"Oyster Thief"

Tracking the origins of invasive *Codium fragile* spp. *tomentosoides* populations in the Northwest Atlantic using molecular biology tools.

(www.dal.ca/~rescheib/ research.html, 4/19/04)

TECH 797
Ocean Projects

Jillian Lynch
Ian Patten

Advisor: Anita Klein

Sea Grant
New Hampshire

Abstract

Codium fragile ssp. *tomentosoides* is an Asiatic coenocytic green algae that has become one of the most invasive macroalgal species in temperate regions worldwide (Trowbridge 1999). This study combines an ecological evaluation of the recent spread of this species with a molecular genetic analysis of several samples from different geographical areas. PCR based genetic markers were developed in this study to trace source populations of the invasive green algae that is rapidly increasing throughout the Northwest Atlantic coastline. These genetic markers will be used to differentiate between new introductions caused by synanthropic activities, as well as natural dispersal mechanisms in existing populations of *Codium fragile*.

Table of Contents

Introduction

Codium fragile (see phylogeny report in Figure 1) is a coenocytic, unicellular organism containing many nuclei within its mass of cytoplasm, green algae that originated from the Pacific Ocean near Japan. These green algae have become one of the most aggressive and invasive macroalgae species in temperate regions around the world (Trowbridge 1999). *Codium fragile* spp. *tomentosoides* has displaced many native species due to its rapid growth. The combination of an ecological and molecular research approach to this invasive problem will lead to improvements of containment policies. In this study specifically, molecular biology tools were used to identify molecular markers that would be useful track new introductions of *Codium fragile* subspecies along coastal New England. This phylogeographic study, based upon microevolutionary and macro-evolutionary processes, will give insight to the genetic variation and similarities between populations from various geographic areas in the Northwest Atlantic.

Codium fragile is dark green in color, ranging from ten to forty centimeters high and consists of repeatedly branching cylindrical segments about 0.5 to 1.0 cm in diameter. The segments look like dark green fingers, therefore giving rise to the common name "Dead Man's Fingers". The species inhabits in the middle and lower intertidal zone as well as subtidal regions of rocky coastlines (http://www.racerocks.com/racerock/eco/taxalab/philipk.htm. *Codium fragile* was initially introduced in the North Atlantic in Holland in the early 1900's and was subsequently reported in the United States in the 1950's. The earliest record of *Codium* in the Northwest Atlantic was in 1956 in the Long Island Sound area and has since spread to the Booth Bay Harbor in Maine by shellfish transplant (Carleton & Scanlon, 1985).

Further dispersal has been tracked to the Isles of Shoals NH/ME, Cape Cod MA, and Nova Scotia Canada (see Figure 2). *Codium fragile* has proved to be very invasive and has displaced several native species because of its rapid growth. For example, when new plants of *Codium* start to grow in mussel, scallop, and oyster beds, the shells of these animals provide a firm attachment surface for the small algae. Once the plant is large enough in size, waves surging through the shellfish beds tend to dislodge the algae and host shellfish from the seabed. *Codium fragile* spp. *tomentosoides* can also smother the shellfish and cause uplifting, drifting, and death of various shellfish because of its buoyant nature, therefore accurately nicknaming the specie "Oyster Thief". *Codium* has also been known to alter nursery habitats for juvenile fish and therefore reducing stock. Once the algae have established a dense population, they can be spread due to ocean currents and synanthropic mechanisms such as shellfish aquaculture and boat fouling. For these invasive problems, *Codium fragile* needs to be evaluated ecologically and phylogeographically, which is the understanding of current geographic distributions of populations or groups of species in terms of their phylogenetic relationships. This will allow a better understanding of the properties allowing this species to expand exponentially.

Other investigators have hypothesized that the rapid spread of *Codium fragile* in the Gulf of Maine over the last 20 years is actually an introduction of a new subspecies, *Codium fragile* ssp. *atlanticum* that may have invaded the Northwest Atlantic after *C. fragile* ssp. *tomentosoides*. Genetic analysis of specimens from different geographic locations may give insight to whether a new subspecies has been introduced, the dispersal

patterns, and possibly aid in the development of prevention and containment methods for this invasive genus of algae.

Limited molecular genetic studies of Codium have been published (Goff, L.J. 1992) and only a small number of gene sequences for this species have been reported (16; http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Nucleotide&cmd=search&term=Codium+fragile, 4/21/04). Therefore we began by examining different methods to isolate DNAs from fresh samples of *C. fragile* spp. *tomentosoides* and dried herbarium specimens. Archival herbarium samples provide a historical record allowing for comparative phylogeographic information to be obtained as *C. fragile* has spread in the Gulf of Maine.

To study this specie at a genetic basis, molecular markers were developed that will allow us to distinguish between new introductions of *C. fragile* ssp. *tomentosoides* or introductions of new subspecies (i.e. *Codium fragile* ssp. *Atlanticum*) caused by the synanthropic activities and natural dispersal mechanisms that have been described. Two genes were used in this study to search for intraspecific variation between different *C. fragile* populations.

The first gene used in this study was the multi-copy nuclear ribosomal internal transcribed spacer (ITS) (see Figure 3). The ITS region is found between the 18s, 5.8s, and 26s subunits of a ribosome. This region is particularly helpful in phylogenetic research because it is highly variable within species (Baldwin et al. 1993). Therefore we predicted the ITS region of *Codium fragile* might have sufficient variation across the geographic distribution of the species, to differentiate populations originating in different locations.

The 18S and 26S of the ribosomal genes are highly conserved across deep evolutionary lineages, therefore it is possible to design primers in the 18S and 26S genes for the Polymerase Chain Reaction (PCR®) which will amplify across the ITS region (White *et al*, 1990). However these primers sites are relatively conserved for all eukaryotic organisms and were specifically designed from fungi species. It is possible in these PCR reactions to amplify contaminating DNA possibly from epiphytic fungi that live among *Codium* or have grown on the sample during drying in herbarium presses.

Specificity problems with the ITS region led us to pursue another gene for phylogeographic studies, one that could be selectively amplified from the *Codium* DNA that was mixed with epiphytic fungi. Ribulose-1,5-bisphosphate carboxylase/oxygenase is a photosynthetic gene not found in fungi, therefore allowing for specific amplification of *Codium* DNA. Primers flanking the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase were used to amplify various portions of the rbcL gene among samples from different geographic locations to study the similarities and differences in the different portions of the gene. Exons, coding regions, are segments of DNA that are transcribed and translated into protein. Introns are found in between the coding sequence and are not transcribed into mRNA. The intron segments of the gene are more variable then protein coding regions. Therefore the *Codium* rbcL was a good candidate for detecting phylogeographically informative variation within *Codium* subspecies.

Protocols, procedures, and rationale used to isolate the DNAs, design primers, amplify target sequences, sequence and analyze DNA are described in the Materials and Methods section of this study's report. Conditions are described for *Codium* DNA extraction from fresh tissues and dried herbarium specimens. Universal primers and

primers designed from a close relative of *Codium* were used to selectively amplify the ITS region. Results showed that contaminating fungal DNAs were selectively amplified with ITS primers. Finally both coding and noncoding regions of the plastid rbcL gene were investigated to see if these might provide a signal from phylogeographic studies.

Materials and Methods

Various samples of *Codium fragile* spp. *tomentosoides* from different geographic locations were obtained with the help of Art Matheson, Jackson Estuarine Laboratory UNH (see Figure 4). Both dried and wet tissue was attained from Booth Bay Harbor, ME, Millstone Point, CT, and Hawthorne Cove, MA.

Genomic DNA Isolation: The samples of *Codium fragile* were finely ground using a microfuge tube pestle and a microfuge tube. The samples were ground in liquid nitrogen to prevent endogenous nuclease activity from degrading the DNA. Once the frozen samples were finely ground, they were stored at –80 °C until DNA was extracted. The following are the protocols for the DNA isolations used in this study.

Puregene DNA Purification Kit:

Genomic DNA was purified from both fresh and dried samples of *Codium fragile* ssp. *tomentosoides* using a Puregene DNA Purification Kit. This kit provides the components and procedures necessary for purifying genomic DNA from plant matter. The protocols for both fresh and dried sample extractions are as followed (Gentra Systems, 2002):

"DNA Purification from 10-30 mg Fresh/Frozen or 5-10 mg Dried Plant Tissue: (Expected Yield: 1-15 mg DNA)

Cell Lysis:

1. Add 10-30 mg fresh or frozen leaf tissue or 5-10 mg dried tissue (finely ground), to a 1.5 ml microfuge tube.
2. Add 300 ml Cell Lysis Solution to the leaf tissue. Vortex 1-3 seconds to wet the tissue.
3. Add 1.5 ml Proteinase K to each sample, which degrades nucleases that may degrade DNA during the cell lysis step.
4. Incubate cell lysate at 65oC for 60 minutes. (After 30 minutes invert tubes 10 times.)

RNase Treatment:

1. Add 1.5 ml RNase A Solution to the cell lysate, which degrades RNA in the sample that could co-purify with the DNA
2. Mix the sample by inverting the tubes 25 times and incubate at 37oC for 30 minutes.

Protein Precipitation:
1. Cool samples to room temperature.
2. Add 100 ml Protein Precipitation Solution to the cell lysate.
3. Mix the Protein Precipitation Solution with the cell lysate by vortexing each tube at high speed for 20 seconds.
4. Place samples on ice for 30 minutes (due to high polysaccharide content).

DNA Precipitation:
1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 1.5 ml microfuge tube containing 300 ml 100% Isopropanol.
2. Mix the sample by inverting gently 50 times.
3. Centrifuge at 13,000-16,000 x g for one minute. The DNA will be visible as a pellet that ranges in color from off-white to light green.
4. Pour off supernatant and drain tube briefly on clean absorbent paper. Add 300 ml 70% Ethanol and invert tubes several times to wash the DNA pellet.
5. Centrifuge at 13,000-16,000 x g for 1 minute. Carefully pour off the ethanol.
6. Invert and drain the tube on clean absorbent paper and allow to air dry for 10-15 minutes.

DNA Hydration:
1. Add 50ml DNA Hydration Solution (50 ml will give a concentration of 100 mg/ml if the total yield is 5 mg)."

CTAB DNA Isolation:

The CTAB method is a DNA isolation procedure that uses a specialized detergent, Cetyltrimethylammonium bromide (CTAB) buffer. This detergent helps proteins and polysaccharides to precipitate out of solution while the DNA remains in solution. The following procedure was followed: (Doyle and Doyle 1990)

1. "Transfer ground sample to prewarmed CTAB isolation buffer (0.75 ml in a 1.5 microfuge tube. Add B– mercapthoethanol to final concentration of 0.2%. Mix by gentle inversion and incubate at 60 °C for 30 minutes.
2. Extract with an equal volume of 25:24:1 phenol, choloroform, isoamyl alcohol. Centrifuge for 10 minutes at 3000 RPM.

3. Transfer upper aqueous phase to a fresh tube. Pipet with large oriface tips to prevent DNA shearing. Extract with 24:1 chloroform, isoamyl alcohol; invert to mix well. Centrifuge for 5 minutes at 3000 RPM.
4. Remove aqueous phase (without interphase layer) to a new tube containing an equal volume of ice- cold isopropanol. Mix by inversion and incubate at room temperature for 15-30 minutes. The DNA should appear as a fine white fibrous material.
5. Using a Pasteur pipet, drawn out to a sealed hook, to spool the DNA. Place in a 1.5 ml microfuge tube containing 1 ml 76% ethanol, 10mM ammonium acetate for 20 – 30 minutes. If the DNA will not spool, centrifuge sample at 7000 rpm for 5 minutes, carefully remove supernatant, and wash with 1 ml 76% ethanol, 10 mM ammonium acetate.
6. Transfer spooled DNA to a new microfuge tube and rinse the pellet with 50 µl TE buffer. Resuspend DNA on rocker platform, overnight at 4 °C. Add more TE if DNA will not dissolve (Stiller and Waaland, 1996)."

Primer Design:

Perhaps the most critical parameter for successful PCR (Polymerase Chain Reaction) is the design of the primers. The primer sequence determines the length of the product by its position on the gene, its melting temperature, and ultimately the yield of the PCR product. Several variables were considered when designing primers for our study including and not exclusive to primer length, melting temperature, specificity, complimentary primer sequences (primer dimmers), G/C content, and 3'-end sequence specificity. Primers were designed using the Primer Select software program, which is part of the suite of DNA analysis programs called DNAstar (Primer Select, DNAstar, Lasergene, Madison WI.). Both Universal Primers and primers designed based on the ribosomal DNAs of related green algae were used for ITS amplification. The sequences of all the primers used in this study are shown in Figure 5.

Primers were designed for the rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase) gene by using the rbcL sequence of *Codium fragile* ssp. *tomentosoides* from the GenBank database (Accession # M67453) found on http://www.ncbi.nlm.nih.gov/. This sequence was analyzed into the Primer Select software and primer pairs were analyzed for length, melting temperature, specificity, formation of internal hair pins and formation of primer dimmers. Primers that span exon 1, intron 1, and exon 2 are listed in Figure 5.

Polymerase Chain Reaction:

PCR is an acronym that stands for polymerase chain reaction. The PCR technique (see Figure 6) is a primer extension reaction for amplifying specific nucleic acids in vitro. PCR allows a short stretch of DNA (usually fewer than 3000 bp) to be amplified to about a million fold so that one can determine its size and nucleotide sequence. The particular stretch of DNA to be amplified, called the target sequence, is identified by a specific pair of DNA primers, oligonucleotides usually about 20 nucleotides in length. There are three major steps in a PCR reaction, which are repeated for 30 or 40 cycles. This is done on an automated thermocycler (Figure 7), which can heat and cool the tubes with the reaction mixture in a very short time. The first step (94 °C) involves an initial denaturation of the double stranded DNA to create single stranded DNA templates. Once the DNA is denatured, the primers align with complementary sequences flanking the target gene. The annealing step (between 60 and 45 degrees Celsius) is carried out at a specific temperature which is a function of the sequences of the primer pairs. The thermostable DNA polymerase then begins to extend the primer, in a fashion that is complementary to the target template sequence. Once there area few bases are added to the primer, the

hydrophobic bonds are sufficiently strong between the template and the extended primer, allowing for the third step of the reaction, extension. The extension of the primer occurs best at 72 °C, which is the temperature optimum for the polymerase. Bases are added (complimentary to the template) to the primer in the 3' side because the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side. After the target region is copied the first round of the PCR, what was one copy of the gene has been increased to two copies of the target. The double stranded DNAs are denatured with a short heating cycle (30 sections at 94 degrees Celsius) and then the cycle is repeated. At each successful cycle there has been a geometric increase in the target sequence relative to the rest of the template DNA. After 30-35 cycles of PCR, the target sequence has been amplified more then a million fold. Thermostable Taq DNA polymerase withstands multiple rounds of heat denaturation of template and newly synthesized DNA with minimal loss of enzymatic activity.

The polymerase chain reaction can be inhibited by molecules (ie. polysaccharides) from DNA isolation processes. Certain modifications to the basic protocol were implemented to increase amplification yield, specificity, and consistency. Multiple magnesium concentrations were used to optimize the PCR reactions because magnesium affects the stability of the primers annealing to the template DNA. Bovine Serum Albumin has also been known to counter PCR inhibitors therefore increasing yield and consistency of the amplifications.

Reaction components and PCR thermocycler profiles are as followed for the ITS-PCR reaction.

ITS PCR Reaction:

10X buffer: 2.5 µl
dNTP: 2.5 µl
Primer Forward: 1.0 µl
Primer Reverse: 1.0 µl
Mg^{2+}: (varied) 1.5-2.5 µl
BSA (200 µg): 0.5 µl
Taq polymerase: 0.2 µl
DNA: 2.0 µl (diluted 1:100)

ITS - PCR profile:

1. Initial Denaturation (94°C): 3 minutes.
2. Denaturation (94 °C): 30 seconds.
3. Annealing (54 °C): 45 seconds.
4. Extension (72 °C): 45 seconds.
    - (35 cycles)

The polymerase chain reaction set up and profile for the rbcL gene was different due to its larger sequence length and the AT rich regions of the sequence. Touchdown PCR was utilized in amplifying the rbcL gene to attempt to enhance the rbcL product. Touchdown PCR involves decreasing the annealing temperature by 0.5 degrees Celsius every cycle to a 'touchdown' annealing temp, which is then used for about 20 more cycles, each increasing in length by one second. This process increases specificity for reactions that are not at the best optimal conditions (i.e. long target sequence, AT rich template making it hard for primers to anneal, or other various potential PCR problems). *Taq* extender, a PCR reagent utilized in touchdown PCR reactions, was used because *taq* polymerase misincorporates nucleotides with an error rate of ~$1/ 10^3$ to $1/ 10^4$ bp, and perhaps stalls at the mismatched base. Taq extender has a DNA proofreading function, therefore helping the polymerase add bases across the entire gene.

Reaction set-up and PCR profile for the rbcL reactions are as followed:

RbcL - PCR Reaction:

Water: 14.3 µl
Taq extender buffer: 2.5 µl
dNTP's: 2.5 µl
Primer Forward: 1.0 µl
Primer Reverse: 1.0 µl
Taq Extender: 0.6 µl
Taq: 0.6 µl
BSA: 0.5 µl
DNA: 2.0 µl (1:100 dilutions)

RbcL – PCR Profile:

1. 94°C – 1 minute
2. 92 °C – 30 seconds
3. 53.4 °C – 45 seconds (-0.5 °C per cycle)
4. 72 °C – 3 minutes
5. (19 cycles of step 2-4)
6. 92 °C – 30 seconds
7. 48.4 °C – 40 seconds (+ 1 second per cycle)
8. 72 °C – 3 minutes
9. (30 cycles of step 6-8)
10. 10 °C hold until store in 4 °C refrigerator

Gel Electrophoresis:

Agarose gel electrophoresis separates DNA fragments according to their size. An electric current is used to move the DNA molecules across an agarose gel, which is a polysaccharide matrix that functions as to separate larger molecules from small molecules as they are transported by the electric current (see Figure 8). The phosphate molecules that make up the backbone of DNA molecules have a high negative charge. When DNA is placed on a field with an electric current, these negatively charged DNA molecules migrate toward the positive end of the field, which in this case is an agarose gel immersed in a buffer bath. Larger DNA fragments migrate slower than smaller fragments through the matrix allowing for the DNA to be separated by size. The agarose gel is stained with ethidium bromide (see Figure 9), which is a DNA intercalating agent

that fluoresces. The ethidium bromide inserts itself in-between the DNA bases allowing for the DNA fragments to be visualized under an ultraviolet transilluminator (Figure 10). Once the fragments are visualized and sized, the bands can be physically cut out of the agarose gel (Low melt™ gels melt at a low temperature which are used for gel electrophoresis prior to DNA sequencing). Purification of the agarose gel is necessary before the PCR product can be sequenced.

Agarase Protocol: (Agarose Degradation)

1. Separate PCR products on a low-melting point agarose gel. (1.2% low-melting gel).
2. Cut out the band as tightly as possible (visualizing under ultraviolet transilluminator) and transfer to a 1.5 ml microfuge tube.
3. Incubate at 65 °C for 10 minutes or until completely liquefied.
4. Place at 37 °C for 5 minutes to equilibrate sample to this temperature.
5. Add 1.5 µl Agarase (Sigma Corp. St. Louis MO) enzyme5 units/ µl) per 100 µl-melted agarose and mix well with end of pipette. Agarase digests the agarose matrix.
6. Incubate at 37 °C for 30 minutes.
7. The product after incubation can be used directly in the cycle sequencing reactions. Volume used depends on yield that is determined from band intensity visualized under UV light before band was excised.

Cycle Sequencing: Sanger Method

The Sanger sequencing method (dideoxynucleotide sequencing) (Figure 11) is a DNA synthesis reaction that utilizes the incorporation of dideoxynucleotides (Figure 12) into the sequence to provide a mixture of all possible lengths of the target sequence. Dideoxynucleotides (ddNTP's) are missing a hydroxyl group (OH) group at the 3' position. This position is normally where one nucleotide attaches to one another to from a chain. If there is no OH group in the 3' position, the additional nucleotide (dNTP) cannot be added to the growing chain, therefore stopping the chain elongation. Once the reaction is complete, and all possible lengths of the target sequence are terminated with a ddNTP, which is linked to a specific colored fluorescent tag) the reaction product is

electrophoresed on a polyacrylamide gel in a ABI Prism Sequencer (Figure 13). A laser at the base of the gel constantly scans the migrating fragments (smaller fragments migrating faster than larger fragments). The laser records the specific wavelength (color) of the ddNTP-Fluorescent dye, therefore creating a simulated gel (Figure 14). The data from this simulated gel is compiled into a chromatogram (Figure 15), which the DNA sequence can be interpreted and analyzed from. The following protocol was used for the cycle sequencing reactions.

1. For each template to be sequenced combine:
   Sequencing reagent premix – 8 µl
   DNA (0.1-0.2 picomole) – 5 µl
   Primer (only one) – 1 µl
   Water – 6 µl
2. Cycle 25 times as followed:
   95°C, 20 seconds
   50°C, 15 seconds
   60°C, 1 minute
3. Remove 20 µl from each termination reaction and transfer to a fresh tube containing 2 µl of sodium acetate/EDTA buffer.
4. Add 80 µl of 95% ethanol, mix, and place on ice for at least 15 minutes.
5. Centrifuge in a micro-centrifuge for 15 minutes. Draw off supernatant.
6. Add 250-500 µl of 70% ethanol to wash pellet (in vacuum centrifuge) for 2-5 minutes.
7. Send sample to Hubbard Center for Genome Studies, UNH, to be separated on an ABI Prism Sequencer; the resulting fluorescence pattern is recorded for further analysis.
8. Analyze using computer based software: Seqman DNAstar
9.

DNA Sequence Analysis: DNAstar (SeqEd, SeqMan, MegAlign)

Various DNA analysis methods were applied using the suite of programs called DNAstar. The sequences were edited, trimmed, and assembled in SeqMan. Chromatograms are evaluated for noise (usually at the beginning and the end of a

sequence). Each amplified fragment is sequenced from each end, and the chromatograms are compared to ensure the sequence is accurately determined. Comparisons were made between the sequences obtained in this study to known Codium sequences with the MegAlign program. The sequences obtained were then analyzed by the BLAST algorithm (Altschul 1990) against all known gene sequences in GenBank (see Figure 16 for sample Entrez/Blast Hit result (http://www.ncbi.nlm.nih.gov/BLAST/, 4/21/04).

Results

DNA Isolation:

DNA was isolated from *Codium fragile* samples from various geographic locations (Figure 4) by means of the Puregene Gentra DNA Isolation Kit and the CTAB Buffer Isolation Method. Intact DNAs are expected to run on an agarose sizing gel as a tight bright band of high molecular weight (example seen in Figure 17 (lane 31 and 36)). If little or no DNA was isolated from the sample then there would be no ethidium fluorescing material in the gel lane (example seen in Figure 17, lane 38). If the DNA were degraded, then it will shown up as a fluorescing smear of lower molecular weight (seen in Figure 17, lanes 28, 29, and 34). The CTAB isolation method was used three times, each resulting in no intact DNA as confirmed with agarose gel electrophoresis. The Puregene Gentra DNA Isolation Kit proved to be the most reliable protocol after numerous trials with both methods. Various modifications of the Puregene protocol were compared with such as using just Proteinase K or RNase, neither, or both. Using both Proteinase K, then RNase as described in the Materials and Methods section resulted in the most reproducible and intact DNA products.

Using the Puregene Protocol, as described in the materials and methods, high molecular weight DNAs were isolated from both fresh and herbarium samples. There were no consistent differences in the quality of DNA from either fresh or herbarium samples. Interestingly some of the older herbarium samples extracted produced high molecular weight DNAs while DNAs extracted from some of the most recent herbarium accessions (<4 years old) were extensively degraded. Overall about 75% of the samples extracted produced DNA was either partially degraded (seen as smeared fluorescence) or fully

degraded (no fluorescence). The partially degraded DNA was used in PCR of the ITS region, but no amplification was obtained from these degraded samples. Other samples with high molecular weight DNAs (~25% of all DNA samples isolated) proved to be amplifiable. **PCR and Sequencing:**

(ITS Amplifications)

ITS amplifications were performed from a number of DNAs isolated from different samples of varied geographic origins, but the amplifications proved to be inconsistent. Since these reactions were so inconsistent, Mg^{2+} optimization curves were run by titrating varied amounts of Mg^{2+} for each PCR reaction. This was also performed for the PCR additive Bovine Serum Albumin. The optimal conditions for the ITS amplification with Universal primers was(100-200 μg/ml BSA. Even with Mg^{2+} and BSA optimization, amplifications remained inconsistent and somewhat template specific. Figure 22 shows the agarose gel from some of the PCR reactions. Those amplifications that produced fragments (shown in Figure 22) were electrophoresed in a low melt gel and sequenced. After several attempts to amplifying and sequencing the ITS region using the Universal ITS primer pair, sequence was obtained. The sequence from the sequencing chromatogram was trimmed and edited using the DNAstar sequencing programs.. These ITS sequences were then BLASTED against Genbank database, where billions of comparisons are made to all the sequences that have been registered in GenBank. The results of the BLAST showed that the sequences amplified had closest homology to various fungal ITS regions. Since these sequences were most closely related to various fungi DNAs, we hypothesized that the ITS primers were not selective enough for the

Codium DNA, and was therefore amplifying contaminating DNA mixed with the C. fragile.

Primers for this region were redesigned against available rDNA sequences for distant evolutionary relatives for *C. fragile.* The limitation to this approach is that no ribosomal gene sequences are available for *Codium* so primers selected are a best 'guess' as what the corresponding *Codium* sequences should be. The new primers differed by one to two bases from the Universal ITS primers (White 1990). , Numerous PCR trials with the new algal primers produced inconsistent DNA amplifications and none specific enough for the *Codium* template. Figure 20 shows one of the PCR amplifications from several samples from Hawthorne Cove, MA and Brave Boat, ME. Multiple bands in the lanes indicates several ITS regions possibly being amplified. Sequencing reactions and analysis of these PCR products proved to be from contaminating DNA templates.

(RbcL PCR Amplification)

Various rbcL primer pairs were developed to amplify either parts of both the exons or from the two exons across the intron of the rbcL gene (see Figure 4) to increase PCR specificity to the *Codium* templates. Exon 1 and exon 2 of the rbcL gene were amplified from CF #36 (Hawthorne Cove, MA), CF #45 (Millstone Point, CT) and the ultra-centrifuged DNA template UC #2 (Brian Teasdale, UNH, Fort Stark NH). Figure 19 is a photograph of PCR products separated by gel electrophoresis using a 1.2% Low Melt gel with the PCR products of correct predicted size for exon 1 and exon 2 of the rbcL gene. Table 1 depicts the primers, DNA template source, and quality sequence (determined length and low percent of mis-pairings) obtained from these PCR reactions. The sequences for each algal accession we sequenced will be submitted to the GenBank

database (http://www.ncbi.nlm.nih.gov/). **Table 1: RbcL 79 and 2500 PCR/Sequenced Samples.**

DNA Source	Sample origin	Primer Pair	Quality Sequence
Ultra-centrifuged #2	Fort Stark, NH	rbcL 79	Yes
Ultra-centrifuged #2	Fort Stark, NH	rbcL 2500	Yes
CF # 36	Hawthorne Cove, MA	rbcL 79	Yes
CF # 45	Millstone Pt., CT	rbcL 79	Yes
CF # 45	Millstone Pt., CT	rbcL 2500	Yes

These sequences were compared to the three rbcL 79 (exon 1) sequences were aligned and analyzed in the DNA analysis computer software. These three sequences were identical to each other as well as to the GenBank accession # M67453. Likewise, the two sequences from the second exon were identical to each other as well as to the GenBank sequence #M67453. This confirmed that the primers designed from #M67453 were amplifying exon 1 and 2 in the *Codium* samples. This data also confirms that these exon regions are conserved within this subspecies from various geographical locations.

Once it was determined that the exon regions were amplifiable, yet provided no genetic variation, primers were deigned to amplify over what should be the more variable region, the intron in the rbcL gene. This proved to be a difficult target for PCR amplification for the following reasons: length (anything that exceeds 2 kbp is amplified with lower efficiency, in part, because the Taq polymerase misincorporates nucleotides with an error rate of ~$1/ 10^3$ to $1/ 10^4$ bp, and perhaps stalls at the mismatched base) and secondly because this gene is relatively AT rich, which means a typical 20 nucleotide primer anneals at a low temperature, and may fall off the template as the PCR profile is raised to 72°, the temperature required for extension. The additive *Taq* extender (Stratagene Corp., La Jolla Ca), which is a proofreading polymerase simply complements

the activity of Taq, was use d to overcome the length problems for amplification. Longer primers (24-26 oligonucleotides) were developed to overcome the problematic annealing temperature of AT rich areas.

To date there has been no success in amplifying across the rbcL intron from the PCR reactions using primers "2.8 and 2.3 rbcL intron". Therefore various troubleshooting experiments will be preformed, to optimize PCR amplification across the 2.5 kilobase pair intron which is AT rich. Adjusting the concentrations of the PCR reagents as well as the temperature and time of the anneal profile and extension steps may lead to conditions allowing amplification of this longer intron region.

Discussion

Codium fragile ssp. *tomentosoides* has become one of the most aggressive and invasive macroalgal species in temperate regions around the world (Trowbridge, 1999). For this reason, we have looked at two genes from the *C. fragile* genome that could provide information allowing us to track the origins of new populations of the algae as it spreads in the Gulf of Maine. DNA isolation, necessary for any genetic experiments was optimized for each new sample studied, as well as for different tissue sources (i.e. fresh material versus archival herbarium specimens. Purgene DNA extractions, with the addendum of Proteinase K and Rnase treatments, provided the most reliable DNA isolations. Never the less, the DNAs from many herbarium and fresh samples appeared to be degraded. Note this was not correlated with the age of the specimen, since both tissue types produced high molecular weight DNA as well as degraded. We speculate DNA degradation may be correlated with how fast the tissue dried during the preparation of the herbarium specimen, and nuclease activity during the grinding and cell lysis steps.

The PCR amplifications for the internal transcribed spacer 1 and 2 (ITS) proved that either epiphytic or contaminating fungal DNAs from most likely the drying stage of herbarium sample preparation was isolated along with the *Codium fragile* DNA. This co-purification problem led to nonspecific amplification of the ITS region, and therefore sequencing the ITS region of various fungal templates. Primers were redesigned, yet still proved to be non-specific for the *Codium* template. Therefore examination was turned to the ribulose-1,5-bisphosphate carboxylase/oxygenase gene as an alternative marker gene for phylogeographic studies.

Ribulose-1,5-bisphosphate carboxylase/oxygenase, a photosynthetic gene that does not exist in fungi species, was used since it should be feasible to amplify the *Codium* gene in a mixed template of algal and fungal DNAs. A number of different primers were designed for various parts of the rbcL gene: rbcL79 flanks part of exon 1, rbcL2500 flanks part of exon 2, and 2.8 and 2.3 rbcL primers are anchored in the exons and span the intron of the gene. Sequences from PCR amplifications of exon 1 and exon 2 were obtained from samples from Hawthorne Cove, MA, Millstone Point, CT, and Fort Stark, NH and were identical analytically compared to each other. This coding region of the gene is subjected to little variation in general, therefore although the sequence obtained from the exon regions in the rbcL gene was of good quality and length, no variable genetic information was obtained. Our focus then turned to amplifying the intron of the rbcL gene because non-coding regions offers more variability that can be compared. The intron is about 2.5 kb in length and is adenine and thymine rich, therefore considerable troubleshooting is necessary to successfully amplify regions designated by the 2.8 and 2.3 rbcL intron primers. With these adjustments the intron region can be successfully amplified and sequenced.

Further amplifications and sequencing of the intron region of the rbcL gene will lead to comparative data that can be applied to the various geographic locations. Troubleshooting for the ITS amplifications may also be beneficial for comparative studies. With the comparative data from the rbcL intron and the ITS region two hypotheses can then be tested using these sequences: 1. Has a second morphologically similar subspecies, *Codium fragile* ssp. *atlanticum* invaded the Canadian waters that may be contributing to the problems in the Gulf of Maine? Have new populations of *C. fragile*

resulted from localized dispersal or repeated introductions from other locations? The genetic and phylogeographic history of *Codium fragile* ssp. *tomentosoides* obtained through the rbcL intron and ITS sequences will eventually provide useful data to assist in designing control and containment plans to reduce further spread of this invasive alga.

Acknowledgements

This work is a result of research sponsored in part, by the National Sea Grant College Program, NOAA, Department of Commerce, under grant #NA16RG1035 through the University of New Hampshire Sea Grant College Program.

We would like to thank the following for their aid and support throughout the academic year:

- Dr. Anita Klein for her priceless time, guidance and direction as research advisor.
- Aaron Wallace for his support and technical advice.
- Brian Teasdale for his aid and counsel.
- Dr. Arthur C. Mathieson for specimen samples.
- Dr. Larry Harris for specimen samples.
- Jon Scott for his technical assistance.

Literature Cited

Altschul, S.F., Gish, W., Miller W., Myer E.W., and Lipman D.J. Basic local alignment search tool. *Journal of Molecular Biology*. 215: 403-410, 1990.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S., and Donoghue, M.J. The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny. *Ann. MO Bot. Gard.* 82: 247-277, 1995.

Carlton, J.T. and Scanlon, J.A., 1985. Progression and dispersal of an introduced alga: *Codium fragile* ssp. *tomentosoides* (Cholorophyta) on the Atlantic coast of North America. Botanica Mar. 28: 155-165.

Doyle, J.J. and Doyle, J.L. Isolation of plant DNA from fresh tissue. Anonymous. Anonymous. *Focus* 12: 13-15, 1990.

Kooistra, W.H., Stam, C.F., Olsen, J.L., and van den Hoek, C. 1992. Biogeography of *Cladophoropsis mebrancea* (Chlorophyta) based on the comparisons of nuclear rDNA ITS sequences. *J. Phycology* 28 (5): 660-668, 1992.

Stiller, J. W. and Waaland, J. R. 1996. *Porphyra rediviva* sp. nov. (Rhodophyta): a new species from northeast Pacific salt marshes. *J. Phycol.* **32**: 32332.

Trowbridge, C.D. 1999. An assessment of the potential spread and options for control of the introduced green macroalgal *Codium fragile* ssp. *tomentosoides* on Australian shoers CSIRO marine organism; CRIMP Consultancy Report, 51 pp. Hobart Tasmania, Australia.

Wattier, R. and Maggs, C.A. 2001. Intraspecific variation in seaweeds: The application of new tools and approaches. Advances in Botanical Research, Vol 35: 171-212.

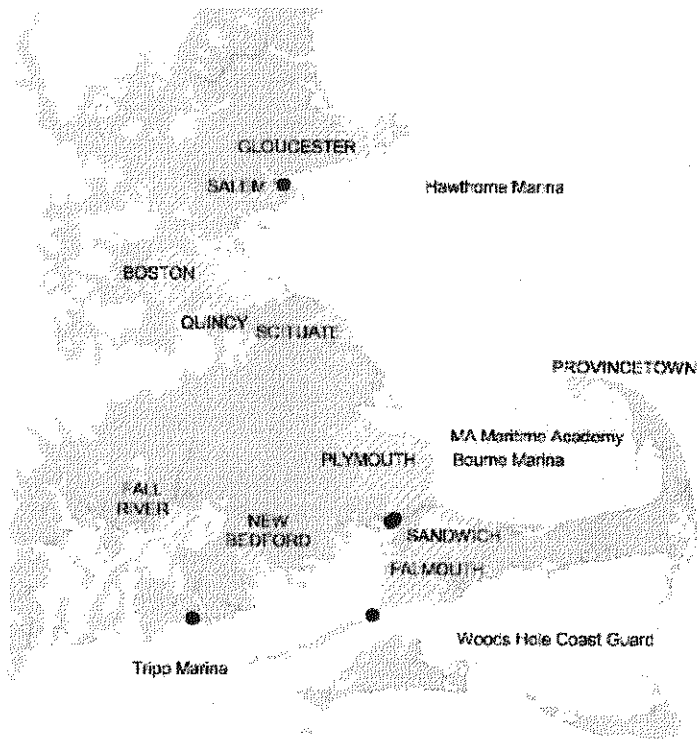
White, T. J., T. Bruns, S. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 In: PCR Protocols: A Guide to Methods and Applications, eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York.©W°

Appendix

Figure 1: Taxonomy Report of *Codium fragile* ssp. *tomentosoides*.

Domain	Eukarya
Kingdom	Protoctista
Phylum or Division	Chlorophyta (Green Algae)
Class	Chlorophyceae
Order	Codiales
Genus	*Codium*
Species Name	*fragile* (Suringar) Hariot subsp. Tomentosoides (van Goor) Silva
COMMON NAME:	Green sea fingers

Figure 2: Dispersal Patterns in Northwest Atlantic (Shoals NH/ME, Cape Cod MA, and Nova Scotia, Canada)



massbay.mit.edu/.../intro/ Codium_fragile.html, 4/10/04

Figure 3: Schematic of Ribosomal Gene with Internal Transcribed Regions. (Note: arrows indicate region of primer annealing.)

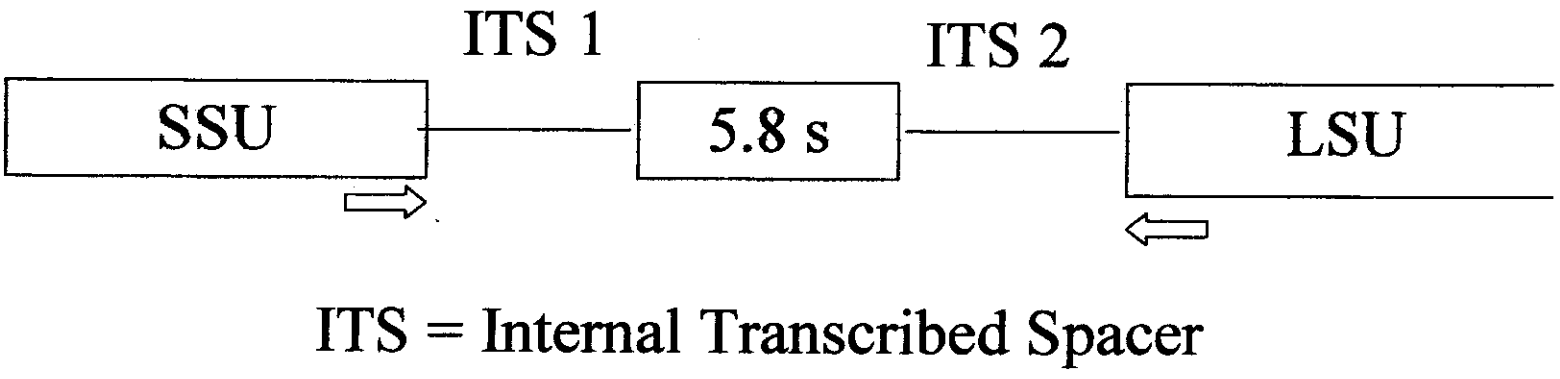


Figure 4: ***Codium fragile*****: Type, Amount, and Origin.**

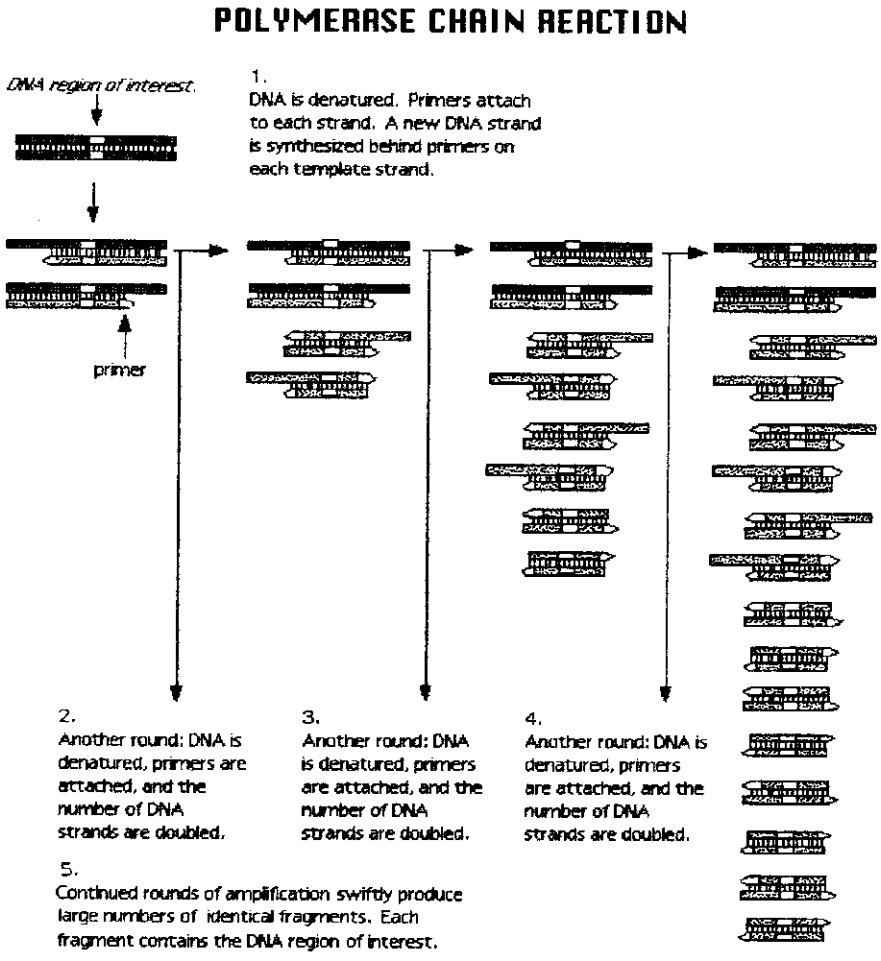
Sample #	Amt. Tissue (fw or dw) (grams)	Herbarium accession #	Collection site	Date of collection
	0.010	68302	Brave Boat HarborME site 4	11/19/97
	0.008	68307	Brave Boat HarborME site 4	11/26/97
	0.0083	68302	Brave Boat HarborME site 4	11/19/97
	0.0118	68302	Brave Boat HarborME site 4	11/19/97
	0.0075	68302	Brave Boat HarborME site 4	11/19/97
	0.0112	68307	Brave Boat HarborME site 4	11/26/97
	0.0094	68307	Brave Boat HarborME site 4	11/26/97
	0.0095	68307	Brave Boat HarborME site 4	11/26/97
	0.0115	68307	Brave Boat HarborME site 4	11/26/97
	0.0114	68302	Brave Boat HarborME site 4	11/19/97
	0.0307	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0314	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0347	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0320	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0277	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0288	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0293	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0308	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.042	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0304	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0331	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0327	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0443	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0434	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0323	Fresh	Hawthorne Cove Marina in Salem, MA	24 September, 2003
	0.0112	68307	Brave Boat HarborME site 4	11/26/97

0.0115	68307	Brave Boat HarborME	site 4	11/26/97
0.0098	68302	Brave Boat HarborME	site 4	11/19/97
0.00127	68302	Brave Boat HarborME	site 4	11/19/97
0.0138	68302	Brave Boat HarborME	site 4	11/19/97
0.0097	68307	Brave Boat HarborME	site 4	11/26/97
0.0122	68307	Brave Boat HarborME	site 4	11/26/97
0.0116	68307	Brave Boat HarborME	site 4	11/26/97
0.0465	Fresh	Millstone Point, CT		
0.0267	Fresh	Millstone Point, CT		
0.0365	Fresh	Hawthorne Cove Marina in Salem, MA		24 September, 2003
0.0261	Fresh	Hawthorne Cove Marina in Salem, MA		24 September, 2003
0.0105	dried	Millstone Point, CT		
0.0151	dried	Millstone Point, CT		
-	Ultracentrifuged	Fort Stark, NH		
0.0131	48695	Montaunk Point, NY		4/29/85
0.0107	48695	Montaunk Point, NY		4/29/85
0.0101	48695	Montaunk Point, NY		4/29/85
0.008	77586	Millstone Point, CT		11/18/03
0.0086	77586	Millstone Point, CT		11/18/03
0.0091	77586	Millstone Point, CT		11/18/03
0.0105	77134	Newport Shipyard, RI		8/6/03
0.0121	77134	Newport Shipyard, RI		8/6/03
0.0108	77134	Newport Shipyard, RI		8/6/03
0.0113	75692	Misquamicut Beach, RI		10/21/01
0.0116	75692	Misquamicut Beach, RI		10/21/01
0.0125	75692	Misquamicut Beach, RI		10/21/01

Figure 5: Primer Sequences

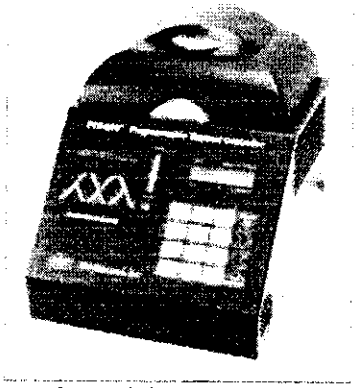
Primer Name	Gene/Region Amplifying	Oligonucleotide Sequence (5' to 3')
ITS 4	ITS region	TCCTCCGCTTATTGATATGC
ITS 5	ITS region	GGAAGTAAAAGTCGTAACAAGG
ITS forward	ITS region	CTCTGAACCTTCGCACGTAGA
ITS reverse	ITS region	TTTCCTCGCCGACGACATCGC
RbcL79 forward	RbcL exon 1	AATTATGCACGTGCTGTTTATGA
Rbcl79 reverse	RbcL exon 1	TTCCCACGTTTTTACTGCTTTAT
RbcL2500 forward	RbcL exon 2	TGCACAACGCGAAAATACTAATA
RbcL2500 reverse	RbcL exon 2	AATCATCTCGCATCAAATCAACA
2.3 rbcL intron for.	Rbcl intron	TAGATTTTTATTTGTTGCGGAAGC
2.3 rbcL intron rev.	RbcL intron	CAAACATGAATACCCCCTGAAGC
2.8 rbcL intron for.	RbcL intron	GCTGCTGTTGCTGCTGAATCT
2.8 rbcL intron rev.	RbcL intron	CCAAACATGAATACCCCCTGAAG

Figure 6: Schematic of Polymerase Chain Reaction



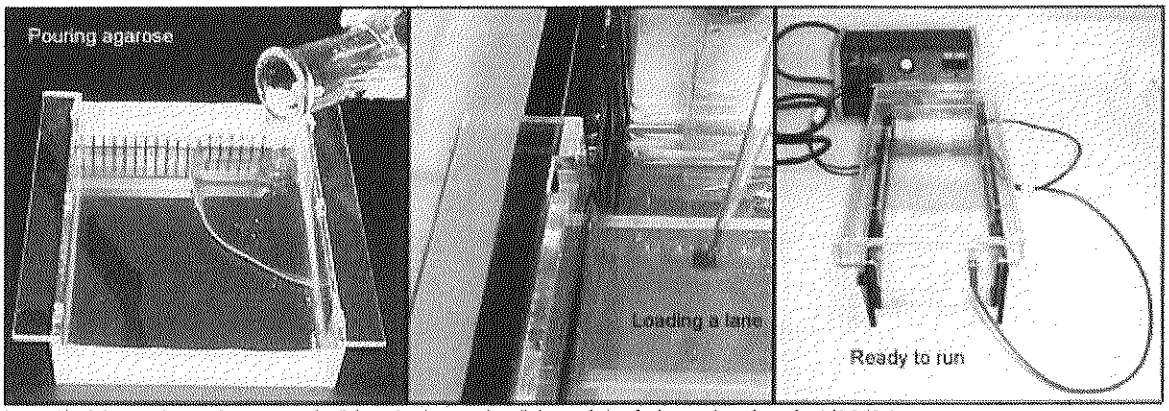
(www.zum.de/.../methoden/ pcr/polymerase-2.html, 4/10/04

Figure 7: Thermocycler (PCT 100)



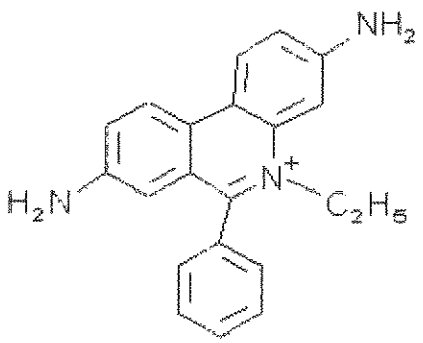
gsrtech.com/p/ ptc100.html, 4/10/04

Figure 8: Agarose Gel Electrophoresis.



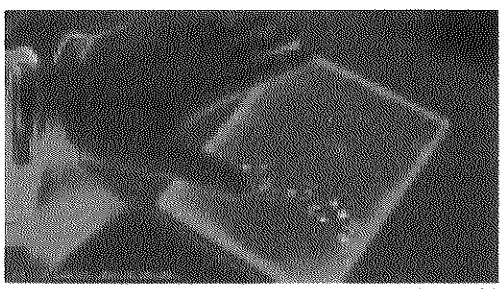
http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/agardna.html, 4/10/04

Figure 9: Ethidium Bromide structure.



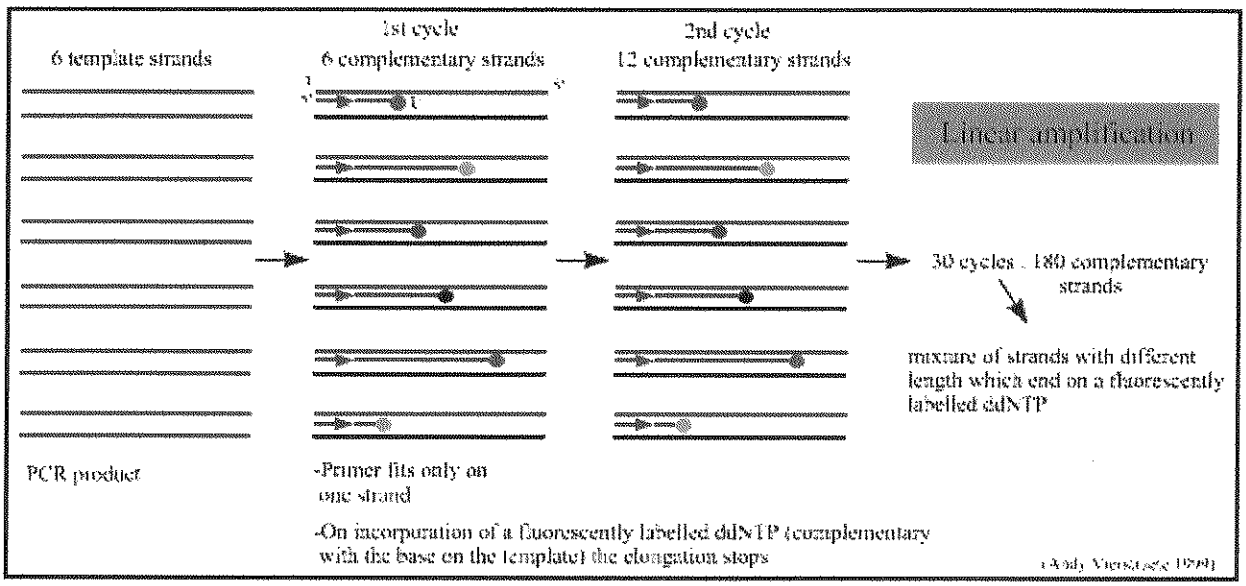
gucfm.georgetown.edu/.../ Ethidium_Bromide.html, 4/10/04

Figure 10: Agarose gel under ultraviolet light.



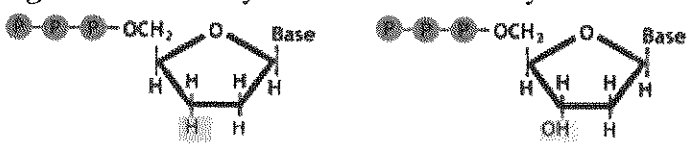
www.usd.edu/biocareers/risticbiol163f2000/ lec4.ht, 4/10/04

Figure 11: Sanger Method for DNA Sequencing



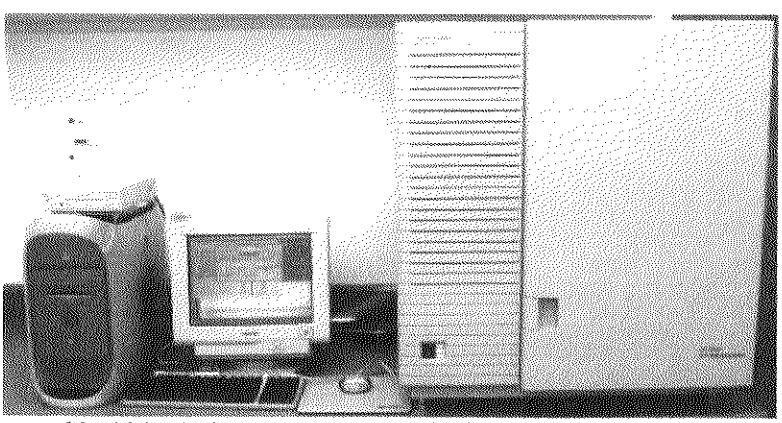
lsvl.la.asu.edu/.../sequencing/ sequencing.html, 4/19/04

Figure 12: Dideoxynucleotide and Deoxynucleotide Structures



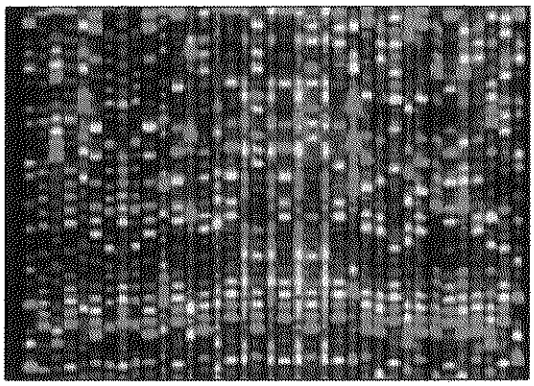
lsvl.la.asu.edu/.../sequencing/ sequencing.html, 4/19/04

Figure 13: ABI Prism Sequencer.



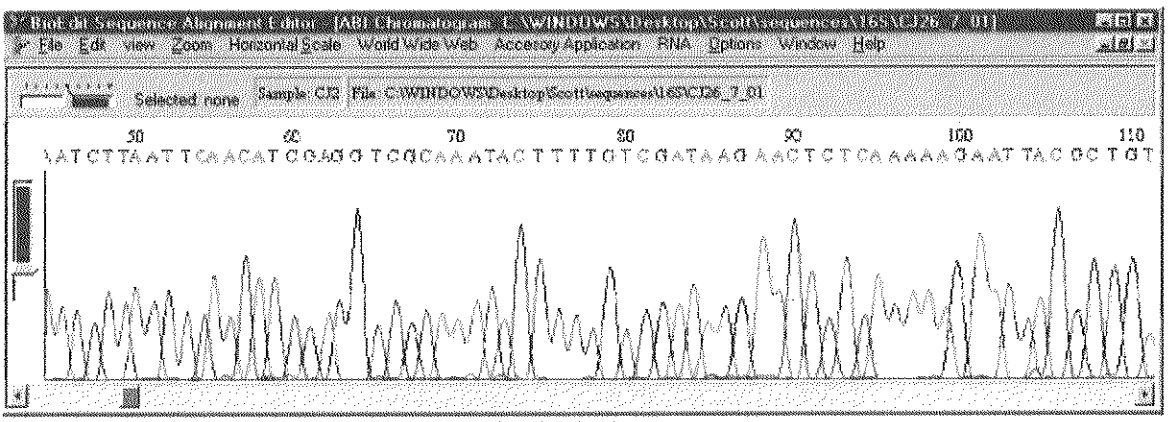
www.hku.hk/patho/ sequencer-2.htm, 4/20/04

Figure 14: Fluorescent tags recorded from ABI sequencer onto simulated gel.



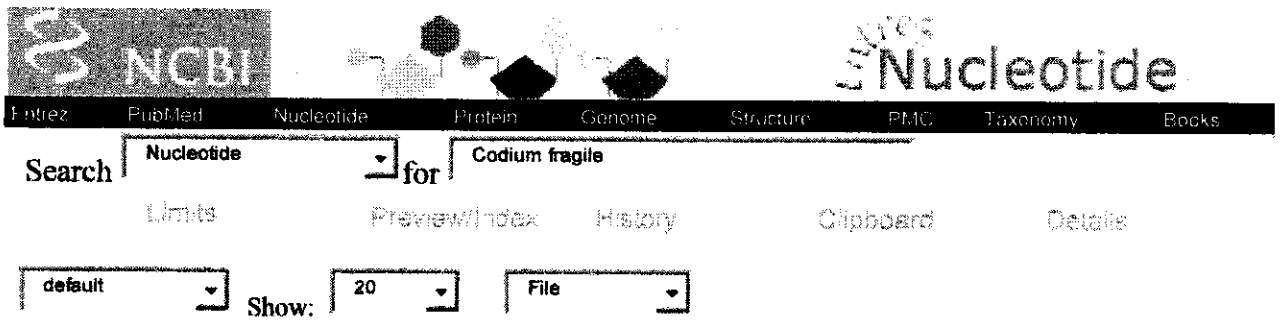
hils.psu.edu/stf/naf/ Sequencing.html, 4/10/04

Figure 15: DNA Sequence Chromatogram



www.mluri.sari.ac.uk/.../ rhizosphere_project.html, 4/21/04

Figure 16: Blast/Entrez Results Example from NCBI (Genbank): (complete rbcL sequence including exon 1, intron 1, and exon 2). Accession # M67453. (http://www.ncbi.nlm.nih.gov:80/entrez/viewer.fcgi?db=nucleotide&val=336614



1: M67453. Codium fragile ch...[gi:336614]

```
LOCUS       CODCPRBCL               3382 bp    DNA     linear   PLN 03-AUG-1993
DEFINITION  Codium fragile chloroplast ribulose-1,5-bisphosphate
            carboxylase/oxygenase large subunit (rbcL) gene, exons 1 and 2.
ACCESSION   M67453
VERSION     M67453.1  GI:336614
KEYWORDS    ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit.
SOURCE      chloroplast Codium fragile
  ORGANISM  Codium fragile
            Eukaryota; Viridiplantae; Chlorophyta; Ulvophyceae; Caulerpales;
            Codiaceae; Codium.
REFERENCE   1  (bases 1 to 3382)
  AUTHORS   Manhart,J.R. and VonderHaar,R.A.
  TITLE     Intron revealed by nucleotide sequence of large subunit of
            ribulose-1,5-bisphosphate carboxylase/oxygenase from codium fragile
            (Chlorophyta): Phylogenetic analysis
  JOURNAL   J. Phycol. 27, 613-617 (1991)
COMMENT     Original source text: Chloroplast Codium fragile (organelle
            Chloroplast Codium fragile) DNA.
FEATURES             Location/Qualifiers
     source          1..3382
                     /organism="Codium fragile"
                     /organelle="plastid:chloroplast"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:3133"
     gene            29..3269
                     /gene="rbcL"
     exon            29..832
                     /gene="rbcL"
                     /number=1
     intron          833..2645
                     /gene="rbcL"
     exon            2646..3269
                     /gene="rbcL"
                     /number=2
ORIGIN
        1 atcgatatat ttagatgaaa tattaaaaat ggttccaaaa actgaaacta aagcaggtgc
       61 aggatttaaa gcaggggtta aagattatcg actaacctat tatacacctg attatcaagt
      121 taaagaaact gacattcttg cagcttttcg tatgactcca caacctggag ttccgccaga
      181 agaatgtgga gctgctgttg ctgctgaatc ttcaactgga acatggacaa cagtttggac
      241 agatggatta acaagtttag atagttataa aggacgttgt tatgatttag aaccggtaaa
      301 aggagaagaa aatcaatata ttgcatatgt tgcttatcct attgatttat ttgaagaagg
      361 atcggttact aatttattta catcaattgt tggaaacgtt tttggattta aagcattacg
      421 agctcttcga ttagaagatt tacggatttc accagcttat gcaaaaacat ttcaaggacc
      481 acctcatggg attgaagtag aacgagataa attaaataaa tatggacgtc cattattagg
      541 ttgtactatt aaacctaaat taggcttatc agctaaaaat tatggacgtg ctgtttatga
      601 atgtttaaga ggtggattag attttactaa agatgatgag aatgtaaatt ctcaaccttt
```

```
 661 tatgcgttgg agagatagat ttttatttgt tgcggaagct atttataaat cacaagcaga
 721 aactggagaa attaaaggac attatttaaa tgcaacagct gggacgtgtg aagaaatgtt
 781 aaaacgtgca caatgtgcga aagaattcgg cgtacctatt atcatgcacg atgtgtgaca
 841 cgaagttact ggttatagta gaaatttggc attttgacaa ctttctgact taagtattag
 901 agtcttctct tttaaagggt gcatgatgaa tacatgtatc aaacacttta aaagaaattt
 961 tattttataa agatcaaata acagcttcgt taggatactt ttagatctat ataagagtag
1021 aatttttatc gtgattttct attaatactt ctttgatctc ggagtgattg atttgatgat
1081 tataaattat atatataaag cagtaaaacg tgggaaactt aaattaagcc tactgggata
1141 tttaaatagt aatatttatt ttttatttaa gttgcggaaa actctgttat gtcttgaaca
1201 ttttttgatt aaagatattc ttctgaaaat caacggaaaa ttacaatcat taggttatac
1261 gtttaactta atttgtatat aaccgaaaaa tgaaaataat ttttttattt gacaaatgag
1321 agtttggtct taaaaatttt ttttatatgc attttaattt ttataactct atattttcat
1381 gggataatat tgattggaat tttattgaaa aacgagttcg aaaattaaaa tatcgaattt
1441 ttttgagtaa aaaaaaaaaa ttcttttata tttcacaaaa atagaaagtt tagtttagaa
1501 agtcactcta ttcgattatt ttctattaaa accatttttt ttactaaaaa aaaaattttt
1561 tttttgtttt atgttaaatc tacttttaat cttatttta agaaattatg taccatttct
1621 ttggaatatt atgaaccttg caatttaaac tttaaatttg aattatttct tgatttttgt
1681 tgtcaaatta ttttatttca tatttttgaa cccgattggt tttgtcgaca aaatatttat
1741 ttctatcatt gtcacagaaa tccacaattc tttttatata aaactattgc ggctttacaa
1801 tttaagaatt atttgaaaaa atggatttta gttttcaata ttcatttttt agattggact
1861 ttttgcttac cagtcaaaat gccagtttta aatttttttt tacagatatg gattacacag
1921 aaacacattc tttttaataa aacaatacag ttttttattt tagaaatttt cgtttttttct
1981 ttttttaaga ttactcaaat tacttttatt tctttttta attctttatt tttctttttt
2041 aaaaatttga aaaaagtgca aggtttgcaa aataaatttt attctatcat aggtggagca
2101 tctacatata aacatctttt ttctagttta gaaggatttc aagtatttta ttggaatttt
2161 caacattttt tgactaagca atctcagaaa catcattaca atatgatttt aattattcaa
2221 cctagtcaat ttattcgaca acatattaaa gatattttta aatatgaatg gagattttta
2281 agaggaaaat ctactttatc tgttatttta cgattaaatc ctattattcg aaaatggaat
2341 ctttttttttt ttaaagccaa attttctagt atatttttaa atcagttaga tcaatggatg
2401 tttgtcaaaa gttggagata tgcacaacgc gaaaatacta ataaatcaaa aaaatggatc
2461 aaaaaacatt attttgggtc ttttaaaatt ttaaatactt ataaatggat ttttggagat
2521 ctcaaaacag ggtatttttt agttaaattt aattggtttt tttttaagct tgagccatat
2581 gcattgagaa atgcacgtat ggttcttagg ggggactttt ttggtaacat tttgacctac
2641 ccgactattt aactggaggt tggacagcta atacaagttt agctcgttac tgtcgagata
2701 atggcttatt attacatata catcgagcta tgcatgcggt tattgatcga caaaaaaatc
2761 atggaatgca ttttcgggtt ttagctaaat ctttacgtat gtctggtgga gatcatttac
2821 attctggaac tgttgtagga aaacttgaag gtgaacgtga agttacttta ggttttgttg
2881 atttgatgcg agatgatttt attacaaaag atagaagtcg tggaatttat tttactcaag
2941 attggggtttc tttacctggt gttatgcctg ttgcttcagg gggtattcat gtttggcata
3001 tgcccgcatt agtagaaatt ttcggagatg atgcttgttt acaatttgga ggaggtactt
3061 taggacatcc atggggaaat gctcctggtg cagctgctaa ccgaattgct tgtgaagctt
3121 gtgttcaagc tagaaatgaa ggaagagatt tagctagaga aggcggagat gttattagag
3181 ctgcttgtac ttggagtcca gaattagcag cggcttgtga agtttggaaa gaaattaaat
3241 ttgaatttga tactattgat actctttaaa ttttttattt gaaaattcat atatatgtct
3301 agacatatat atgaattttt agagtaacat tatagtttga gaaatatatt tatagggaat
3361 tacaccatcc agttgagcga ta
```

Figure 17: DNA extractions (CF # 28, 29, 31, 34, 36, 38) (1.2% Agarose Gel)
(MW)(28)(29)(31)(34)(36)(38)(MW)

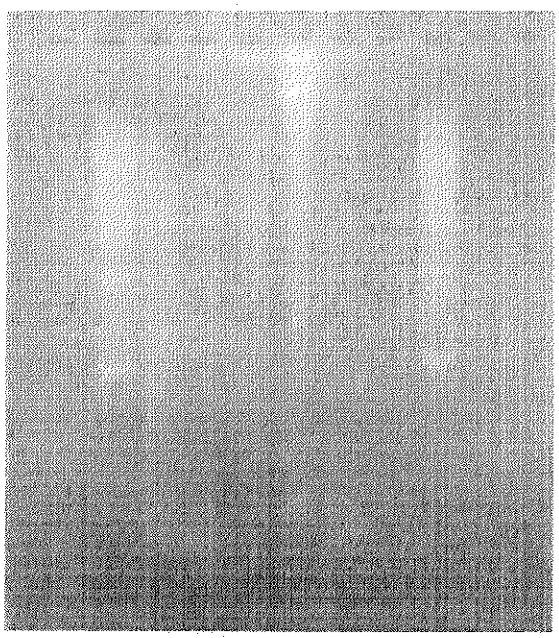


Figure 18: PCR with DNA samples 19, 20, 21, 25, 26, 27. (ITS 4 and 5) Multiple Bands indicate multiple DNA templates in reactions.

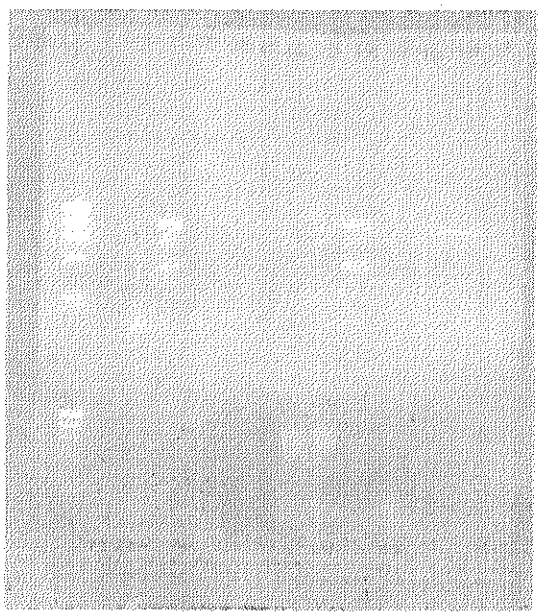
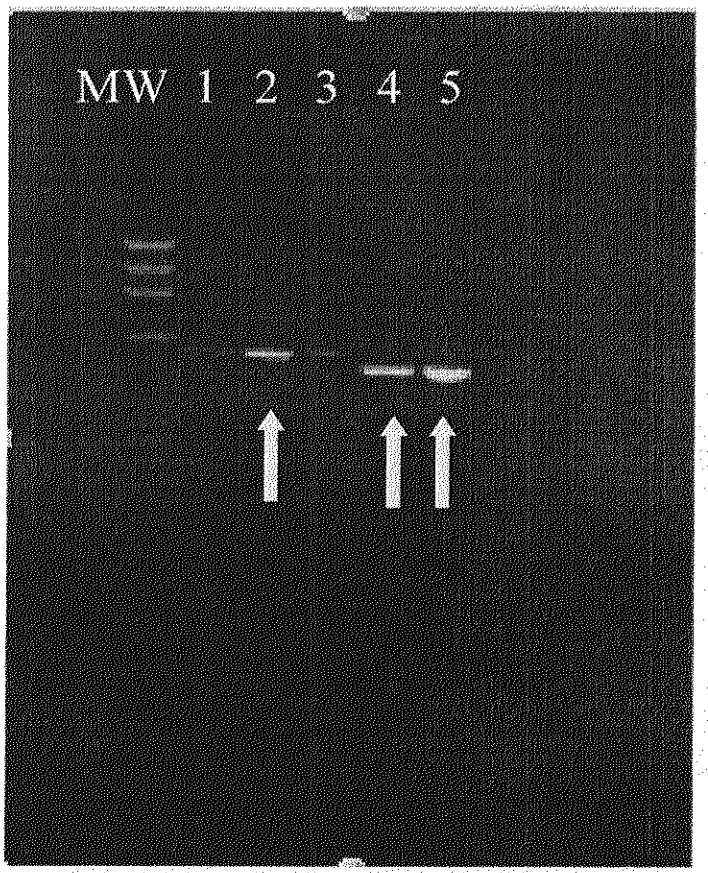


Figure 19: RbcL exon primers: Sequenced Samples (1/2% Low Melt Gel)



1: CF 36, [2.0] Mg^{2+}, 1.0 µl BSA- rbcL79 (exon 1)
2: *CF 36, [2.0] Mg^{2+}, 1.0 µl BSA- rbcL79 (exon 1)
3: UC 2, [1.5] Mg^{2+}, 0.5 µl BSA- rbcL79 (exon 1)
4: *UC 2, [2.0] Mg^{2+}, 0.5 µl BSA- rbcL2500 (exon 2)
5: *UC 2, [2.0] Mg^{2+}, 0.5 µl BSA- rbcL2500 (exon 2)