52 with an average of 14. Sixteen clones contained between 16 and 49 dinucleotide repeats with sufficient flanking sequence to design primers for PCR amplification of the locus. An initial screening of 16 primer pairs using six geographically distant *Porphyra umbilicalis* isolates along with four related species (*P. linearis, P. purpurea, P. leucosticta,* and *P. amplissima*) demonstrated that all microsatellite containing loci isolated in this study were monomorphic in *P. umbilicalis*. However, several microsatellite primer sets supported amplification of fragments with size variation in other species. The present study demonstrated the advantages and problems associated with a size-selective hybrid capture method for isolating microsatellite loci in *P. umbilicalis* and the unexpected level of monomorphism exhibited by each locus between different populations of this alga in comparison to similar microsatellite isolation studies of other marine algae.

Introduction

The study of the impact of Pleistocene ice ages on the distribution and population genetic structure of North Atlantic flora and fauna has been a relatively new area of investigation (Hewitt 1996, 2000, 2001; Vendramin *et al.*, 1998). The usual approach to deciphering the effect of history on current biogeographical distributions has been to investigate patterns of genetic structure and gene flow found within and among extant populations at both a regional and local scale. Although patterns of postglacial expansion, glacial refugia and genetic structure have been described for many terrestrial species, most marine studies have concentrated on benthic invertebrates and fish (Brown *et al.*, 2001; Wares 2001a, 2002; Wares and Cunningham 2001). To date there have only been three population genetic studies of marine algae in relation to the Last Glacial Maximum (LGM), occurring 18,000-20,000 years ago, with all three primarily concentrating on Baltic Sea populations (*Fucus serratus* L., Coyer *et al.*, 2003; *Ceramium tenuicorne* (Kützing) Waern, Gabrielson *et al.*, 2002; *Phycodrys rubens* (L.) Batters, van Oppen *et al.*, 1995).

Porphyra umbilicalis Kützing is a prominent red algal species along the North Atlantic coast, occurring in Northern Europe, Greenland, the Northeastern United States, and Canada (see Chapter 2). In Chapter 3, I compared the restriction fragment length polymorphism patterns for the *rbc*L gene in Northwest and Northeast Atlantic *P. umbilicalis* individuals and confirmed these samples were conspecific on both coasts. Hence, *P. umbilicalis* populations constitute a continuous, amphi-Atlantic species.

Porphyra umbilicalis is an excellent species for addressing population and phytogeography questions related to the effect of the Pleistocene glaciation. First, the

species is considered to have a cosmopolitan distribution and is found in most open coastal and estuarine habitats throughout the North Atlantic (Mathieson and Hehre, 1986; Brodie *et al.*, 1996). Second, many of its current habitats were covered during the Pleistocene glaciations and these regenerated populations may thus provide insights into historical recolonization patterns. Recent studies have suggested ice expansion during the last glaciation forced many seaweeds southward into small ice-free refugia (Hoek and Breeman, 1990; Dawson, 1992), causing extreme population bottlenecks. Van Oppen *et al.* (1995) examined this hypothesis using the red alga *Phycodrys rubens*, comparing nuclear ribosomal DNA internal transcribed spacer (ITS) sequences and found significant divergence between eastern Atlantic and North Sea populations. Additionally, they found distinct thermal responses between these two populations and concluded that they were once isolated, even though the present-day distribution is continuous in the North Atlantic and Arctic oceans. By extending the conclusions of van Oppen *et al.* (1995), the North Atlantic populations of *Porphyra umbilicalis* may have experienced a similar history of "population bottlenecks." Third, the species is primarily an obligate rocky-intertidal organism requiring a hard substrata to attach and survive. Based on this requirement for a rocky substratum, the lack of available refugia along the coastlines of North America may have alternatively caused a continental extripation of this species, with current North American distributions coming from European donor populations. Understanding the allelic diversity that exists within *Porphyra umbilicalis* can provide valuable information on the historical sequence of fragmentation and expansion, as well as the pattern of gene exchange between existing populations.

Currently, the genetic structure of seaweed populations is poorly understood (Coyer *et al.*, 1997). Previous population studies involving the red alga *Porphyra* have employed isozymes to study the genetic structure of *P. yezoensis* Ueda (Miura *et al.*, 1978; Fujio *et al.*, 1985). Both studies showed extensive variation at isozyme loci, suggesting the existence of subpopulations in *Porphyra yezoensis.* Fujio *et al.* (1985) further hypothesized that extensive selfing and asexual propagation may explain the high levels of genetic differentiation and variability in these haploid plants. Hence, they concluded that more detailed studies concerning population structure were warranted. Unfortunately, isozyme studies in marine macroalgae have not always been definitive due to a combination of unreliable markers and/or a low level of polymorphism (Innes, 1984; Sosa *et al.* 1996; Williams and di Fiori, 1996). A variety of hypervariable markers have been employed with macroalgae for population genetic and phylogeographic studies. Randomly Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), and Intersimple Sequence Repeats (ISSRs) are based on PCR amplification of small amounts of DNAs. While these three types of markers are relatively inexpensive means to screen for polymorphisms between individuals and populations, they all suffer from problems of reproducibility. RAPDs and AFLPs are also dominant alleles that are not as informative as codominant genetic markers for various measures of population differentiation as some other markers. Another significant problem with RAPDs, ISSRs and AFLPs is that small amounts of contaminating DNA templates (i.e. from endophytes or epiphytes) may amplify as 'rare' alleles, confounding the population statistics (Wattier and Maggs 2001).

More recently, population studies with the brown algae *Fucus* (Coyer *et al.*, 2002) and *Postelsia palmaeformis* Ruprecht (Whitmer, 2002), plus the red alga *Gracilaria gracilis* (Stackhouse) Steentoft, L.M. Irvine *et* Farnham (Wattier, *et al.*, 1997) have shown that microsatellites are an appropriate genetic tool to characterize population structure and to address many taxonomic and phylogenetic issues within morphologically or genetically diverse algal species. Microsatellites are expected to be very useful tools for accessing post-glacial recolonization pathways (Vendramin *et al.,* 1998) as well as provide fundamental knowledge about marine species conservation. Microsatellites are short stretches (1-8bp) of DNA arrayed as tandem repeats that are scattered throughout the genomes of prokaryotic and eukaryotic organisms (Jarne and Lagoda 1996). Microsatellite variation is assumed to follow a mechanism of adding and subtracting a single repeat to or from the current allele with equal probability. Such a mechanism of microsatellite variation has been described as the stepwise mutation model, that considers similar-sized alleles as less-different in terms of mutational steps than alleles with larger differences in size and that this process of mutation has a memory (Jarne and Lagoda, 1996). Based on this, genetic distances and population parameters based on microsatellite markers may be correctly estimated. Simulation studies of microsatellites have provided linear relationships between genetic distance based on the size differences of the microsatellite and the time of divergence (Di Rienzo et al., 1994, Feldman et al., 1997). While they are time-consuming and expensive to develop, microsatellite markers are codominant, reproducible, and permit high sample throughput (Wattier and Maggs 2001).

Several researchers in the Klein laboratory at the University of New Hampshire have utilized diverse molecular methods, including allele specific polymerase chain reaction (PCR) of the ribosomal small subunit (SSU; Klein *et al.* 2003), restriction fragment length polymorphisms (RFLP, Teasdale *et al.* 2002), and *rbc*L gene sequences, to distinguish many North Atlantic *Porphyra* taxa, including *P. umbilicalis* (Klein *et al.*, 2003; see also Chapter 1). As mentioned previously, the usefulness of these methods in evaluating intra-specific population genetics is limited due to the low levels of sequence variation in these conservative genes. Recent work with ribosomal group-I introns (see Chapter 2) and internal transcribed spacers regions (Chapter 3) have shown increased levels of nucleotide variation that may be useful for large-scale biogeographic analyses. However, these loci are still inadequate for high-resolution studies of population genetic structures.

The objective of this study was to isolate microsatellite-containing loci from *Porphyra umbilicalis*. If successful, these markers would then be used to differentiate between Northwest and Northeast Atlantic populations in order to better understand the population genetic structure of red algae within the North Atlantic.

Materials and Methods

Sample collection and DNA extraction

Porphyra umbilicalis blade tissue was collected from one individual at Jaffrey Point New Hampshire. The samples were rinsed and cleaned of visible epiphytes. The blades were incubated for two weeks in a 20 gallon tank at 4°C in artificial seawater (Instant Ocean® from Aquarium Systems Inc., Mentor, OH) containing $0.18 mg \bullet L^{-1}$

germanium dioxide according to Markham and Hagmeir (1982) to reduce the diatom load. The DNAs were extracted from 1 gram tissue fragments using a modified CTAB extraction protocol by Apt and Grossman (1993), which included a CsCl ultracentrifugation step for DNA purification. The DNAs were confirmed as *Porphyra umbilicalis* using a restriction fragment length polymorphism assay developed for species discrimination between all known Northwest Atlantic *Porphyra* species (see Chapter 1).

Microsatellite Isolation

A rapid microsatellite isolation protocol used for the *Tilapia* Genome (Carleton *et al.*, 2002) was adapted for *Porphyra umbilicalis*. Genomic DNA was initially cut by the restriction enzyme *Sau*3AI. The genomic DNA digestion was separated by size using agarose gel electrophoresis and the gel region containing the 400-900 base pair fragments was excised. Adapters (A=5'-GATCGTCGACGGTACCGAATTCT-3';B=5'-GTCAAGAATTCGGTACCGTCGAC-3') were ligated to the ends of the size-specific DNA fragments using T4 DNA polymerase, which were then used as annealing sites for PCR primers. PCR was performed increasing the number of copies of all DNAs for the desired fragment size. The DNA was then denatured and hybridized to a biotinylated probe as the initial step in isolating the microsatellites. The probes selected for this work contained complimentary dinucleotide motifs (either $[AT]_{15}$, $[GA]_{15}$. or $[GT]_{15}$), which were chosen because of their prevalence in eukaryotic genomes and because dinucleotide repeats have higher mutation rates than other microsatellite motifs (Lagercrantz *et al.*, 1993). The hybridized probe/microsatellite complexes were isolated from background DNAs using streptavidin-bound magnetic beads (Dynal Corp., Oslo, Norway), according

to manufacturer's instructions. The selected DNA was again amplified by PCR to increase the copy number of microsatellite-containing DNAs and cloned into a pGEM-T bacterial plasmid vector (Promega, Madison, WI) using high efficiency JM109 (Promega) competent cells for transformation according to manufacturer's directions. A transformation efficiency of 1.8-4.5 X 10^8 cfu/μg DNA was calculated from all three microsatellite isolations when using a 1:1 molar ratio of insert DNA to vector. Blue/white screening identified bacterial colonies containing inserts. Insert-containing colonies were cultured overnight in 4 ml of LB (Luria-Bertrani) medium. QIAprep® Spin Miniprep Kits (Qiagen, Valencia, CA) were used to isolate the plasmids according to manufacturer's instructions.

The plasmid DNAs containing the microsatellite inserts were sequenced using the ABI DYEnamic™ ET terminator cycle sequencing kit on an ABI 377 Automated Sequencer (Applied Biosystems, Foster City, CA) at the UNH Sequencing Facility. The forward and reverse M13 primers as well as SP6 and T7 primers were used. Sequence analysis was performed using the SeqEd software program (vers. 1.0.3; Applied Biosystems) in order to identify microsatellite loci. The Lasergene Software PrimerSelect© was used to develop primer pairs for microsatellite sequences with >16 uninterrupted repeats. The polymerase chain reaction was performed using unlabeled primers for selected microsatellite containing loci and the products were separated by electrophoresis on 3% agarose gels.

Screening microsatellites for polymorphism

Template DNA from six *Porphyra umbilicalis* individuals (Klein Laboratory *Porphyra* DNA Accessions: 103, 163, 426, 451, 499, 1009), the closely related species *P. linearis* Grev. (# 710) and three other *Porphyra* species: *P. leucosticta* Thuret *in* Le Jol. (#593), *P. purpurea* (Roth) C. Agardh (#555), and *P. amplissima* (Kjell.) Setch. *et* Hus *in* Hus (#465) were used to screen for microsatellite polymorphisms. The geographic locations for each DNA accession used in the screening are given in Table 1. The DNAs were extracted directly from haploid blades as described previously in Chapters 2 and 3.

Polymerase chain reactions (50 μL total volume) contained 1 μL of each genomic DNA template, 50 mM KCl, 10mM Tris-HCl, 2.0 mM $MgCl_2$, 0.8mM dNTPs, 0.4μM of each primer, and 0.8U *Taq* polymerase (Promega). Each PCR was performed with a PTC-100™ thermocycler (MJ Research, Watertown, MA). All reactions were performed using a standard Hot Start protocol (D'Aquila *et al.*, 1991). The cycling parameters included an initial denaturation step of 5 minutes at 94°C, followed by 39 cycles of 30 seconds at the annealing temperature (Table 2), a 30-second extension at 72°C, and denaturation for 30 seconds at 94°C, with a final extension at 60°C for 90 minutes to promote uniform A-tailing of amplicons (Applied Biosystems, 1995). Amplicons were separated by electrophoresis on 3% agarose gels at ≈4V/cm, and than stained with Ethidium bromide and photographed.

Based on results from the high resolution agarose gels, three primer pairs sets (clone #: B12, D131, and D134) were chosen for GeneScan analysis. Flourescent-labeled forward primers were ordered for each primer set (Table 1). Following PCR, 10μL from each reaction was loaded onto a 3% agarose gel and separated by electrophoresis at

≈4V/cm for 1.5 hours. Based upon the brightness of each band, samples were diluted from 0-5 fold with TE. For allele sizing, 1.25μL from each diluted reaction was mixed with 1.75μL 5:1 deionized formamide: loading dye and 0.25μL GeneScan™-500 ROX™ size standard (Applied Biosystems, Warrington, UK), denatured for 2 minutes at 94°C, and immediately placed on ice. One microliter of each sample mixture was loaded onto a 6% denaturing polyacrylamide gel. Electrophoresis was carried out for six hours on an ABI373A automated DNA sequencer. Gels were analyzed using ABI GeneScan™ software version 3.1.

Results

Microsatellite-containing loci

Size-selective microsatellite isolation was performed three times during the course of this study. The first partial library was probed with GT_{15} dinucleotide probe and contained 195 positive clones from which 81 clones were sequenced based on the size or presence of the insert. The second library was again enriched by hybridization with GT_{15}; it contained 125 positive clones of which 48 were sequenced. Finally, a third partial library was probed with a combination of a GA_{15} and TA_{15} oligonucleotides, of which 54 inserts were sequenced. Of the 183 clones sequenced from all three partial genomic libraries, ~22.4% (41 clones) contained simple or compound microsatellites with 8 or more repeats. Of these 41 clones, only 16 (39%) had sufficient length (≥16 uninterupted repeats) and flanking sequence to design primers.

Screening of microsatellite loci

Primers were designed for the 16 loci. While all the primer pairs supported amplification of plasmid DNA, only 10 amplified genomic DNA (Table 2, Appendix E). The 10 primer pairs supported amplification of DNA from six geographically disperse accessions of *Porphyra umbilicalis* and for at least three of four other *Porphyra* taxa screened (Table 2). Figure 1 is an example of the amplification results using the B71 primer pair. The B71 primer pair produced a fragment in the predicted size range for *P. umbilicalis* templates as well as the amplification of the closely related *Porphyra* taxon, *P. linearis*. The primer set developed for microsatellite loci TA18 is the only example that supported fragment amplification in all species, resulting in what appears to be different size bands in the other species (Figure 2). Small differences in some banding patterns prompted a secondary screening with three loci (TA18, D134, GA5) using higher resolution polyacrylamide gels with fluorescent-labeled primers to accurately verify polymorphism that might exist. However, TA18 amplified fragments from six different P. umbilicalis templates ran with a uniform size distribution, indicating these samples were monomorphic for the TA18 locus as seen in the polyacrylamide GeneScan gel picture for loci TA18 (Figure 3). Thus GeneScan analysis established that the ambiguous banding patterns seen in the agarose gels were probably caused by the overloading of DNA in the individual lanes.

Discussion

The development of microsatellite loci has been accomplished in the red alga *Gracilaria gracilis* (Wattier *et al.*, 1997; Luo *et al.*, 1999), the fucoids (Coyer *et al.*,

2002; Olsen *et al.*, 2002; Engel *et al.,* 2003), kelps (Billot *et al.* 1998; Whitmer 2002; Wallace *et al.*, submitted), and members of the green algae (Van der Strate *et al.* 2000; Alström-Rapaport and Leskinen, 2002). The abundance and polymorphism of microsatellite-containing loci in all of these studies have been less than those found in higher plants and animals (Wang *et al.*, 1994; Wattier and Maggs, 2001; Olsen *et al.*, 2002). The present study utilized a size-selective dinucleotide enriched library to improve the isolation of microsatellites from *Porphyra umbilicalis.*

The present study of single-locus microsatellite genetic markers in *Porphyra umbilicalis* identified 10 microsatellite-containing loci with no allelic variation. Previous studies have shown that allelic polymorphisms in microsatellites have been linked to the number of uninterrupted repeats in the sequenced clone; in addition it has been observed that a low number of repeats are associated with lower levels of polymorphism (Weber, 1990; Yang *et al.*, 1994; Valdes *et al.*, 1993). My results could not confirm these observations because both uninterrupted and interrupted microsatellites with repeat motifs ≥ 16 were shown to be monomorphic; this may be related to the low number of loci screened or to differences in the mutation rate for microsatellites in *Porphyra* versus other organisms.

Of the 16 pairs of primers that were developed for microsatellite screening, six did not amplify genomic DNA but did amplify in the cloned vector DNA. The most plausible explanation for this result is that the positive clone may contain chimeric DNA, a PCR artifact. During the PCR amplification steps that are used to enrich for single sequence repeats (SSRs), the DNA polymerase may jump between different microsatellite-containing templates to create a chimeric DNA (Bradley and Hillis, 1997).

The Carleton *et al.* (2002) protocol for microsatellite cloning addressed some possible solutions associated with the formation of chimeras (use of a proofreading DNA polymerase, reduction of PCR cycles during microsatellite enrichment). However, although the recommended modifications of the protocol were employed in the present study, the level of putative chimeric (6/16) sequences among the *Porphyra* microsatellite-containing loci suggests that chimeric clones are still being produced in the hybrid-capture microsatellite cloning procedure.

The number of insert-containing clones sequenced in this study (183) was in the same order of magnitude to most other published microsatellite isolation studies involving marine algae (Table 3). Of the insert-containing clones, 22.4% had microsatellite-containing loci containing motifs of ≥ 16 repeats. Similar studies by Wallace *et al.* (unpublished), Billot *et al.* (1998), and Luo *et al.* (1999) exhibited similar or lower rates of microsatellite detection in their studies. In several of the studies that reported details of microsatellite cloning, chimeric clones were also problematic (i.e., Wallace *et al.*, submitted; Luo *et al.* 1999, Engel *et al.*, 2003). A noticeable difference between the present study and the other macroalgal microsatellite isolation studies, with the exception of Wallace *et al.* (unpublished), is the number of primers that were developed for microsatellite-containing inserts. Based on the present study's criteria to restrict primer development to clones with a repeat motif ≥16 dinucleotide repeats and the avoidance of imperfect or "interrupted" microsatellite sequences for primer design, a large number of sequences were omitted from further investigation. Interestingly, many of the microsatellite studies in algae have isolated the majority of their polymorphic microsatellites from imperfect repeat motifs (*i.e.,* Billot *et al.*, 1998; Luo *et al.*, 1999;

Engel *et al.,* 2003) or have an equal amount of both perfect and imperfect repeat motifs (*i.e.,* Wattier *et al.,* 1997; Olsen *et al.*, 2002; Wallace *et al.*, unpublished).

The main difference in this study versus other macroalgal microsatellite studies (Table 3) is the failure to detect polymorphic loci in *Porphyra umbilicalis*. Although it is unclear why my isolation method resulted in primarily monomorphic loci, I have two hypotheses: 1) the number of microsatellite sequences for primer development and screening needed to be increased; and 2) *P. umbilicalis* is uniquely lacking a large number of polymorphic microsatellite regions within its genome. Regarding the first hypothesis, prior studies with algae have shown that polymorphic microsatellites occur in the three major algal divisions (Rhodophyta, Chlorophyta, Phaeophyta) and are therefore likely to exist in *Porphyra umbilicalis*. A re-evaluation and analysis of smaller and imperfect microsatellite motifs may increase the chances of isolating polymorphic loci. For the second hypothesis, if the DNA polymerases of *P. umbilicalis* have inherently slower mutation rates, then the frequency of polymorphisms would also show a reduction compared to microsatellite mutation rates in other organisms. With respect to the second hypothesis, it is interesting to note that the level of intraspecific mitochondrial *cox2-3* spacer variation was much lower for *Porphyra* than has been observed in the Florideophyceae (Chapter III).

Although I isolated microsatellite-containing loci from *Porphyra umbilicalis*, their monomorphic nature prevented any application to population or biogeographic analysis. Alternative methods for microsatellite enrichment of dinucleotide repeats are suggested for continued work with *Porphyra* with the following goals: (1) to reduce the level of chimeric sequences (possible by avoiding PCR-based approaches for isolating

microsatellite-containing loci); (2) to utilize both imperfect and shorter repeats when pooling sequences for primer development. Alternatively, primers could be designed for any trinucleotide repeats found within the Expressed Sequence Tag (EST) expression library database of *P. yezoensis* and screened for polymorphism. It is worthwhile to note the primers for several of these loci (B71, GA5, TA18) that amplified bands of different sizes in other *Porphyra* taxa may be useful tools for molecular identification of *Porphyra* species.

Table 1. List of *Porphyra* used to screen microsatellite loci DNA ID= numbering system used for genomic DNA accessions in Dr. Klein's laboratory at the University of New Hampshire.

Organism	DNA ID	Sample location	Date
Porphyra umbilicalis	103	Fort Williams, Portland Head, Maine	01/28/1996
Porphyra umbilicalis	163	Two Lights State Park, Maine	01/28/1996
Porphyra umbilicalis	426	Schooner Point, Mount Desert Island, Maine	02/22/1996
Porphyra umbilicalis	451	West Quoddy Head, Maine	02/24/1996
Porphyra umbilicalis	499	East Point, Nahant, MA	10/19/1996
Porphyra umbilicalis	1009	Dover Point, NH	02/23/1998
Porphyra linearis	710	Rye Harbor, NH	unknown
Porphyra leucosticta	593	Rachel Carson Salt Pond Reserve, Bristol, Maine	02/02/1996
Porphyra purpurea	555	Lighthouse Cove, Dipper Harbor, NB, Canada	11/02/1996
Porphyra amplissima	465	(cultured conchocelis)- Univ. of Connecticut	n.a.

Table 2. Characteristics of the ten monomorphic microsatellites from *Porphyra umbilicalis* including DNA ID, primer sequences, band size, repeat motif, screening method, and other species that showed amplification products. The nucleotide sequence for each clone is given in Appendix E.

Clone ID	Forward Primer (5'→3')	Reverse Primer (5'→3')	Expected product size	Microsatellite	Annealing Temperature	Screening method	Other *Porphyra* species[a]
B63	GCCGGATTGCGTGGTTATG	AGCCCTGAGTGCCTTGTGC	184	GT_{16}	53	agarose[b]	
B71	GTTGATGCTTGTGGCTTGAGAGG	CGGATTGTGCGGTGATGTG	132	CA_{22}	53.0	agarose	1
B73	GCGAGTGGCATAGAATGACT	CAGCACGTGTACCCCTACTG	161	GT_{16}	58	agarose	
D131	GGTCGCGCCCGCTATTTTG	CATGACCTGCCGCCTGTGAG	174	CT_{16}	51.5	agarose, polyacrylamide[c]	
D134	GGCAACGACCGGTCGAAACACATC	CTGCGCCGGACGGGGCATTCTAC	183	AC_{21}	49.5	agarose, polyacrylamide[c]	
GA5	CCGGATTGTCTGTGCTTCTCT	GCATCGCTCTCCACACTATCAT	302	GA_{18}	53.5	agarose	1,2
TA17	AAATTCTTGCCTTTGCTCCTT	GCCACGCCAGACCAAATTGAC	176	$TC_{24}AC_8TC_{14}$	52.0	agarose	
TA18	GAGACGGCTTAATTTGCGATG	AGGGTGAGCGCGCTCTCTTTC	291	GA_{38}	58.0	agarose, polyacrylamide[c]	1,2,3,4
TA45-50-54	TGTCGTCGTGACAAGTCGC	ACCCTAACTCTAACCCTCCC	320	GT_{49}	55.0	agarose	
TA56	CGCGGAGTTCTAATAGTTGTG	ACGCGGGCCAAGGTGTATTTC	311	GT_{32}	55.9	agarose	

a. Four other *Porphyra* species were used to screen probably microsatellite loci. 1=*P. linearis*; 2=*P. leucosticta*; 3=*P. purpurea* and 4=*P. amplissima*.
b. 3% Metaphor agarose gels
c. 6% denaturing polyacrylamide gels

Table 3. The results of the microsatellite isolation method for *Porphyra umbilicalis* and other studies involving marine algae. Note:? = undetermined or not disclosed.

	This study	**Wallace *et al.* (in submission)**	**Wattier *et al.*, 1997**	**Billot *et al.*, 1998**	**Luo *et al.*, 1999**	**C. Alstrom-Rapaport and Leskinen, 2002**	**Olsen *et al.*, 2002**	**Engel *et al.*, 2003**
Species of origin	*Porphyra umbilicalis* (Rhodophyta)	*Fucus spiralis* (Phaeophyta)	*Gracilaria gracilis* (Rhodophyta)	*Laminaria digitata* (Phaeophyta)	*Gracilaria gracilis* (Rhodophyta)	*Enteromorpha intestinalis* (Chlorophyta)	*Ascophyllum nodosum* (Phaeophyta)	*F. vesiculosus, F. serratus, A. nodousm* (Phaeophyta)
Total Clones Sequenced	183	293	66	216	225	6	300	96
No. sequenced clones containing repeat motifs ≥ x	41 (x=16)	63 (x=16)	?	48 (x=10)	23 (x=?)	5 (x=8)	?	59 (x=?)
No. of primer pairs developed	16	12	4	42	23	5	70	28
No. of putative chimeric sequences or inadequate amplification	6	4	?	?	7	0	?	6
No. of simple microsatellites	9	5	2	3	3	4	3	2
No. of imperfect microsatellites	1	3	2	7	6	1	3	8
No. of monomorphic microsatellites	10	4	2	?	7	0	?	3
No. of polymorphic microsatellites	0	4	2	10	9	5	6	9

Figure 1. A representative result of monomorphic microsatellite loci in test samples. The analysis below shows a 3% agarose gel with the amplification results of microsatellite loci B71. Lane 1 = ΦX174DNA/*Hae* III with the sizes of some fragments labeled in base pairs; 2-7 = *Porphyra umbilicalis*; 8 = *P. linearis*; 9 = *P. leucosticta*; 10= *P. purpurea*; 11 = P. *amplissima*; 12 = negative control (Tris EDTA).

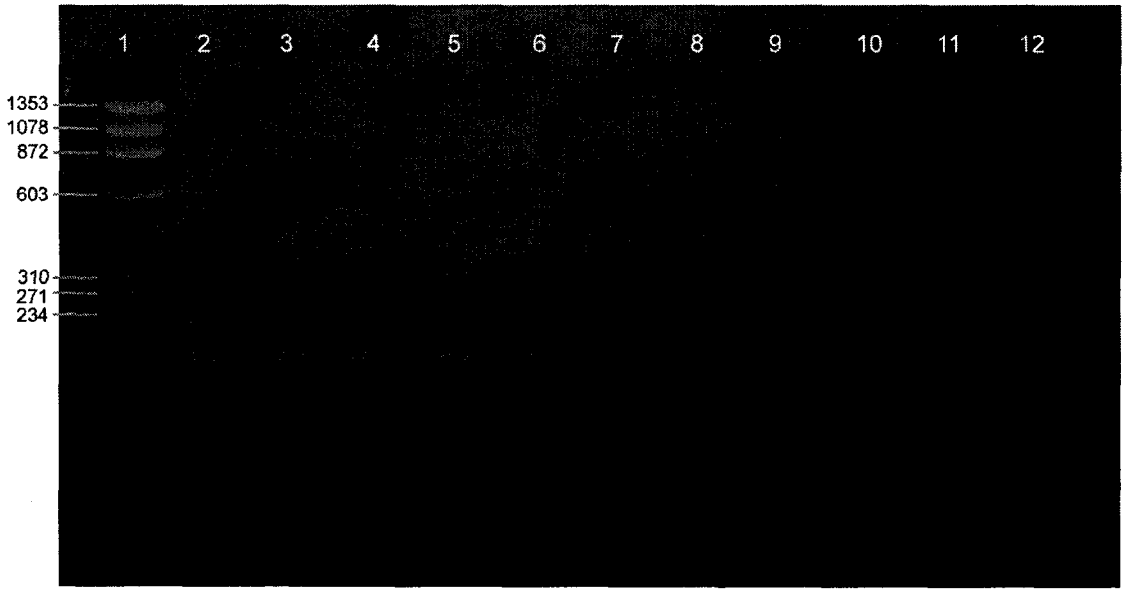


Figure 2. A 3% agarose gel that show PCR amplification patterns of the TA18 microsatellite locus. . Lane 1 = ΦX174DNA/*Hae* III with the sizes of some fragments labeled in base pairs; 2-8 = *Porphyra umbilicalis*; 9 = *P. linearis*; 10 = *P. leucosticta*; 11= *P. purpurea*; 12 = P. *amplissima*; 13 = negative control (Tris EDTA).Note: *Lane 2 corresponds to P. umbilicalis plasmid DNA.*

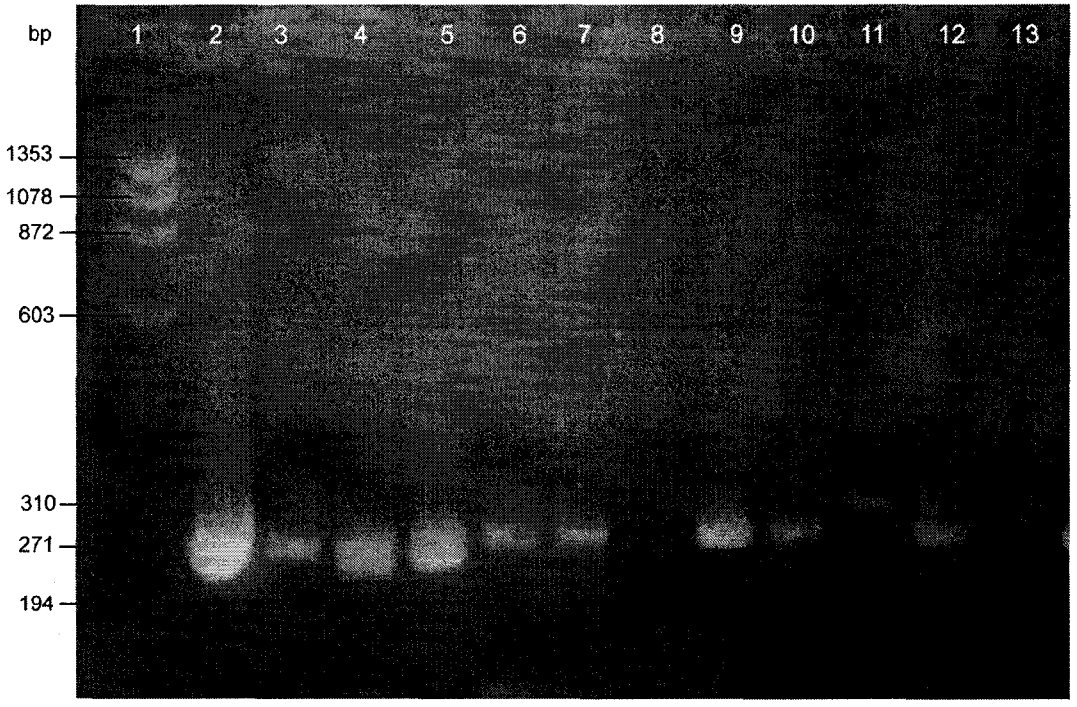
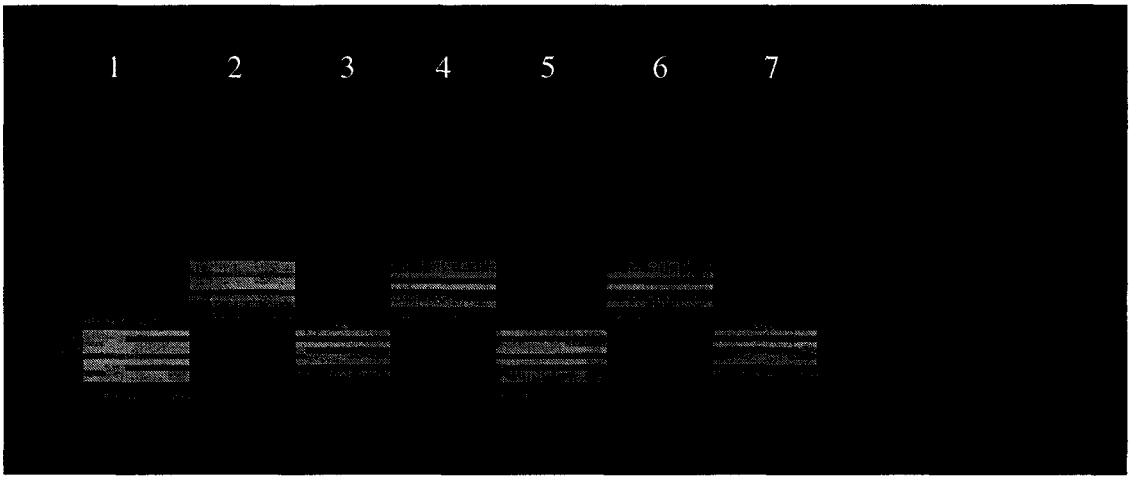


Figure 3. A section from a polyacrylamide GeneScan gel showing the amplification results from microsatellite loci TA18 in six previously screened *Porphyra umbilicalis* individuals and the original plasmid isolated DNA. The lanes are staggered with odd numbers pre-run for 5 minutes to avoid contamination by nearby even lanes. Therefore, all lanes in this picture are showing the same size band (~291 bp). *blue* bands = microsatellite loci, *red* bands= ROX size standard. Lane 1 = isolate # 103; 2 = 163; 3 = 426; 4 = 499; 5 = 1009; 6 = 451; 7 = positive control (plasmid DNA).



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APPENDIX A

DNA	Species	Location	Collection date
1	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
2	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
3	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
4	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
5	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
6	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
7	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
8	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
9	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
10	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
11	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
12	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
13	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
14	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
15	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
16	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
17	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
18	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
19	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
20	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
21	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
22	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
23	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
24	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
25	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
26	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
27	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
28	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
29	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
30	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
31	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
32	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
33	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
34	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
35	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
36	*P. 'leucosticta'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
37	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
38	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
39	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
40	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
41	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
42	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
43	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
44	*P. 'amplissima'*	Gove Point, Cobscook Bay, Lubec, ME	7/8/95
45	*P. 'linearis'*	Seapoint, ME	2/14/96
46	*P. 'linearis'*	Seapoint, ME	2/14/96
47	*P. 'linearis'*	Seapoint, ME	2/14/96

48	*P. 'linearis'*	Seapoint, ME	2/14/96
49	*P. 'linearis'*	Seapoint, ME	2/14/96
50	*P. 'linearis'*	Seapoint, ME	2/14/96
51	*P. 'linearis'*	Seapoint, ME	2/14/96
52	*P. 'linearis'*	Seapoint, ME	2/14/96
53	*P. 'linearis'*	Bristol, ME	2/18/96
54	*P. 'linearis'*	Bristol, ME	2/18/96
55	*P. 'linearis'*	South Bristol, ME	2/18/96
56	*P. 'linearis'*	South Bristol, ME	2/18/96
57	*P. 'linearis'*	South Bristol, ME	2/18/96
58	*P. 'linearis'*	South Bristol, ME	2/18/96
59	*P. 'linearis'*	South Bristol, ME	2/18/96
60	*P. 'linearis'*	South Bristol, ME	2/18/96
61	*P. 'umbilicalis'*	Sand Beach, Mount Desert Island, ME	2/22/96
62	*P. 'umbilicalis'*	Sand Beach, Mount Desert Island, ME	2/22/96
63	*P. 'umbilicalis'*	Bagaduce Falls, ME	3/22/96
64	*P. 'umbilicalis'*	Bagaduce Falls, ME	3/22/96
65	*P. 'umbilicalis'*(lin)	Red Point, Swans Island, ME	3/12/96
66	*P. 'umbilicalis'*(lin)	Red Point, Swans Island, ME	3/12/96
67	*P. 'purpurea'*	Leighton Cove, Whiting, ME	3/27/96
68	*P. 'purpurea'*	Leighton Cove, Whiting, ME	3/27/96
69	*P. 'amplissima'*	cultured conchocelis	N/A
70	*P. 'amplissima'*	cultured conchocelis	N/A
71	*P. 'amplissima'*	cultured conchocelis	N/A
72	*P. 'amplissima'*	cultured conchocelis	N/A
73	*P. 'carolinsus'*	Waterford, CT	11/3/95
74	*P. 'carolinsus'*	Waterford, CT	11/3/95
75	*P. 'carolinsus'*	Waterford, CT	12/3/95
76	*P. 'carolinsus'*	Waterford, CT	12/3/95
77	*P. 'amplissima'*	Eastport, ME	7/8/95
78	*P. 'amplissima'*	Eastport, ME	7/8/95
79	*P. 'amplissima'*	Eastport, ME	7/8/95
80	*P. 'amplissima'*	Eastport, ME	7/8/95
81	*P. 'amplissima'*	Eastport, ME	7/8/95
82	*P. 'amplissima'*	Eastport, ME	7/8/95
83	*P. 'amplissima'*	Eastport, ME	7/8/95
84	*P. 'amplissima'*	Eastport, ME	7/8/95
85	*P. 'amplissima'*	Eastport, ME	7/8/95
86	*P. 'amplissima'*	Eastport, ME	7/8/95
87	*P. 'amplissima'*	Eastport, ME	7/8/95
88	*P. 'amplissima'*	Eastport, ME	7/8/95
89	*P. 'amplissima'*	Eastport, ME	7/8/95
90	*P. 'amplissima'*	Eastport, ME	7/8/95
91	*P. 'amplissima'*	Eastport, ME	7/8/95
92	*P. 'amplissima'*	Eastport, ME	7/8/95
93	*P. 'amplissima'*	Eastport, ME	7/8/95
94	*P. 'amplissima'*	Eastport, ME	7/8/95
95	*P. 'amplissima'*	Eastport, ME	7/8/95
96	*P. 'amplissima'*	Eastport, ME	7/8/95
97	*P. 'amplissima'*	Eastport, ME	7/8/95
98	*P. 'amplissima'*	Eastport, ME	7/8/95
99	*P. 'amplissima'*	Eastport, ME	7/8/95
100	*P. 'amplissima'*	Eastport, ME	7/8/95
101	*P. 'amplissima'*	Eastport, ME	7/8/95
102	*P. 'umbilicalis'*	Fort Williams, Portland Head, ME	1/27/96
103	*P. 'umbilicalis'*	Fort Williams, Portland Head, ME	1/27/96
104	*P. 'umbilicalis'*	Fort Williams, Portland Head, ME	1/27/96
105	*P. 'umbilicalis'*	Fort Williams, Portland Head, ME	1/27/96
106	*P. 'umbilicalis'*	Fort Williams, Portland Head, ME	1/27/96
107	*P. 'umbilicalis'*	Fort Williams, Portland Head, ME	1/27/96
108	*P. 'umbilicalis'*	Fort Williams, Portland Head, ME	1/27/96
109	*P. 'umbilicalis'*	Fort Williams, Portland Head, ME	1/27/96
110	*P. 'linearis'*	Newagen, South Port Island, ME	2/18/96
111	*P. 'linearis'*	Newagen, South Port Island, ME	2/18/96
112	*P. 'linearis'*	Newagen, South Port Island, ME	2/18/96
113	*P. 'linearis'*	Newagen, South Port Island, ME	2/18/96
114	*P. 'linearis'*	Newagen, South Port Island, ME	2/18/96
115	*P. 'linearis'*	Newagen, South Port Island, ME	2/18/96
116	*P. 'linearis'*	Newagen, South Port Island, ME	2/18/96
117	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96

118	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
119	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
120	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
121	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
122	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
123	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
124	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
125	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
126	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
127	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
128	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
129	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
130	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
131	*P. 'umbilicalis'*	Camp Ellis, ME	2/6/96
132	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
133	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
134	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
135	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
136	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
137	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
138	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
139	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
140	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
141	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
142	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
143	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
144	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
145	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
146	*P. 'linearis'*	Newagen, South Port Island, ME	2/19/96
147	*P. 'leucosticta'*	Eastport, ME	7/8/95
148	*P. 'leucosticta'*	Eastport, ME	7/8/95
149	*P. 'leucosticta'*	Eastport, ME	7/8/95
150	*P. 'leucosticta'*	Eastport, ME	7/8/95
151	*P. 'leucosticta'*	Eastport, ME	7/8/95
152	*P. 'leucosticta'*	Eastport, ME	7/8/95
153	*P. 'leucosticta'*	Eastport, ME	7/8/95
154	*P. 'leucosticta'*	Eastport, ME	7/8/95
155	*P. 'leucosticta'*	Eastport, ME	7/8/95
156	*P. 'leucosticta'*	Eastport, ME	7/8/95
157	*P. 'leucosticta'*	Eastport, ME	7/8/95
158	*P. 'leucosticta'*	Eastport, ME	7/8/95
159	*P. 'leucosticta'*	Eastport, ME	7/8/95
160	*P. 'leucosticta'*	Eastport, ME	7/8/95
161	*P. 'leucosticta'*	Eastport, ME	7/8/95
162	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
163	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
164	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
165	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
166	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
167	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
168	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
169	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
170	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
171	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
172	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
173	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
174	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
175	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
176	*P. 'umbilicalis'*	Two Lights State Park, ME	1/22/96
192	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
193	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
194	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
195	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
196	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
197	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
198	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
199	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
200	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
201	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
202	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96

203	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
204	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
205	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
206	*P. 'umbilicalis'*	Cape Elizabeth, ME	1/26/96
222	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
223	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
224	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
225	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
226	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
227	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
228	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
229	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
230	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
231	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
232	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
233	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
234	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
235	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
236	*P. 'umbilicalis'*	Higgins Beach, ME	1/26/96
268	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
269	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
270	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
271	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
272	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
273	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
274	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
275	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
276	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
277	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
278	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
279	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
280	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
281	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
282	*P. 'umbilicalis'*	Parson's Beach, ME	2/4/96
310	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	
311	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	
312	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	
313	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	
314	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	
315	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	
316	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	
317	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	
318	*P. 'leucosticta'*	Jamestown, RI	6/13/96
319	*P. 'leucosticta'*	Jamestown, RI	6/13/96
320	*P. 'leucosticta'*	Jamestown, RI	6/13/96
321	*P. 'leucosticta'*	Jamestown, RI	6/13/96
322	*P. 'leucosticta'*	Jamestown, RI	6/13/96
323	*P. 'leucosticta'*	Jamestown, RI	6/13/96
324	*P. 'leucosticta'*	Jamestown, RI	6/13/96
325	*P. 'leucosticta'*	Jamestown, RI	6/13/96
326	*P. 'leucosticta'*	Jamestown, RI	6/13/96
327	*P. 'leucosticta'*	Jamestown, RI	6/13/96
328	*P. 'leucosticta'*	Jamestown, RI	6/13/96
329	*P. 'leucosticta'*	Jamestown, RI	6/13/96
330	*P. 'leucosticta'*	Jamestown, RI	6/13/96
331	*P. 'leucosticta'*	Jamestown, RI	6/13/96
332	*P. 'leucosticta'*	Jamestown, RI	6/13/96
333	*P. 'leucosticta'*	Jamestown, RI	6/13/96
334	*P. 'leucosticta'*	Jamestown, RI	6/13/96
335	*P. 'leucosticta'*	Jamestown, RI	6/13/96
336	*P. 'leucosticta'*	Jamestown, RI	6/13/96
337	*P. 'leucosticta'*	Jamestown, RI	6/13/96
338	*P. 'leucosticta'*	Jamestown, RI	6/13/96
339	*P. 'leucosticta'*	Jamestown, RI	6/13/96
348	*P. 'leucosticta'*	Jamestown, RI	6/13/96
349	*P. 'leucosticta'*	Jamestown, RI	6/13/96
350	*P. 'leucosticta'*	Jamestown, RI	6/13/96
351	*P. 'leucosticta'*	Jamestown, RI	6/13/96
352	*P. 'leucosticta'*	Jamestown, RI	6/13/96
353	*P. 'leucosticta'*	Jamestown, RI	6/13/96

354	*P. 'leucosticta'*	Jamestown, RI	6/13/96
355	*P. 'leucosticta'*	Jamestown, RI	6/13/96
356	*P. 'leucosticta'*	Jamestown, RI	6/13/96
357	*P. 'leucosticta'*	Jamestown, RI	6/13/96
358	*P. 'leucosticta'*	Jamestown, RI	6/13/96
359	*P. 'leucosticta'*	Jamestown, RI	6/13/96
360	*P. 'leucosticta'*	Jamestown, RI	6/13/96
361	*P. 'leucosticta'*	Jamestown, RI	6/13/96
362	*P. 'leucosticta'*	Jamestown, RI	6/13/96
378	*P. 'leucosticta'*	Charlestown, RI	6/13/96
379	*P. 'leucosticta'*	Charlestown, RI	6/13/96
380	*P. 'leucosticta'*	Charlestown, RI	6/13/96
381	*P. 'leucosticta'*	Charlestown, RI	6/13/96
382	*P. 'leucosticta'*	Charlestown, RI	6/13/96
383	*P. 'leucosticta'*	Charlestown, RI	6/13/96
384	*P. 'leucosticta'*	Charlestown, RI	6/13/96
385	*P. 'leucosticta'*	Charlestown, RI	6/13/96
386	*P. 'leucosticta'*	Charlestown, RI	6/13/96
387	*P. 'leucosticta'*	Charlestown, RI	6/13/96
388	*P. 'leucosticta'*	Charlestown, RI	6/13/96
389	*P. 'leucosticta'*	Charlestown, RI	6/13/96
390	*P. 'leucosticta'*	Charlestown, RI	6/13/96
391	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
392	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
393	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
394	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
395	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
396	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
397	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
398	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
399	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
400	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
401	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
402	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
403	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
404	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
405	*P. 'miniata'*	Fink Cove, Nova Scotia, Canada	6/23/96
419	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
420	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
421	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
422	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
423	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
424	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
425	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
426	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
427	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
428	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
429	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
430	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
431	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
432	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
433	*P. 'umbilicalis'*	Schooner Point, Mount Desert Island, ME	2/22/96
449	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
450	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
451	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
452	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
453	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
454	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
455	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
456	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
457	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
458	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
459	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
460	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
461	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
462	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
463	*P. 'umbilicalis'*	West Quoddy Head, ME	2/24/96
464	*P. 'amplissima'*	cultured blades	N/A
465	*P. 'amplissima'*	cultured conchocelis	N/A
466	*P. 'amplissima'*	cultured conchocelis	N/A

467	*P. 'amplissima'*	cultured conchocelis	N/A
468	*P. 'amplissima'*	cultured conchocelis	N/A
469	*P. 'amplissima'*	cultured conchocelis	N/A
470	*P. 'amplissima'*	cultured blades	N/A
471	*P. 'umbilicalis'*	cultured blades	N/A
472	*P. 'umbilicalis'*	cultured blades	N/A
473	*P. 'umbilicalis'*	cultured blades	N/A
474	*P. 'linearis'*	cultured conchocelis	N/A
475	*P. 'linearis'*	cultured conchocelis	N/A
476	*P. 'yezoensis'*	cultured conchocelis	N/A
477	*P. 'yezoensis'*	cultured blades	N/A
478	*P. 'yezoensis'*	cultured conchocelis	N/A
479	*P. 'umbilicalis'*	cultured blades	N/A
480	*P. 'umbilicalis'*	cultured blades	N/A
481	*P. 'umbilicalis'*		
482	*P. 'umbilicalis'*		
483	*P. 'umbilicalis'*	cultured blades	N/A
484	*P. 'umbilicalis'*	cultured blades	N/A
485	*P. 'linearis'*	cultured conchocelis	N/A
486	*P. 'linearis'*	cultured conchocelis	N/A
487	*P. 'linearis'*		
488	*P. 'linearis'*		
489	*P. 'purpurea'*	Thomas Point, Great Bay, Newington, NH	9/25/96
490	*P. 'purpurea'*	Thomas Point, Great Bay, Newington, NH	9/25/96
491	*P. 'purpurea'*	Thomas Point, Great Bay, Newington, NH	9/25/96
492	*P. 'purpurea'*	Thomas Point, Great Bay, Newington, NH	9/25/96
493	*P. 'purpurea'*	Thomas Point, Great Bay, Newington, NH	9/25/96
494	*P. 'purpurea'*	Thomas Point, Great Bay, Newington, NH	9/25/96
495	*P. 'purpurea'*	Thomas Point, Great Bay, Newington, NH	9/25/96
496	*P. 'purpurea'*	Thomas Point, Great Bay, Newington, NH	9/25/96
498	*P. 'umbilicalis'*	East Point, Nahant, MA	10/18/96
499	*P. 'umbilicalis'*	East Point, Nahant, MA	10/18/96
500	*P. 'umbilicalis'*	East Point, Nahant, MA	10/18/96
501	*P. 'umbilicalis'*	East Point, Nahant, MA	10/18/96
503	*P. 'umbilicalis'*	East Point, Nahant, MA	10/18/96
504A	*P. 'umbilicalis'*	East Point, Nahant, MA	10/18/96
504B	*P. 'umbilicalis'*	Seawall, Mount Desert Island, ME	2/23/96
505	*P. 'umbilicalis'*	Seawall, Mount Desert Island, ME	2/23/96
506	*P. 'umbilicalis'*	Seawall, Mount Desert Island, ME	2/23/96
507	*P. 'umbilicalis'*	Seawall, Mount Desert Island, ME	2/23/96
508	*P. 'umbilicalis'*	Seawall, Mount Desert Island, ME	2/23/96
509	*P. 'purpurea'*	Starboard, ME	2/24/96
510	*P. 'purpurea'*	Starboard, ME	2/24/96
511	*P. 'purpurea'*	Starboard, ME	2/24/96
512	*P. 'purpurea'*	Starboard, ME	2/24/96
513	*P.' purpurea'*	Starboard, ME	2/24/96
514	*P. 'linearis'*	Red Point Swan's Island, ME	3/12/96
515	*P. 'linearis'*	Red Point Swan's Island, ME	3/12/96
516	*P. 'linearis'*	Red Point Swan's Island, ME	3/12/96
517	*P. 'linearis'*	Red Point Swan's Island, ME	3/12/96
518	*P. 'linearis'*	Red Point Swan's Island, ME	3/12/96
519	*P. 'yezoensis'*		
520	*P. 'yezoensis'*		
521	*P. 'yezoensis'*		
522	*P. 'yezoensis'*		
523	*P. 'purpurea'*	R. Friedman Field Station, Cobscook Bay, ME	3/24/96
524	*P. 'purpurea'*	R. Friedman Field Station, Cobscook Bay, ME	3/24/96
525	*P. 'purpurea'*	R. Friedman Field Station, Cobscook Bay, ME	3/24/96
526	*P. 'purpurea'*	R. Friedman Field Station, Cobscook Bay, ME	3/24/96
527	*P. 'purpurea'*	R. Friedman Field Station, Cobscook Bay, ME	3/24/96
528	*P. 'purpurea'*	R. Friedman Field Station, Cobscook Bay, ME	3/24/96
529	*P. 'purpurea'*	R. Friedman Field Station, Cobscook Bay, ME	3/24/96
530	*P. 'purpurea'*	R. Friedman Field Station, Cobscook Bay, ME	3/24/96
532	*P.' purpurea'*	R. Friedman Field Station, Cobscook Bay, ME	3/24/96
533	*P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
534	*P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
535	*P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96

536 *P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
537 *P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
538 *P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
539 *P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
540 *P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
541 *P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
542 *P. 'purpurea'*(leuc)	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
543 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
544 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
545 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
546 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
547 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
548 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
549 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
550 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
551 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
552 *P. 'purpurea'*	Ross Island Shore, Grand Harbor, New Brunswick, Canada	11/2/96
553 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
554 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
555 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
556 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
557 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
558 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
559 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
560 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
561 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
562 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
563 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
564 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
565 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
566 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
567 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
568 *P. 'purpurea'*	Lighthouse Cove, Dipper Harbor, New Brunswick, Canada	11/1/96
569 *P. 'purpurea'*	Herring Cove, Nova Scotia, Canada	9/28/96
570 *P. 'purpurea'*	Herring Cove, Nova Scotia, Canada	9/28/96
571 *P. 'purpurea'*	Herring Cove, Nova Scotia, Canada	9/28/96
572 *P. 'purpurea'*	Herring Cove, Nova Scotia, Canada	9/28/96

573	*P. 'purpurea'*	Herring Cove, Nova Scotia, Canada	9/28/96
574	*P. 'purpurea'*	Herring Cove, Nova Scotia, Canada	9/28/96
575	*P. 'purpurea'*	Herring Cove, Nova Scotia, Canada	9/28/96
576	*P. 'purpurea'*	Herring Cove, Nova Scotia, Canada	9/28/96
577	*P. 'purpurea'*	Avonport, Nova Scotia, Canada	9/30/96
578	*P. 'purpurea'*	Avonport, Nova Scotia, Canada	9/30/96
579	*P. 'purpurea'*	Avonport, Nova Scotia, Canada	9/30/96
580	*P. 'purpurea'*	Avonport, Nova Scotia, Canada	9/30/96
581	*P. 'purpurea'*	Avonport, Nova Scotia, Canada	9/30/96
582	*P. 'purpurea'*	Avonport, Nova Scotia, Canada	9/30/96
583	*P. 'purpurea'*	Avonport, Nova Scotia, Canada	9/30/96
584	*P. 'purpurea'*	Avonport, Nova Scotia, Canada	9/30/96
585	*P. 'leucosticta'*	Pemaquid Point	8/8/96
586	*P. 'leucosticta'*	Pemaquid Point	8/8/96
587	*P. 'leucosticta'*	Pemaquid Point	8/8/96
588	*P. 'leucosticta'*	Pemaquid Point	8/8/96
589	*P. 'leucosticta'*	Two Lights State Park, ME	8/17/96
590	*P. 'leucosticta'*	Two Lights State Park, ME	8/17/96
591	*P. 'leucosticta'*	Two Lights State Park, ME	8/17/96
592	*P. 'leucosticta'*	Two Lights State Park, ME	8/17/96
593	*P. 'leucosticta'*	Rachel Carson Salt Pond Preserve, Bristol, ME	8/9/96
594	*P. 'leucosticta'*	Rachel Carson Salt Pond Preserve, Bristol, ME	8/9/96
595	*P. 'leucosticta'*	Rachel Carson Salt Pond Preserve, Bristol, ME	8/9/96
596	*P. 'leucosticta'*	Rachel Carson Salt Pond Preserve, Bristol, ME	8/9/96
597	*P. 'umbilicalis'*	Reid State Park, ME	8/8/96
598	*P. 'umbilicalis'*	Reid State Park, ME	8/8/96
599	*P. 'umbilicalis'*	Reid State Park, ME	8/8/96
600	*P. 'umbilicalis'*	Reid State Park, ME	8/8/96
601	*P. 'purpurea'*	Orr's Island, ME	2/3/96
602	*P. 'purpurea'*	Orr's Island, ME	2/3/96
603	*P. 'purpurea'*	Orr's Island, ME	2/3/96
604	*P. 'purpurea'*	Orr's Island, ME	2/3/96
605	*P. 'purpurea'*	Orr's Island, ME	2/3/96
606	*P. 'purpurea'*	Orr's Island, ME	2/3/96
607	*P. 'purpurea'*	Orr's Island, ME	2/3/96
608	*P. 'purpurea'*	Orr's Island, ME	2/3/96
609	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96
610	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96
611	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96
612	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96
613	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96
614	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96
615	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96
616	*P. 'linearis'*	Rachel Carson Salt Pond Preserve, Bristol, ME	2/1/96
617	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96
618	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96
619	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96
620	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96
621	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96
622	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96
623	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96
624	*P. 'umbilicalis'*	Peak's Island, Casco Bay, ME	3/23/96
625	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96
626	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96
627	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96
628	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96
629	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96
630	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96
631	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96
632	*P. 'linearis'*	Old Soaker, Mount Desert Island, ME	2/22/96
633	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
634	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
635	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
636	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
637	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
638	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
639	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
640	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
641	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96
642	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96

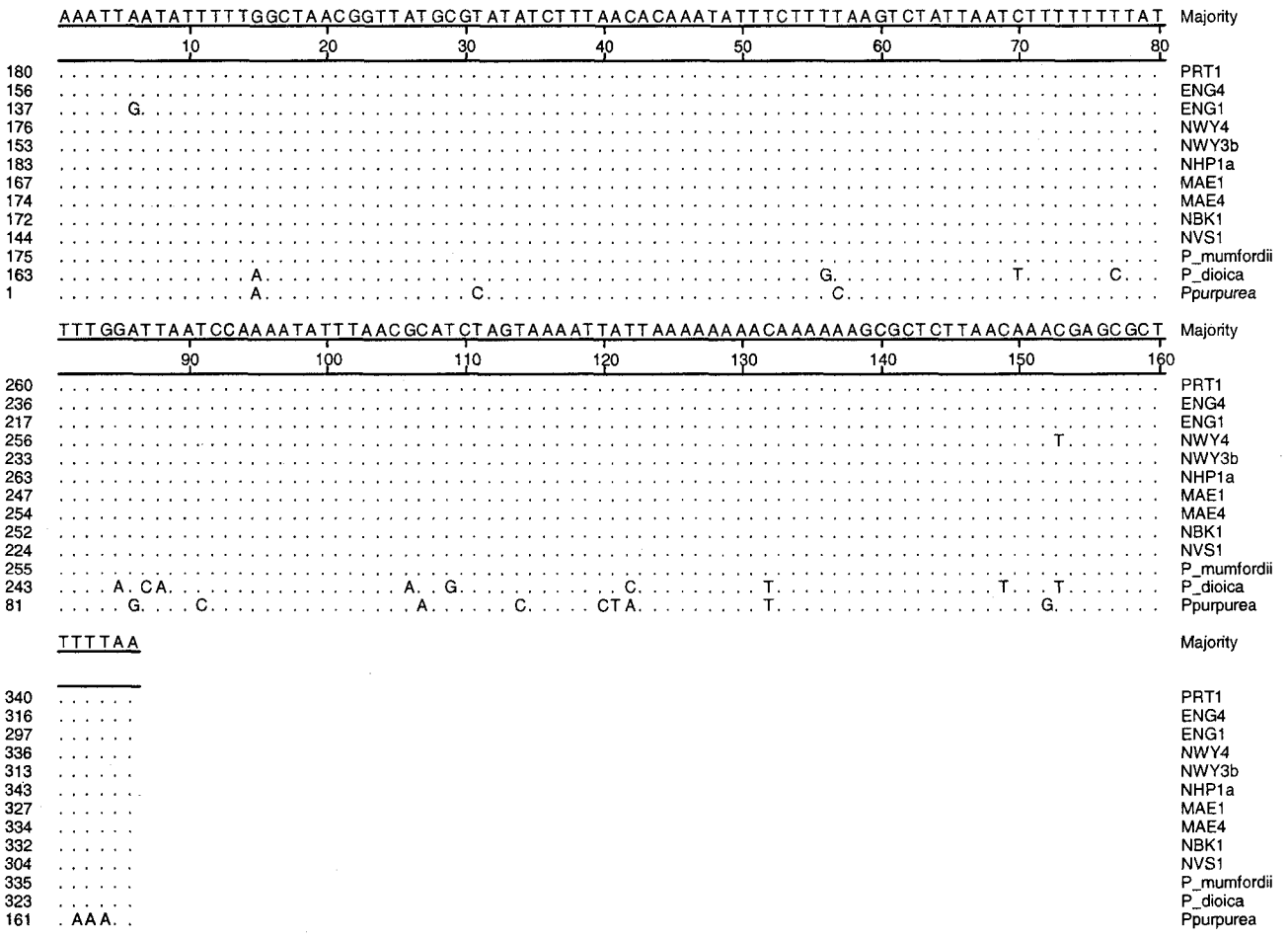
643	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96
644	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96
645	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96
646	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96
647	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96
648	*P. 'linearis'*	Pemaquid Point, Bristol, ME	2/1/96
649	*P. 'linearis'*	Reid State Park, ME	2/10/96
650	*P. 'linearis'*	Reid State Park, ME	2/10/96
651	*P. 'linearis'*	Reid State Park, ME	2/10/96
652	*P. 'linearis'*	Reid State Park, ME	2/10/96
653	*P. 'linearis'*	Reid State Park, ME	2/10/96
654	*P. 'linearis'*	Reid State Park, ME	2/10/96
655	*P. 'linearis'*	Reid State Park, ME	2/10/96
656	*P. 'linearis'*	Reid State Park, ME	2/10/96
657	*P. 'umbilicalis'*	Pine Point, ME	2/3/96
658	*P. 'umbilicalis'*	Pine Point, ME	2/3/96
659	*P. 'umbilicalis'*	Pine Point, ME	2/3/96
660	*P. 'umbilicalis'*	Pine Point, ME	2/3/96
661	*P. 'umbilicalis'*	Pine Point, ME	2/3/96
662	*P. 'umbilicalis'*	Pine Point, ME	2/3/96
663	*P. 'umbilicalis'*	Pine Point, ME	2/3/96
664	*P. 'umbilicalis'*	Pine Point, ME	2/3/96
665	*P. 'linearis'*	Rye Harbor, NH	2/3/96
666	*P. 'linearis'*-	Rye Harbor, NH	2/3/96
667	*P. 'linearis'*	Rye Harbor, NH	2/3/96
668	*P. 'linearis'*	Rye Harbor, NH	2/3/96
669	*P. 'linearis'*	Rye Harbor, NH	2/3/96
670	*P. 'linearis'*	Rye Harbor, NH	2/3/96
671	*P. 'linearis'*	Rye Harbor, NH	2/3/96
672	*P. 'linearis'*	Rye Harbor, NH	2/3/96
673	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96
674	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96
675	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96
676	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96
677	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96
678	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96
679	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96
680	*P. 'purpurea'*	Yarmouth Harbor, Nova Scotia, Canada	9/28/96
681	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
682	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
683	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
684	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
685	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
686	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
867	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
688	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
689	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
690	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
691	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
692	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
693	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
694	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
695	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
696	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
697	*P. 'linearis'*/thick form	Rye Harbor, NH	
698	*P. 'linearis'*/thick form	Rye Harbor, NH	
699	*P. 'linearis'*/thick form	Rye Harbor, NH	
700	*P. 'linearis'*/thick form	Rye Harbor, NH	
701	*P. 'linearis'*/thick form	Rye Harbor, NH	
702	*P. 'linearis'*/thick form	Rye Harbor, NH	
703	*P. 'linearis'*/thick form	Rye Harbor, NH	
704	*P. 'linearis'*/thick form	Rye Harbor, NH	
705	*P. 'linearis'*/thin form	Rye Harbor, NH	
706	*P. 'linearis'*/thin form	Rye Harbor, NH	
707	*P. 'linearis'*/thin form	Rye Harbor, NH	
708	*P. 'linearis'*/thin form	Rye Harbor, NH	
709	*P. 'linearis'*/thin form	Rye Harbor, NH	
710	*P. 'linearis'*/thin form	Rye Harbor, NH	
711	*P. 'linearis'*/thin form	Rye Harbor, NH	
712	*P. 'linearis'*/thin form	Rye Harbor, NH	

713	*P. 'umbilicalis'*	Pine Point, ME	2/6/96
714	*P. 'umbilicalis'*	Pine Point, ME	2/6/96
715	*P. 'umbilicalis'*	Pine Point, ME	2/6/96
716	*P. 'umbilicalis'*	Pine Point, ME	2/6/96
717	*P. 'umbilicalis'*	Pine Point, ME	2/6/96
718	*P. 'umbilicalis'*	Pine Point, ME	2/6/96
719	*P. 'umbilicalis'*	Pine Point, ME	2/6/96
720	*P. 'umbilicalis'*	Pine Point, ME	2/6/96
721	*P. 'umbilicalis'*	Eastport, ME	7/8/95
722	*P. 'umbilicalis'*	Eastport, ME	7/8/95
723	*P. 'umbilicalis'*	Eastport, ME	7/8/95
724	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
725	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
726	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
727	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
728	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
729	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
730	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
731	*P. 'umbilicalis'*	Campobello Bridge, ME and Canada	10/7/95
732	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
733	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
734	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
735	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
736	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
737	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
738	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
739	*P. 'purpurea'*	Stonington Town Deck, Deer Isle, ME	3/25/96
740	*P. 'umbilicalis'*	Parson's Beach, ME	4/7/97
741	*P. 'umbilicalis'*	Parson's Beach, ME	4/7/97
742	*P. 'umbilicalis'*	Parson's Beach, ME	4/7/97
743	*P. 'umbilicalis'*	Parson's Beach, ME	4/7/97
744	*P. 'umbilicalis'*	Parson's Beach, ME	4/7/97
745	*P. 'umbilicalis'*	Parson's Beach, ME	4/7/97
746	*P. 'umbilicalis'*	Parson's Beach, ME	4/7/97
747	*P. 'umbilicalis'*	Parson's Beach, ME	4/7/97
748	*P. 'umbilicalis'*	Herring Cove, Nova Scotia, Canada	9/28/96
749	*P. 'umbilicalis'*	Herring Cove, Nova Scotia, Canada	9/28/96
750	*P. 'umbilicalis'*	Herring Cove, Nova Scotia, Canada	9/28/96
751	*P. 'umbilicalis'*	Herring Cove, Nova Scotia, Canada	9/28/96
752	*P. 'umbilicalis'*	Herring Cove, Nova Scotia, Canada	9/28/96
753	*P. 'umbilicalis'*	Herring Cove, Nova Scotia, Canada	9/28/96
754	*P. 'umbilicalis'*	Herring Cove, Nova Scotia, Canada	9/28/96
755	*P. 'umbilicalis'*	Herring Cove, Nova Scotia, Canada	9/28/96
756	*P. 'leucosticta'*	New Meadows Rivers @ Lehman Hway, West Bath, ME	3/29/97
757	*P. 'leucosticta'*	New Meadows Rivers @ Lehman Hway, West Bath, ME	3/29/97
758	*P. 'leucosticta'*	New Meadows Rivers @ Lehman Hway, West Bath, ME	3/29/97
759	*P. 'leucosticta'*	New Meadows Rivers @ Lehman Hway, West Bath, ME	3/29/97
760	*P. 'leucosticta'*	New Meadows Rivers @ Lehman Hway, West Bath, ME	3/29/97
761	*P. 'leucosticta'*	New Meadows Rivers @ Lehman Hway, West Bath, ME	3/29/97
762	*P. 'leucosticta'*	New Meadows Rivers @ Lehman Hway, West Bath, ME	3/29/97
763	*P. 'leucosticta'*	New Meadows Rivers @ Lehman Hway, West Bath, ME	3/29/97
764	*P. 'amplissima'*	Cape Elizabeth Light, ME	4/2/97
765	*P. 'amplissima'*	Cape Elizabeth Light, ME	4/2/97
766	*P. 'amplissima'*	Cape Elizabeth Light, ME	4/2/97
767	*P. 'amplissima'*	Cape Elizabeth Light, ME	4/2/97
768	*P. 'amplissima'*	Cape Elizabeth Light, ME	4/2/97
769	*P. 'amplissima'*	Cape Elizabeth Light, ME	4/2/97
770	*P. 'amplissima'*	Cape Elizabeth Light, ME	4/2/97
771	*P. 'amplissima'*	Cape Elizabeth Light, ME	4/2/97
772	*P. 'amplissima'*	Five Islands on Sheepscot River	6/19/96
773	*P. 'amplissima'*	Five Islands on Sheepscot River	6/19/96
774	*P. 'amplissima'*	Five Islands on Sheepscot River	6/19/96

775	*P. 'amplissima'*	Five Islands on Sheepscot River	6/19/96
776	*P. 'amplissima'*	Five Islands on Sheepscot River	6/19/96
777	*P. 'amplissima'*	Five Islands on Sheepscot River	6/19/96
778	*P. 'amplissima'*	Five Islands on Sheepscot River	6/19/96
779	*P. 'amplissima'*	Five Islands on Sheepscot River	6/19/96
780	*P. 'amplissima'*	Damariscotta River, Christmas Cove, S. Bristol, ME	5/18/96
781	*P. 'amplissima'*	Damariscotta River, Christmas Cove, S. Bristol, ME	5/18/96
782	*P. 'amplissima'*	Damariscotta River, Christmas Cove, S. Bristol, ME	5/18/96
783	*P. 'amplissima'*	Damariscotta River, Christmas Cove, S. Bristol, ME	5/18/96
784	*P. 'amplissima'*	Damariscotta River, Christmas Cove, S. Bristol, ME	5/18/96
785	*P. 'amplissima'*	Damariscotta River, Christmas Cove, S. Bristol, ME	5/18/96
786	*P. 'amplissima'*	Damariscotta River, Christmas Cove, S. Bristol, ME	5/18/96
787	*P. 'amplissima'*	Damariscotta River, Christmas Cove, S. Bristol, ME	5/18/96
788	*P. 'umbilicalis'*	Cape Elizabeth Light, ME	4/2/97
789	*P. 'umbilicalis'*	Cape Elizabeth Light, ME	4/2/97
790	*P. 'umbilicalis'*	Cape Elizabeth Light, ME	4/2/97
791	*P. 'umbilicalis'*	Cape Elizabeth Light, ME	4/2/97
792	*P. 'umbilicalis'*	Cape Elizabeth Light, ME	4/2/97
793	*P. 'umbilicalis'*	Cape Elizabeth Light, ME	4/2/97
794	*P. 'umbilicalis'*	Cape Elizabeth Light, ME	4/2/97
795	*P. 'umbilicalis'*	Cape Elizabeth Light, ME	4/2/97
796	*P. 'leucosticta'*	Montauk Point, NY	5/7/97
797	*P. 'leucosticta'*	Montauk Point, NY	5/7/97
798	*P. 'leucosticta'*	Montauk Point, NY	5/7/97
799	*P. 'leucosticta'*	Montauk Point, NY	5/7/97
800	*P. 'leucosticta'*	Montauk Point, NY	5/7/97
801	*P. 'leucosticta'*	Montauk Point, NY	5/7/97
802	*P. 'leucosticta'*	Montauk Point, NY	5/7/97
803	*P. 'leucosticta'*	Montauk Point, NY	5/7/97

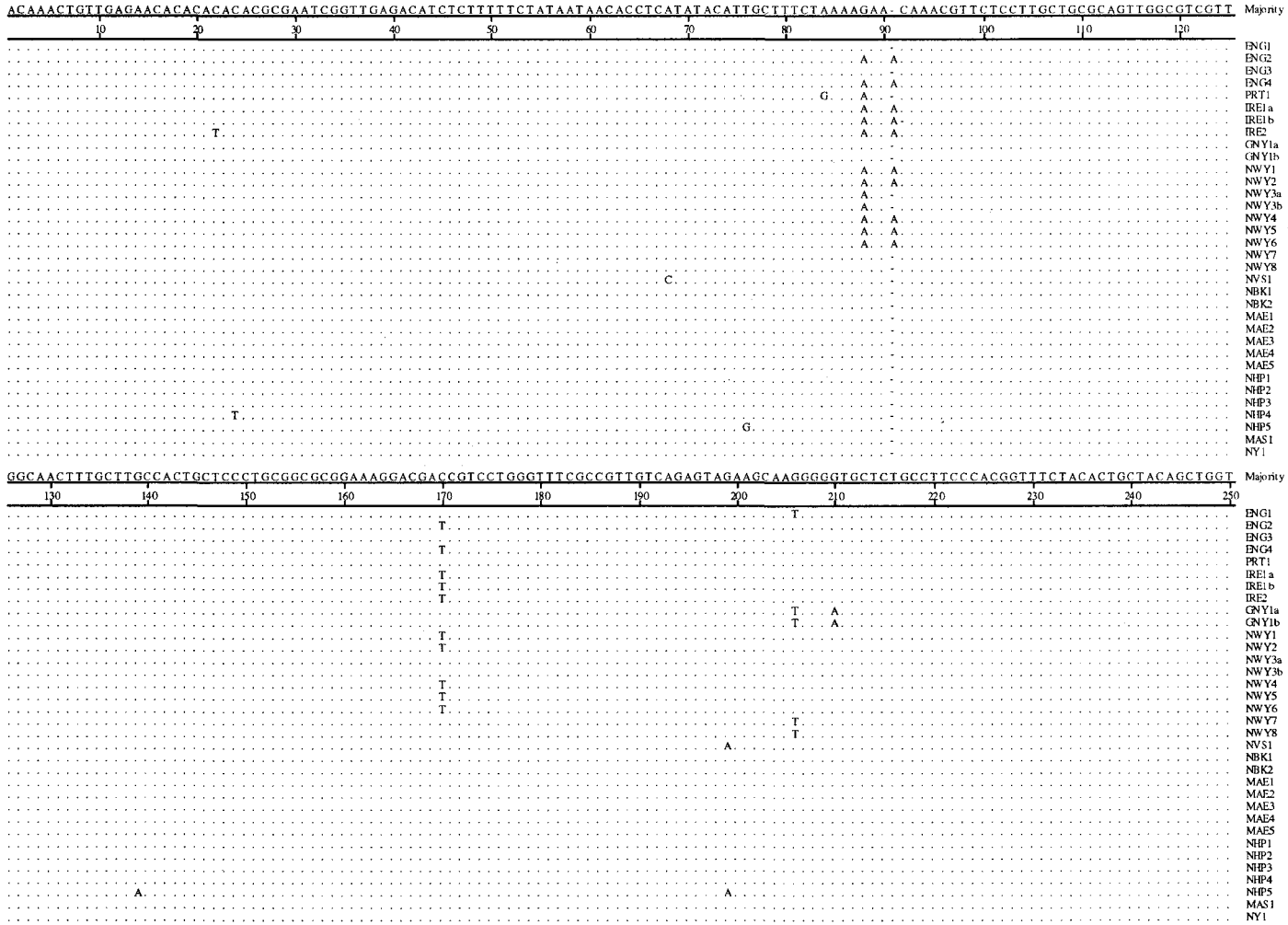
Appendix B.

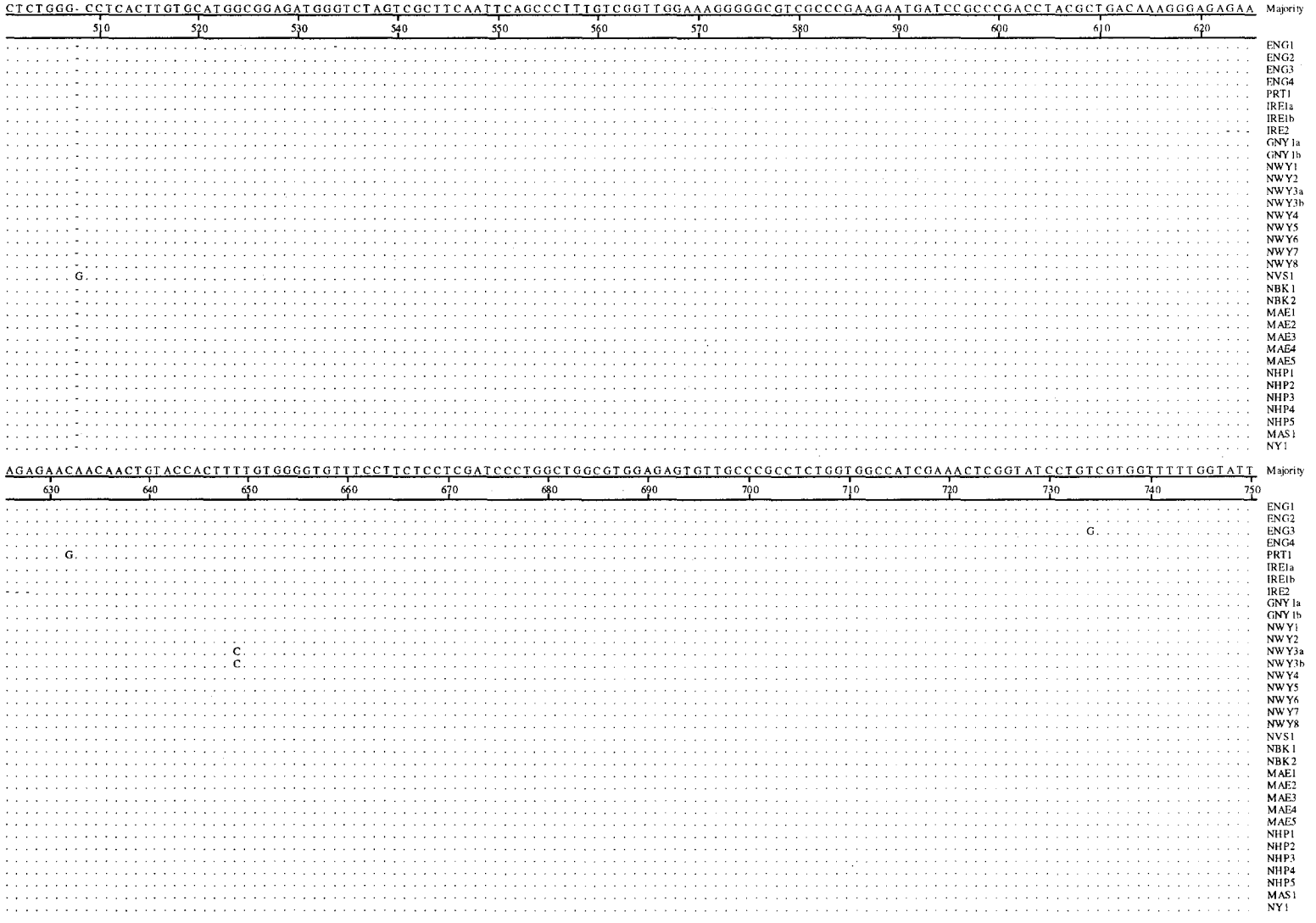
A nucleotide alignment of the cytochrome oxidase 2-3 (COX) spacer region between ten different isolates of *Porphyra umbilicalis* and *P. mumfordii, P. dioica*, and *P. purpurea*.

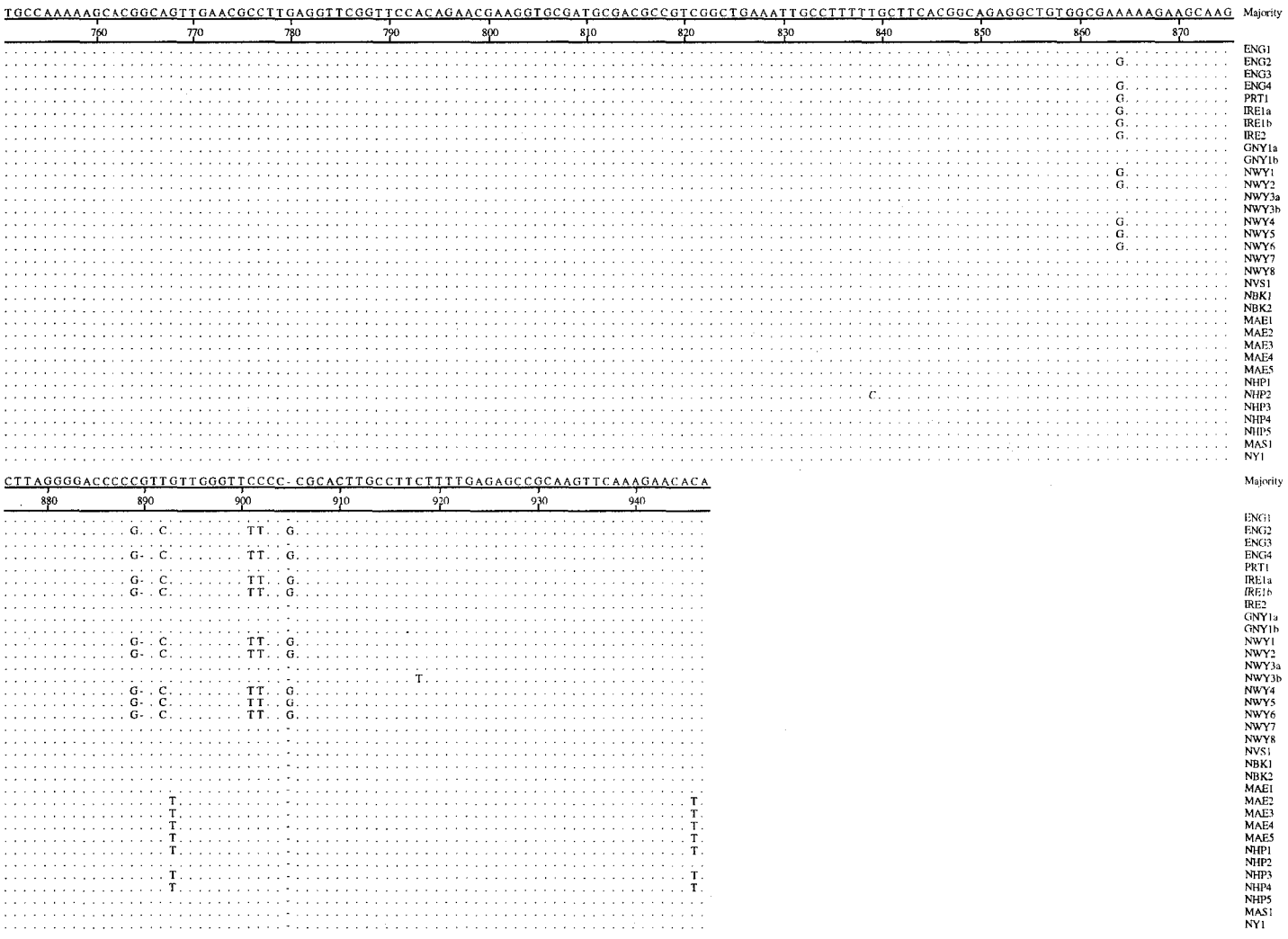


Appendix C.

Alignment and variation of the ITS1-5.8S-ITS2 Region of *Porphyra umbilicalis* from different geographical regions.



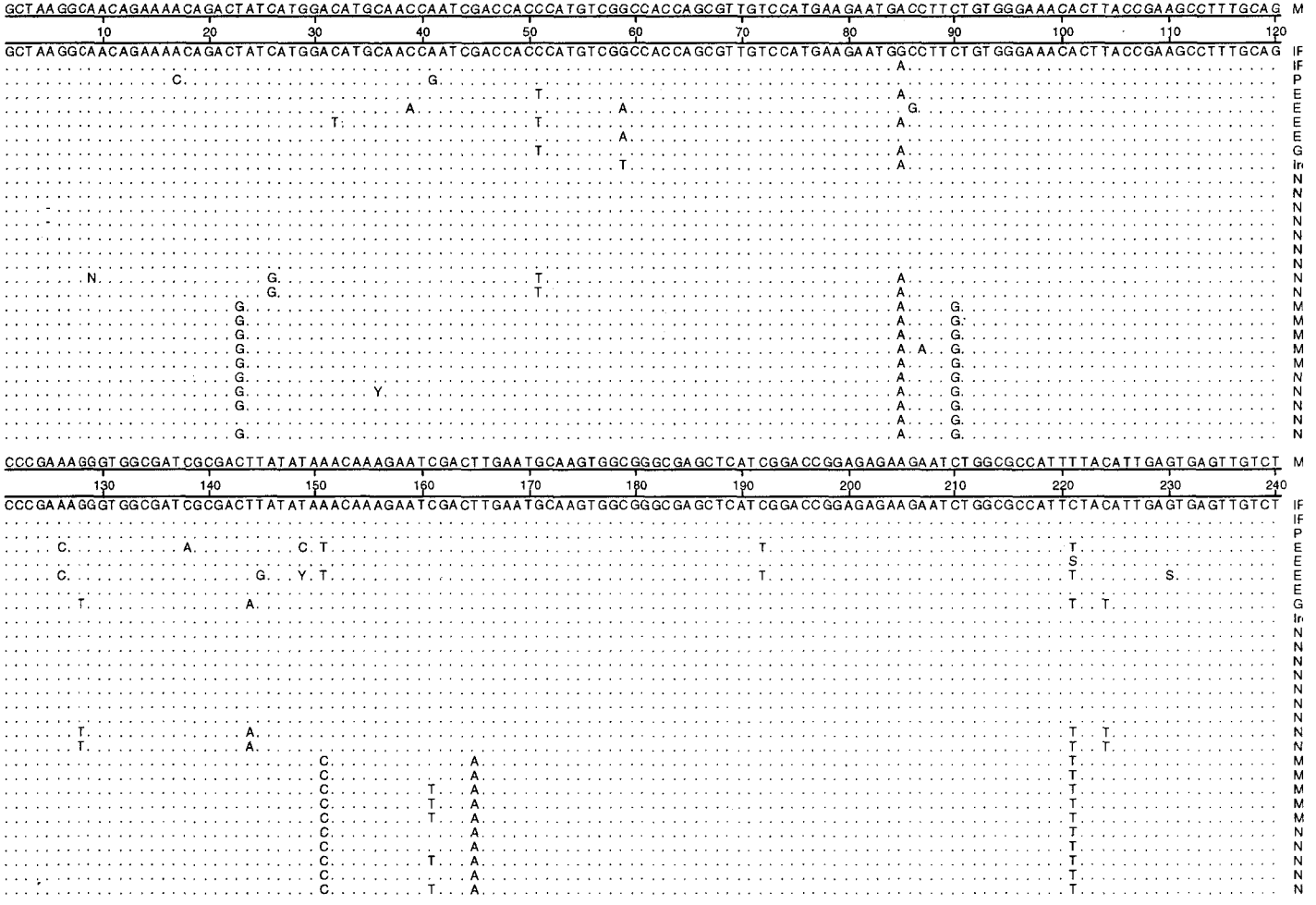


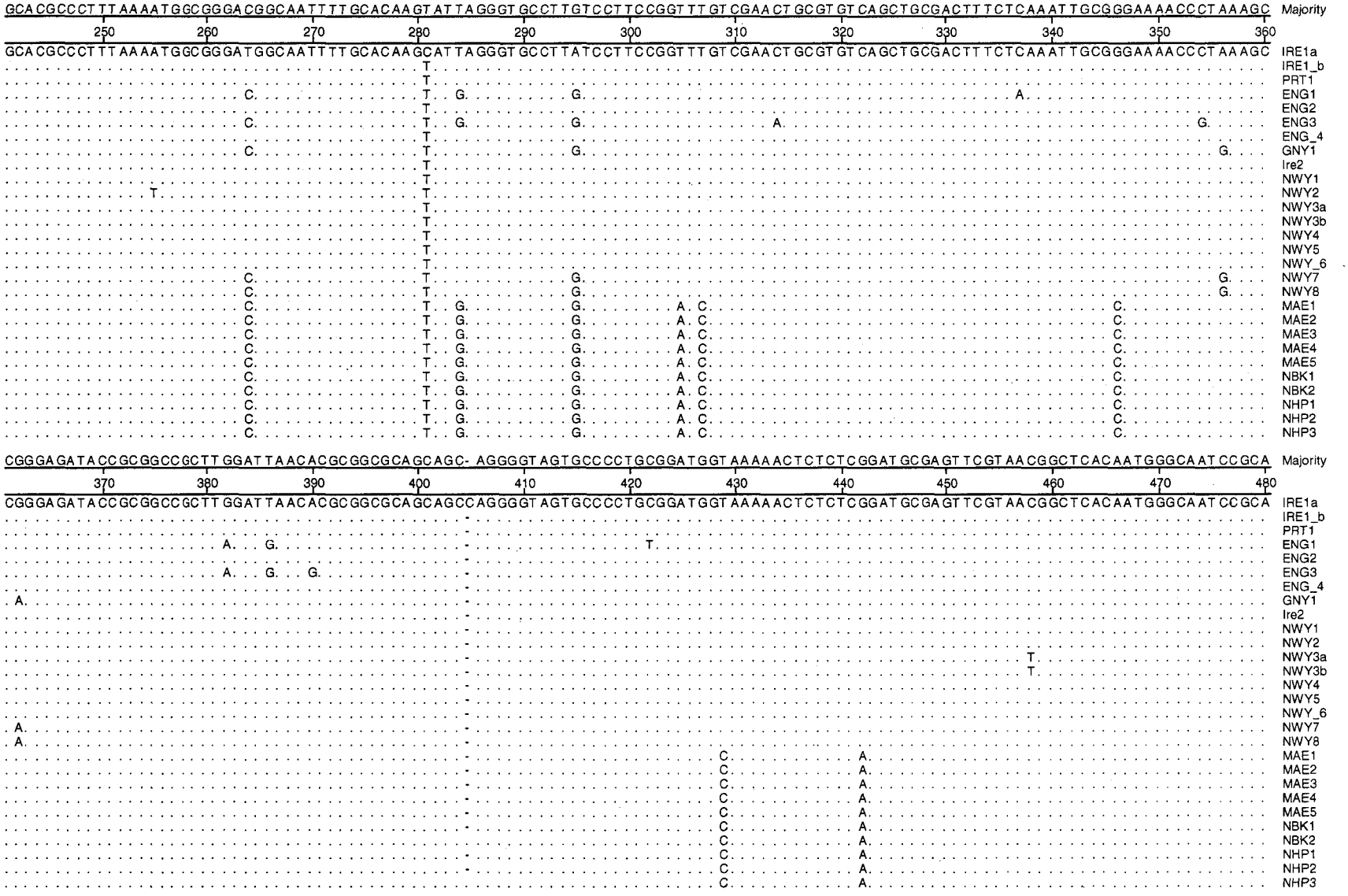


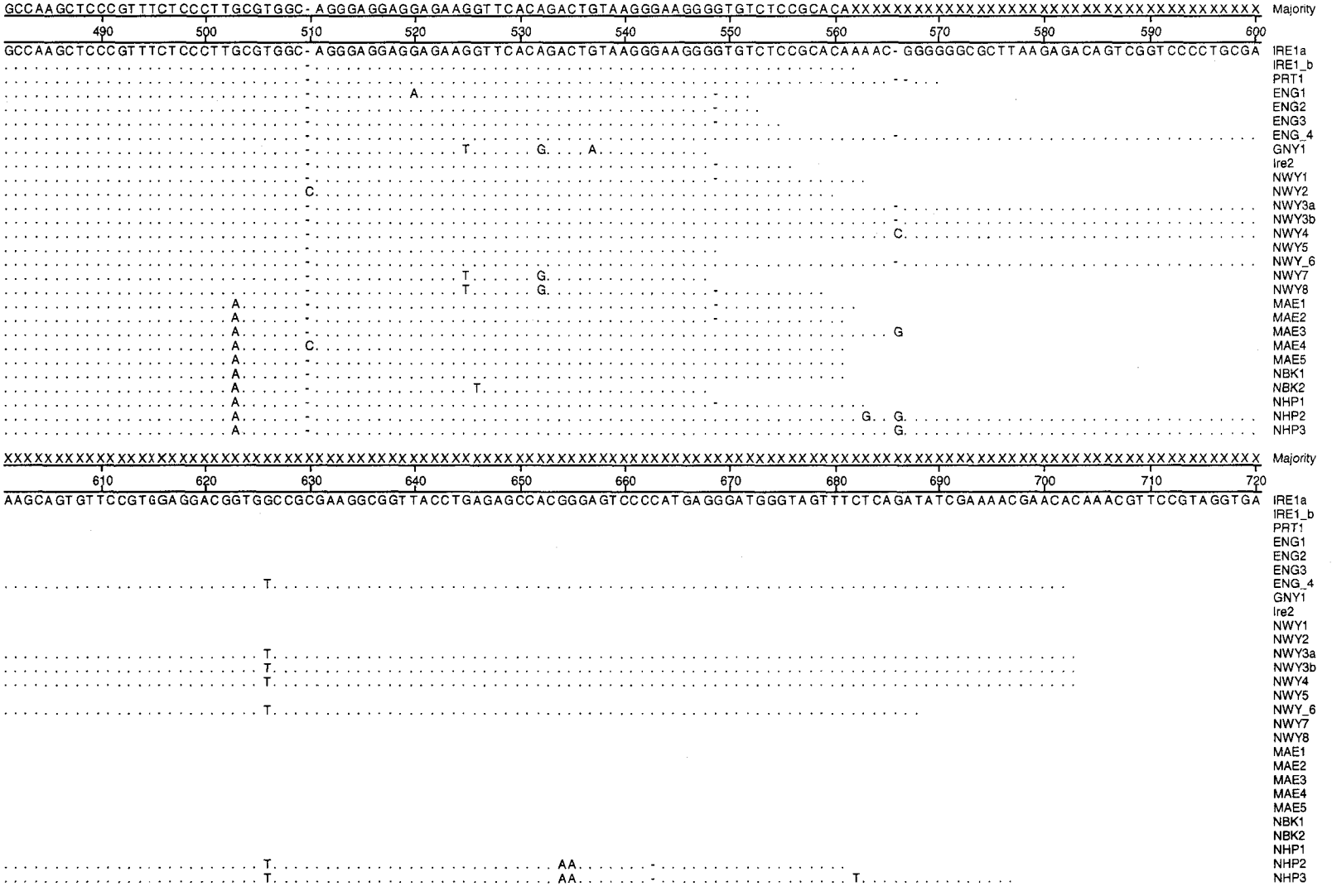
Code representations: (.) = same as consensus; (-) indicates insertion/deletion, shaded area indicates 5.8S.

Appendix D

18S ribosmal Group I intron sequence alignment in geographically distinct isolates of *Porphyra umbilicalis*.







APPENDIX E

B63 (CA-20)
GCCGGATTGCGTGGTTATGTGCAATTTAATAAATACACACACACACACACACACACACACACACACACACACACATTG
GAAAAAACGAAGACAAGAAAGTGTTAGTGCAGTAGCTACCGCCCGAGATAATCTAGAGAATAGATTTTCAAGGAAGC
AGAGAGGGAAGCACAAGGCACTCAGGGCT

B71 (GT-24)
GTTGATGCTTGTGGCTTGAGAGGCGGATGACCATCTATGAGGTTATGTGTACATTTGTGTGTGTGTGTGTGTGTGTGT
GTGTGTGTGTGTGTGTGTGTGTGTATTCAATTGCACATCACCGCACAATCCG

B73 (CA8-CT-CA14)
GCGAGTGGCATAGAATGACTAGGATGACACGGCCGGATTGCGTGGTTATGTGCAGTTTAATAAATACAACACACACAC
ACACTCACACACACACACACACACACACACACACACGCACACACACAGAGGACACACAACACCCCAGTAGGGGTACACG
TGCTGCTG

D131 (GT11)
GGTCGCGCCCGCTATTTTGTTGTTCCTCGCCTTGGCGAGTTTCTCTGCATTGTGTGTATTGTCATATATTTCGTGTGTGTT
TGTGTGTGTGTGTGTGTGTGTgtTTGTGCGAGCCTTTTTGTCTGCCTCGTTTGGTGATTCGTGTTTATTGTCTGCCTTGTGT
GTCTCGCCGTGCCTCACAGGCGGCAGGTCATG

D134 (GT-20)
GGCAACGACCGGTCGAAACACATCCCCAGATTCGGAGCAGCACCCATCTCCGCAACGTACTTGCCGGGCGCTCGCCGC
CGCGCGGTCCGAAATCTTCGGCTCAGTTCCCACTCAGCGGGAAACCAGCCGAAGATTTGTGTGTGTGTGTGTGTGTG
TGTGTGTGTGTGTGTGTGTGGCGTGACGGTGGTGGGGGAGGTTGCGGTGGTGGGAGTGTGCAAGCGAAGCAGCGACTG
CCGCAGAGCCGAAAAAGTAGAATGCCCCGTCCGGCGCAG

GA5 (GA-58)
CCGGATTGTCTGTGCTTCTCTCGTCTCTCAACAATCCATCCATTATCATTGGAACARAGWYTSTGCCARARARAAMWAR
WGtAWGAGAGAGAGAGAGAGAGAGagagagagagagagagagagagagagagAGAGAGcAGAGAGAGAGAGAGAGAGAGAGAGA
GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGagagagagaTAGAAGTACGAAGAAAGAAAWACAGAAAGTAGTAGGAT
GTACCTGCGCTGAGACTGTTTAATAGAAACGAAGAGTTCCATGATAGTGTGGAGAGCGATGC

TA17
AAATTCTTGCCTTTGCTCCTTTTTATAAAAGTATATTATATTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
CTCTCTCACACACACACACACACTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCCCTCTCTCTCTATAAAATCGTCAATTTG
GTCTGGC

TA18
GAGACGGCTTAATTTGCGATGGAAAGTAXAAGTTTCCCACGACAAACACGTCGGGCGCTATGCGGGGGCAAGGGCGCG
GGGATGTGTGTGTACGTGTGTGTGTGTGTGCGCGCGCGCGTGTGTGATGGGGGCAAGAGAGAGAGAGAGAGAGAG
AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAAAGAGAGAGAGAGAG
GGAGGGAGGGGGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAAAGAGAGCGCGCTCACCCT

TA45
TCGTCGTGACAAGTCGCCTCATCGCGTTTCGGAATGGCTTCCGCGAGCCGGCCAAGGTGCgtGTGTGTGTGTGTGTGTGT
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGtGgT
GTGTGGTGTGTGGTGTGTGGTGTGTGTGTGCGTGTGTATGTGTGTTTGTTTGTGTGTGTGCGTGTGTGTGTGTGTG
TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTTAGGGCTAGGGTTAGGGTTAGGGAGGGTTAGAGTTAGGGTTA
GGGT

TA56
CGCGGAGTTCTAATAGTTGTGTGTGCGCGCAAGCCTGTGTGTTTGTTTGGTTGTGTGTTTGCGTGTTTGTGTGTGTGTGT
GTGTGTGCGCGCGTGTGTGAGGGGGTGCGCACGTGGTTTTTTTTTTGTGTCTGTGTATGTATGTATGTATGTGTGTGTAT
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCGCGCGCGCGCGCGCGT
GCTACGTGTAGCGCGTGCGCGATAAAGAGACTGCGAGAGAATTTGAGAGCGAAATACACCTTGGCCCGCGT