“The Invader”

Optimizing genetic approaches for species identification of *Grateloupia turuturu.*

http://www.flickr.com/photos/9053334@N06/2896133513

TECH 797

Undergraduate Ocean Research Projects

Usaila Ahmad

Allison Baldio

Matthew Mackenzie

Adviser: Anita Klein

Table of Contents

Abstract

Grateloupia turuturu is an invasive red algae, native to the western Pacific. Along with being the largest known red alga, it is widely considered to be a top threat for local marine habitats. Among the effects *G. turuturu* can have on a regional ecosystem are: disruption of shoreline ecology; sequestering of nutrients vital to local organisms; and the overall displacement of native organisms. *G. turuturu* was originally discovered in Boston Harbor by Mathieson *et al.* in 2007. The alga has also been present in Narragansett Bay, Rhode Island for 14 years. Using molecular techniques we sought to determine whether this introduction into Boston Harbor is in fact *G. turuturu* and not one of several closely related species. The *RbcL* gene has been shown to distinguish *G. turuturu* from similar species. To do this we tested and compared several published protocols for red alga DNA isolation, sought to optimize the polymerase chain reaction for several sets of primers, to amplify segments of the *RbcL* gene. We then will sequence these amplified segments. After sequencing we will compare the results of Boston Harbor with different locations including Narragansett Bay, and herbarium samples from Europe that are confirmed to be *G. turuturu*. This will give us a better understanding of the source of *G. turuturu* in Boston Harbor.

Introduction

Grateloupia turuturu is a red alga, originating from the Asiatic region of the Pacific Ocean. Widely believed to be the largest red algae on the planet, *G. turuturu* has increasingly become one of the most aggressive red macro algae and a substantial threat to native flora. It can have a large impact in shoreline ecology and nutrient availability resulting in a disruption of the status quo environment for native species. *G. turuturu* has spread throughout the world establishing itself in the southern Pacific Ocean and the Atlantic Ocean (c.f. Mathieson et. al. 2007). These introduction patterns are consistent with global shipping routes, leading to the hypothesis that *Grateloupia turuturu* is transported in ballast ship water.

Grateloupia turuturu is part of the genus *Grateloupia* that contains 51 species, under the family Halymeniaceae (Fredericq et. al. 2002). *Grateloupia turuturu* can grow up to 15cm wide and one meter long and consists of elongated fronds of a red hue. (c.f. Mathieson et al.2007).

G. turuturu was first discovered in North America in 1994 in Narragansett Bay, Rhode Island (Bohnsack et. al. 1997). Thirteen years later, in 2007, *G. turuturu* was found to have invaded the southern region of the Gulf of Maine including Boston Harbor. The Narragansett population was believed to be introduced to the area by transport in ship ballast water and has similar genetic structure to European samples (Marston et. al. 2002). The origin of introduction to Boston Harbor is under investigation, with potential spread from Narragansett Bay through the Cape Cod Canal or alternatively a separate introduction through ship ballast water.

Identification of the particular species of *Grateloupia* is important, due to the fact that in European populations, such as the one found in the Than Lagoon, there are as many as three different species of *Grateloupia* (Verlaque et al. 2005). To study the introduction into Boston Harbor on a molecular genetics level we needed to target genes that would show good interspecific differentiation. Our analysis of previous studies (Fredericq et al. 2002) conducted on red algae led us to target the *RbcL* gene for amplification. *RbcL* codes for the large subunit of the enzyme Ribulose-1,5-bisphosphate carboxylase, commonly known as Rubisco, a key photosynthetic gene involved in catalyzing the Calvin Cycle.

A majority of our time was spent on optimizing DNA extraction methods, which are documented in the Materials and Methods section. Different PCR conditions tested are also listed. For both DNA extraction and PCR amplification, agarose gel electrophoresis was used to evaluate the results of procedures.

Materials and Methods

Various samples of *Grateloupia* from different geographic locations were obtained with the help of Professor Art Mathieson, Department of Biological Sciences and Jackson Estuarine Lab UNH (see Table 1).

Tissue Preservation

The fresh collected tissue was first cleaned of visible epiphytes, and measured and weighed within a week of collection. When stored at 4°C for longer periods, the tissue began to degrade releasing red photosynthetic pigments in the stagnant water. Then, per the instructions of multiple extraction protocols, tissue was stored using several different methods. These preservation methods included silica desiccation, flash freezing, and freeze drying.

Genomic DNA Isolation

Our initial DNA isolation approach was focused on a commercial kit, the DNeasy Plant Mini Kit (QIAGEN). However in the past, our lab has had difficulty using the kit to isolate genomic DNA from marine algae. These algae contain a different spectrum of polysaccharides and pigments than do terra firma plants, which the kit is specifically optimized for. Since high quality, genomic DNA would not be obtained from the kit, we decided it was best to test several published protocols specifically for red algae DNA extractions.

We identified three published protocols for extracting genomic DNA from algae, allowing us to test multiple approaches in hopes of finding an optimal extraction technique (Table 2). We also tested the DNeasy Plant Mini Kit.

DNeasy Plant Mini Kit (QIAGEN):
(http://www.qiagen.com/Products/GenomicDnaStabilizationPurification/DNeasyPlantSystem/DNeasyPlantMiniKit.aspx#Tabs=t0)

The DNeasy Plant Mini kit is designed to isolate pure DNA, quick and easy. The typical yield of DNA is between 3 – 30 µg. when starting with 30mg of tissue. This commercial kit provides all of the reagents and spin columns necessary for rapid isolation of DNA. The protocol for extracting DNA is as follows (QIAGEN, 2010):

QIAGEN DNeasy Plant Mini Kit Protocol

Important Points

Perform all centrifugation steps at room temperature (15-25°C)
If necessary, redissolve any precipitates in Buffers AP1 and AP3/E concentrate
Ensure that ethanol has been added to Buffers AW and AP3/E
Preheat a water bath or heating block to 65°C

1. Disrupt the sample material (<100 mg wet weight or <20 mg lyophilized tissue) using the TissueRuptor, the TissueLyser, or a mortar and pestle.
2. Add 400 µl Buffer AP1 and 15 µl Proteinase K. Invert 25 times and incubate at 55 °C until top layer is translucent – optimize yourself.
3. Add 4 µl RNase A. Vortex and incubate for 10 min at 65°C.
   Note: Do not mix Buffer AP1 and RNase A before use
4. Add 130 µl Buffer AP2. Mix and incubate for 5 min on ice.
   Recommended: Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm).
5. Pipette the lysate into a purple QIAshredder Mini spin column in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x g (14,000 rpm)
6. Transfer the flow-through fraction into a new tube without disrupting the pellet. Add 1.5 volumes of Buffer AP3/E, and mix by pipetting.
7. Transfer 650 µl of the mixture into a white DNeasy Mini spin column in a 2 ml collection tube. Centrifuge for 1 min at >6000 x g (8000 rpm). Discard flow-through. Repeat this step with the remaining sample.
8. Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW, and centrifuge for 1 min at >6000 x g. Discard flow-through.
9. Add another 500 µl Buffer AW. Centrifuge for 2 min at 20,000 x g.
   Note: Remove the spin column from the collection tube carefully so the column does not come into contact with the flow-through.
10. Transfer the spin column to a new 1.5 or 2 ml microcentrifuge tube, and add 50 µl Buffer AE for elution. Incubate for at least 5 minutes, 10 is better. Centrifuge for 1 min at >6000 x g, repeat.

DNA Isolation Protocol for Red Seaweed (Rhodophyta)
Remi A Wattier, Paulo A. Prodohl, and Christine A. Maggs

The DNA isolation method by Wattier et. al (2000) is specifically designed red algae and yields about 5 µg of high molecular weight DNA (from 10mg starting tissue) with no RNA. The following procedure was followed (Wattier et. al 2000):

UPDATED PROTOCOL:

DAY 1:

1) Grind 10 mg of freeze dried plant material with liquid nitrogen in a 1.5 mL microcentrifuge tube
2) Keep on ice until desired number of samples are ground
3) Add 1.5 mL of complete extraction buffer (100mM Tris HCl, 50 mM EDTA, 500mM NaCl, 20% SDS) warmed to 37^{o}C and vortex
4) Incubate tubes horizontally at 37°C for 30 min with shaking (120 shakes/min)
    a. Shaking reduces aggregation of material and enhances DNA release
    b. If no water bath with shaking is available, hand shaking can be used.
    c. Vigorously invert the tubes 5 times, as often as possible
5) Centrifuge at 13,000 rpm for 15 min
6) Transfer 1.3 mL of supernatant to a new tube
7) Add 10 µL of ribonuclease A stock solution
8) Incubate at 37° for 30 mins
    a. This step allows for RNA degradation
9) Transfer tubes into ice and incubate for 30 mins
10) Centrifuge at 13000 rpm for 15 min at 4°C (either put centrifuge in fridge or cold room)
11) Transfer 1 mL of supernatant to a new tube
12) Add 700 µL of -20° cold isopropanol and gently mix by inverting the tubes a few times (DNA precipitates at this step, often forming visible "jelly-fish-like" filaments)
13) Incubate tubes at -20°C overnight

DAY 2:

14) Centrifuge at 13000 rpm for 30 min at 4°C
15) Discard supernatant and wash the DNA pellet 3 times with 1 mL of -20°C cold 70% ethanol, and centrifuge at 3000 rpm for 10 min after each wash
16) Air-dry pellets and resuspend in 500 µL of TE buffer.

Nucleic Acid Extraction from Seaweed Tissues for Polymerase Chain Reaction Chain Reaction

Yong Ki-Hong, Chul Hyun Sohn, Ki Wan Lee, and Hyung Geun Kim

The nucleic acid extraction method by Hong et. al (1997) was developed for rapid isolation of seaweed DNA that is suitable for the polymerase chain reaction (PCR), while

utilizing lithium chloride (LiCl) to prevent polysaccharide contamination inhibiting PCR.

The following procedure was followed (Hong et. al 1997):

1.) 0.1 g of partially dried tissue, ground with liquid nitrogen
2.) Heated in 4 mL of extraction solution (0.8 M LiCl, 0.6% sarcosyl, 10 mM EDTA, 0.2% PVPP, 5% β-mercaptoethanol, pH 9.0)for 10 min at 55°C
3.) Shake gently at 4°C for 1 hour (in cold room)
4.) Precipitate directly by addition of 0.1 volumes of 3 M sodium acetate (pH 5.4) and 2 volumes of 100% EtOH
5.) Resuspend the precipitate in 100 mL of TE (10 mM tris-HCl, 1 mM EDTA, pH 8.0)
6.) Nucleic Acid solution is spun for 10 min in a microfuge
7.) Supernatant used for determination of nucleic acid amount, per, and impurities

An Efficient Method for DNA Isolation from Red Algae
Zimin Hu, Xiaoqi Zeng, Aihua Wang, Cuijuan Shi, and Delin Duan

The DNA isolation method by Hu et. al (2004) is designed to be simple and efficient for the total genomic DNA extraction for red algae. It should result in the isolation of about 0.1 µg of high quality DNA. The following procedure was followed (Hu et. al 2004):

DAY 1

1) 0.12 g samples were ground with liquid nitrogen
2) Add the β-mercaptoethanol to the extraction buffer
3) Place samples in 1.5 mL tube with 0.7 mL extraction buffer and incubate at 37°C for 1 hour.
    a. Invert at regular intervals
4) Add equal volume of ice-cold potassium Acetate (5.0 M, pH 7.5) and mix gently.
5) Leave on ice for 20 mins. Centrifuge at 10600 x g for 15 min in the cold room.
6) Collect aqueous phase and extract with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Mix thoroughly and centrifuge at 10600 x g for 10 min at room temperature
7) Transfer the upper aqueous phase to a new tube and extract with an equal volume of chloroform:isoamyl alcohol (24:1). Mix thoroughly and centrifuge at 10600 x g for 10 min.
8) Collect supernatant and add a final concentration of 0.1 mg/ml RNase and incubate at 37°C for 1 hour
9) Extract with phenol:chloroform:isoamyl alcohol (25:24:1). Mix thoroughly and centrifuge at 10600 x g for 10 mins at room temperature
10) Transfer the upper aqueous phase to a new tube and extract with equal volume chloroform:isoamyl alcohol (24:1). Mix thoroughly and centrifuge at 10600 x g for 10 min
11) Collect the upper aqueous phase and add 2/3 volume of ice-cold isopropanol
12) Store at -20°C (1 hr to overnight)

DAY 2

13) Precipitate DNA by microcentrifugation at 18000 x g for 20 min.
14) Remove supernatant, and precipitated DNA was washed with cold 70% ethanol , for 3 times
15) Vacuum dry pellet for 10-15 min
16) Resuspend in 100 µL TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.5) and store at -20°C

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an incredibly useful molecular biology technique used for the amplification of nucleic acids *in vitro*. PCR allows for the amplification of a particular nucleic acid sequence (up to 10,000 bp) resulting in an increase of > 10^6 fold in copies of the sequence. This affords a researcher the ability to determine the size of a DNA region and its nucleotide sequence. This target sequence known as the template is amplified from two directions using short oligonucleotide sequences called primers (Fig. 1). To achieve said amplification, approximately 30-40 cycles (varying depending on the experiment) of four steps must be completed. The first step is a denaturation of the double stranded DNA and is usually conducted at 94^oC. After this denaturation the temperature is decreased to allow the annealing of the oglionucleotide primers to the target sequence. This step is a major variable in optimizing a PCR reaction, and generally the optimum temperature is somewhere between 45^oC and 60^oC. The next step is one of extension. In this step thermostable DNA polymerase extends the primer sequence, and thus the attached target sequence. This is best done at 72^o C, the optimal temperature for Taq DNA Polymerase and results in the copying of the target sequence. Taq polymerase is unique in that it is thermostable, having been isolated from bacteria found in hot springs, this allows it to function at such a high temperature.

This sequence of events is repeated multiple times (30-40) resulting in millions of copies of the original sequence.

Inhibition is a significant problem when trying to optimize a PCR reaction. Inhibition is quite common in algae specimens due to polysaccharides and photosynthetic pigments that extract and may copurify with the DNA template PCR Amplification can be optimized by modifying: Magnesium ion concentration, DNA template concentration, and the annealing temperature. Our initial starting conditions along with modifications attempted for optimization are listed below (see Table 3 for primer sequences):

Initial PCR Conditions: Based on work done by Fredericq et. al. 2002

- 4 min at 96°C for denaturation
- 35 cycles of 60 s at 94°C, 60 s 42°C and 90 s at 72°C
- Final 10 min extension cycle at 72°C
- Soak cycle at 10°C

Conditions for DNA Template Variation:

- 4 min at 96°C for denaturation
- 35 cycles of 60 s at 94°C, 60 s 42°C and 90 s at 72°C
- Final 10 min extension cycle at 72°C
- Soak cycle at 10°C
- Vary amount of DNA: 1 ul, 3 ul, and 1 ul 1:10 dilution
- 4 min at 96°C for denaturation
- 35 cycles of 60 s at 94°C, 60 s 42°C and 90 s at 72°C
- Final 10 min extension cycle at 72°C
- Soak cycle at 10°C
- *gradient (saved as fredgrad)
- 8 diff temp
- 3 diff concentrations: 1 ul, 3 ul, 1 ul 1:10 dilution

Gel electrophoresis:

Genomic DNA was run on a 0.6% agarose gel at 7-8 Volts/cm for approximately three hours, and stained with ethidium bromide (0.5 μg/ml). The DNA standard used as a comparison was a 21 kB ladder (Bioline) or λ *Hind*III marker (Bioline).

PCR amplicons were run on a 1.2% agarose gel at 7-8 Volts/cm for approximately two hours, and stained with ethidium bromide (0.5 µg/ml). The DNA standard used for comparison was a 2.0 kb ladder (Bioline)

Results

We were unsuccessful in extracting DNA from samples using the tissue preservation methods based on desiccation by silica gel. We substituted a freeze drying preservation method for protocols using silica gel as a desiccant.

Initial attempts at extraction of genomic DNA from *G. turuturu* tissue were unsuccessful with the Hong et al. (1997) and Hu et al. (2004) protocols (Fig. 2). Results were initially positive for isolation through the Wattier et al. (2000) protocol (Figure 2) as seen by the high molecular weight DNA band found above the 21 kb marker; however the results were not reproducible without protocol modification (Figure 3). After multiple trials the Wattier et al. (2000) protocol was the only method consistently producing high molecular weight genomic DNA (Figure 4); we determined that the other two protocols were not viable in application to *G. turuturu*.

DNA Amplification proved to be just as difficult to optimize as extraction. We based our amplification on the conditions in the Fredericq et al. (2002) paper. There were several inconsistencies in the materials and methods regarding to conditions for DNA amplification in this paper as compared to other PCR protocols. Once calculating proper reagent concentrations, we began amplification experiments. Initial attempts based solely on the reaction conditions presented in the paper were unsuccessful (Figure 5), and we determined we needed to attempt to optimize several different variables. Our first variable tested was DNA template concentration, testing 1µl, 3 µl and 1µl of a 1:10 dilution. However none of these concentrations resulted in amplification (Figure 6).

Next we decided to test another variable, the primer annealing temperature. The annealing temperature of primers is based on several factors including primer length and G-C content, and often needs to be optimized. We set up PCR with an annealing

temperature gradient, testing temperatures between 42^{o}C and 50^{o}C on a 2^{o}C interval. None of these temperatures produced amplification.

Next we decided to test a variable that many often run into trouble with, the magnesium concentration. Free magnesium ions have a significant impact on the viability of a PCR reaction. Mg^{2+} ions are a required co-factor for Taq DNA polymerase, so a low concentration will prevent enzymatic activity. However an excessive concentration of Mg^{2+} ions can have severe detrimental effects including decreasing the specificity of the reaction's primers and the stabilization of double stranded DNA thus preventing denaturization in the first step of PCR. We tested three different concentrations of Mg^{2+} ions: 2.0 mM, 2.5 mM, and 3.0 mM. However changing this variable did not result in amplification of *RbcL* from the DNA template we had prepared.

Next we tested whether the template itself might inhibit PCR amplification. We designed an experiment utilizing another alga, *Ulva*, which has a well-established protocol for amplification that has consistently produced positive results. In this experiment *Ulva* DNA and *Ulva* specific primers are used as a positive control. To separate reaction tubes we added our *G. turuturu* samples in addition to the *Ulva* samples. In theory if the positive controls containing *Ulva* DNA yielded positive results and reactions containing *G. turuturu* DNA did not yield positive results it would show that the *G. turuturu* samples contain a PCR inhibitor. The results of this experiment, (compare lanes C and D) demonstrated that the *Grateloupia* template contained inhibitors of PCR (Figure 7)

Discussion

Grateloupia turuturu has proved to be a challenging species to work with. Our initial attempts at tissue preservation, involved the use of silica gel beads to act as a desiccant. The hope was to use this method to prepare samples for the protocol described in the Wattier et al. (2000). However this proved problematic as *G. turuturu* is rather frail and thin and wraps itself tightly to the silica beads. This makes it near impossible to obtain tissue not contaminated with silica, which inhibits downstream applications. To counter this problem we turned to the process of freeze-drying, this method proved to be viable and allowed us to continue with the Wattier et al (2000) protocol. We applied this preservation method to the Hu et al. (2002) protocol as well, while the Hong et al.(1997) protocol utilized flash frozen tissue.

Each protocol that we chose for tissue extraction gave us a unique variable we decided we should test. The Wattier et al. (2000) protocol is more or less a basic genomic DNA extraction protocol using a Tris-HCl buffer to maintain pH, ethylenediaminetetraacetic acid (EDTA) to sequester free magnesium ions, and NaCl. To precipitate extracted DNA the protocol uses a differential precipitation method involving both Isopropanol and Ethanol.

The Hu et al. (2004) protocol uses a standard extraction buffer along with a detergent Sodium dodecyl sulfate (SDS) which aids in the lysing of cells, Polyvinylpyrrolidone which is used to absorb polyphenols that can inhibit the DNA polymerase enzyme used in PCR, and β-mercaptoethanol which stabilizes thiol-ester bonds in certain proteins. The Hu et al. (2004) protocol precipitates the extracted DNA using 3 M Sodium acetate and 100% ethanol.

The Hong et al. (1997) protocol utilizes aspects of each of the previous two protocols. It uses EDTA, PVP, and β-mercaptoethanol, but in addition adds several other reagents. The first is lithium chloride (LiCl), which is used to soften the plant cellular wall and aid in the release of genomic DNA. The second is the detergent Sarcosyl, which aids in the lysing of cells to better release genomic DNA. To precipitate the DNA, the protocol utilizes Isopropanol.

As we were extracting DNA we noticed that the end products of all three protocols had a slight pink/redish tint. At the time the reason did not dawn on us, but it is quite evident now that the pinkish hue was due to the presence of photosynthetic pigments still present even after extraction.

Of the four DNA extraction methods we used, only the Wattier et al. (2000) protocol was reproducible, producing high molecular weight DNA that was visible by gel electrophoresis. However these DNA samples were not stable at 4°C. Fresh DNA extractions using the Wattier et al. (2000) method were prepared, to proceed to PCR amplification.

After numerous attempts at amplification of the *RbcL* gene using the published protocol by Fredericq et al. (2002) as well as several attempts of optimization for multiple variables, PCR attempts were unsuccessful. With so many variables not producing any positive results we began to believe that perhaps the DNA template we were using, produced through the Wattier et al. (2000) protocol still contained contaminants. By analyzing PCR amplicons via gel electrophoresis with a positive control, e.g. *Ulva* amplicon, we demonstrated that the *Grateloupia* template carried strong PCR inhibitors.

We have demonstrated several methods for DNA extraction, established on other red algae, are not useful for *Grateloupia turuturu*. Future steps include altering the starting amount of tissue in the commercial Plant DNeasy kit to optimize it for use with *G. turuturu* tissue samples. It appears that the recommended starting value of 25 mg of *Grateloupia turuturu* plant tissue is simply too much and that better results are produced when using < 3mg (pers. comm. Will Schmidt, LSU).

Acknowledgements

This work is the result of research sponsored in part, by the National Sea Grant College Program, NOAA, Department of Commerce, under grant #NA06OAR4170109 through the New Hampshire Sea Grant College Program

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

📖 Dr. Anita S. Klein
- For her immense support and guidance throughout this project.

📖 Dr. Art C. Mathieson
- For taking us to Narragansett Bay, RI to collect *G. turuturu* and for collecting samples from Cape Cod.

📖 Chris Benton
- For aid and counsel in lab.

📖 Jeremy Nettleton
- For providing us with *Ulva* DNA and primers to amplify a ribosomal spacer region

📖 Jennifer Bedsole
- For ordering the supplies we needed to use to make this research project possible.

📖 TECH 797 Undergraduate Ocean Research Projects

Literature Cited

Gavio, Brigitte, and Suzanne Fredericq. "*Grateloupia turuturu* (Halymeniaceae,Rhodophyta) Is the Correct Name of the Non-Native Species in the Atlantic Known as Grateloupia Doryphora." *European Journal of Phycology* 37 (2002): 349-59.

Hong, Yong-Ki, Chul H. Sohn, Ki W. Lee, and Hyung G. Kim. "Nucleic Acid Extraction from Seaweed Tissues for Polymerase Chain Reaction." *Journal of Marine Biotechnology* 5 (1997): 95-99.

Hu, Zimin, Xiaoqi Zeng, Aihua Wang, Cuijuan Shi, and Delin Duan. "An Efficient Method for DNA Isolation from Red Algae." *Journal of Applied Phycology* 16 (2004): 161-66.

Mathieson, Arthur C., Clinton J. Dawes, Judith Pederson, Rebecca A. Gladych, and James T. Carlton. "The Asian red seaweed *Grateloupia turuturu* (Rhodophyta) invades the Gulf of Maine." *Biological Invasions* 10 (2008): 985-988.

M. F. Marston and M. Villalard-Bohnsack. Genetic variability and potential sources of *Grateloupia doryphora* (Halymeniaceae, Rhodophyta) an invasive species in Rhode Island waters (USA). *J.Phycol.* 38 (4):649-658, 2002

M. Verlaque, P. M. Brannock, T. Komatsu, M. Villalard-Bohnsack, and M. Marston. The genus *Grateloupia* C. Agardh (Halymeniaceae, Rhodophyta) in the Than Lagoon (France, Mediterranean): a case study of marine plurispecific introductions. *Phycologia* 44 (5):477-496, 2005.

M. Villalard-Bohnsack and M. M. Harlin. The appearance of *Grateloupia doryphora* (Halymeniaceae, Rhodophyta) on the northeast coast of North America. *Phycologia* 36 (4):324-328, 1997

Wattier, Remi A., Prodohl, Paulo A., Maggs, Christine A. "DNA Isolation Protocol for Red Seaweed (Rhodophyta)." *Plant Molecular Biology Reporter* (2000): 275-81.

Appendix

Table 1. Location, measurement, and preservation method of *G. turuturu* samples collected.

Sample #	Location	Multiples (if any)	Length (cm)	Width (Cm)	Fresh Weight (g)	Preservation Method
1	Narragansett Bay, RI Beaver Tail	-	14.0	4.0	1.00	Pressed
2	Narragansett Bay, RI Beaver Tail	-	15.5	2.5	0.65	Silica
3	Narragansett Bay, RI Beaver Tail	-	12.1	3.3	1.30	Pressed
4	Narragansett Bay, RI Beaver Tail	-	11.0	1.5	0.30	Frozen
5	Narragansett Bay, RI Beaver Tail	-	19.5	2.0	0.66	Silica
6	Narragansett Bay, RI Beaver Tail	-	10.0	2.8	0.20	Frozen
7	Narragansett Bay, RI Beaver Tail	-	12.0	5.0	1.30	Silica
8	Narragansett Bay, RI Beaver Tail	-	10.0	3.0	0.63	Frozen
9	Narragansett Bay, RI Beaver Tail	-	15.3	2.5		Frozen
10	Narragansett Bay, RI Beaver Tail	-	12.0	5.0	2.14	Frozen
11	Narragansett Bay, RI Beaver Tail	-	8.0	5.0	1.51	Frozen
12	Narragansett Bay, RI Beaver Tail	-	14.5	4.5	1.15	Frozen
13	Narragansett Bay, RI Beaver Tail	-	16.5	4.5	1.98	Frozen
14	Narragansett Bay, RI Beaver Tail	-	15.6	11.4	2.20	Frozen
15	Narragansett Bay, RI Beaver Tail	-	16.6	13.2	4.40	Frozen
16	Narragansett Bay, RI Beaver Tail	-	10.2	5.1	0.90	Frozen
17	Narragansett Bay, RI Beaver Tail	-	16.2	3.5	0.70	Frozen
18	Narragansett Bay, RI Beaver Tail	-	23.2	11.1	4.93	Frozen
19	Narragansett Bay, RI Beaver Tail	-	14.1	5.5	1.60	Frozen
20	Narragansett Bay, RI Beaver Tail	-	9.4	8.9	1.40	Frozen
21	Narragansett Bay, RI Beaver Tail	-	13.7	6.9	1.21	Frozen
22	Narragansett Bay, RI Beaver Tail	-	9.0	4.9	0.80	Frozen
23	Narragansett Bay, RI Beaver Tail	-	10.3	8.5	1.10	Frozen
24	Narragansett Bay, RI Beaver Tail	-	9.5	9.9	2.50	Frozen
25	Narragansett Bay, RI Beaver Tail	-	11.5	4.7	1.90	Frozen

26	Narragansett Bay, RI Beaver Tail	-	9.5	3.6	0.80	Frozen
27	Narragansett Bay, RI Beaver Tail	-	8.9	2.5	0.40	Frozen
28	Narragansett Bay, RI Beaver Tail	-	10.0	5.6	0.50	Frozen
29	Narragansett Bay, RI Beaver Tail	-	12.9	3.8	0.70	Frozen
30	Narragansett Bay, RI Beaver Tail	-	10.5	2.5	0.50	Frozen
31	Narragansett Bay, RI Beaver Tail	-	9.2	1.9	0.28	Frozen
32	Narragansett Bay, RI Beaver Tail	-	9.0	2.1	0.21	Frozen
33	Narragansett Bay, RI Beaver Tail	-	8.1	1.8	0.21	Frozen
34	Narragansett Bay, RI Fort Getty	A-K	38.5	6.5	11.07	Frozen
35	Narragansett Bay, RI Fort Getty	A-J	47.0	7.5	11.60	Frozen
36	Narragansett Bay, RI Fort Getty	A-C	25.0	5.0	3.20	Frozen
37	Narragansett Bay, RI Fort Getty	A-D	17.0	9.0	4.80	Frozen
38	Narragansett Bay, RI Fort Getty	A-E	27.0	7.0	9.70	Frozen
39	Narragansett Bay, RI Fort Getty	-	20.0	4.0	2.60	Frozen
40	Narragansett Bay, RI Fort Getty	A-F	21.5	9.5	6.60	Frozen
41	Narragansett Bay, RI Fort Getty	-	39.9	9.9	9.62	Frozen
42	Narragansett Bay, RI Fort Getty	-	5.6	6.7	2.30	Frozen
43	Narragansett Bay, RI Fort Getty	-	5.5	4.0	0.80	Frozen
44	Narragansett Bay, RI Fort Getty	-	11.2	4.9	1.00	Frozen
45	Narragansett Bay, RI Fort Getty	-	11.9	10.4	2.60	Frozen
46	Narragansett Bay, RI Fort Getty	-	28.9	8.6	8.00	Frozen
47	Narragansett Bay, RI Fort Getty	-	21.3	10.4	4.00	Frozen
48	Narragansett Bay, RI Fort Getty	-	14.9	5.6	3.33	Frozen
49	Narragansett Bay, RI Fort Getty	-	21.1	10.4	8.20	Frozen
50	Narragansett Bay, RI Fort Getty	-	13.9	10.9	4.40	Frozen
51	Narragansett Bay, RI Fort Getty	-	6.6	4.5	1.10	Frozen
52	Narragansett Bay, RI Fort Getty	-	5.2	5.9	1.43	Frozen
53	Narragansett Bay, RI Fort Getty	A-C	33.4	4.6		Frozen
54	Narragansett Bay, RI Fort Getty	-	16.4	7.2	3.36	Frozen

55	Narragansett Bay, RI Fort Getty	A-D	17.0	6.5	4.08	Frozen
56	Narragansett Bay, RI Fort Getty	A-C	21.0	4.5	3.13	Frozen
57	Narragansett Bay, RI Fort Getty	A-E	18.3	11.2	4.59	Frozen
58	Narragansett Bay, RI Fort Getty	-	14.0	6.8	2.35	Frozen
59	Narragansett Bay, RI Fort Getty	-	13.2	3.9	1.00	Frozen
60	Narragansett Bay, RI Fort Getty	-	16.0	4.0	1.63	Frozen
61	Narragansett Bay, RI Fort Getty	A-C	18.0	5.3	2.98	Frozen
62	Narragansett Bay, RI Fort Getty	A-D	14.5	7.5	3.90	Frozen
63	Narragansett Bay, RI Fort Getty	A-C	13.5	5.5	3.20	Frozen
64	Narragansett Bay, RI Fort Getty	A-B	16.5	5.0	1.95	Frozen
65	Narragansett Bay, RI Fort Getty	A-B	20.4	4.0	2.25	Frozen
66	Narragansett Bay, RI Fort Getty	-	17.5	6.0	4.98	Frozen
67	Narragansett Bay, RI Fort Getty	A-G	30.0	8.0	7.80	Frozen
68	Narragansett Bay, RI Fort Getty	A-B	20.3	4.1	2.00	Frozen
69	Narragansett Bay, RI Fort Getty	A-B	15.4	10.2	2.30	Frozen
70	Narragansett Bay, RI Fort Getty	-	13.0	2.5	0.58	Frozen
71	Narragansett Bay, RI Fort Getty	-	12.0	4.0	1.60	Frozen
72	Narragansett Bay, RI Fort Getty	-	17.0	5.0	3.00	Frozen
73	Narragansett Bay, RI Fort Getty	-	19.0	3.5	1.20	Frozen
74	Cape Cod, MA Massachusetts Maritime Academy	A-B	21.0	3.5	2.15	Frozen
75	Cape Cod, MA Massachusetts Maritime Academy	A-B	26.0	4.0	2.25	Frozen
76	Cape Cod, MA Massachusetts Maritime Academy	-	18.0	4.5	1.70	Frozen
77	Cape Cod, MA Massachusetts Maritime Academy	-	17.0	3.0	1.70	Frozen
78	Cape Cod, MA Massachusetts Maritime Academy	A-E	22.0	5.0	4.80	Frozen
79	Cape Cod, MA Massachusetts Maritime Academy	-	11.5	2.5	0.90	Frozen
80	Cape Cod, MA Massachusetts	-	13.5	3.5	1.10	Frozen

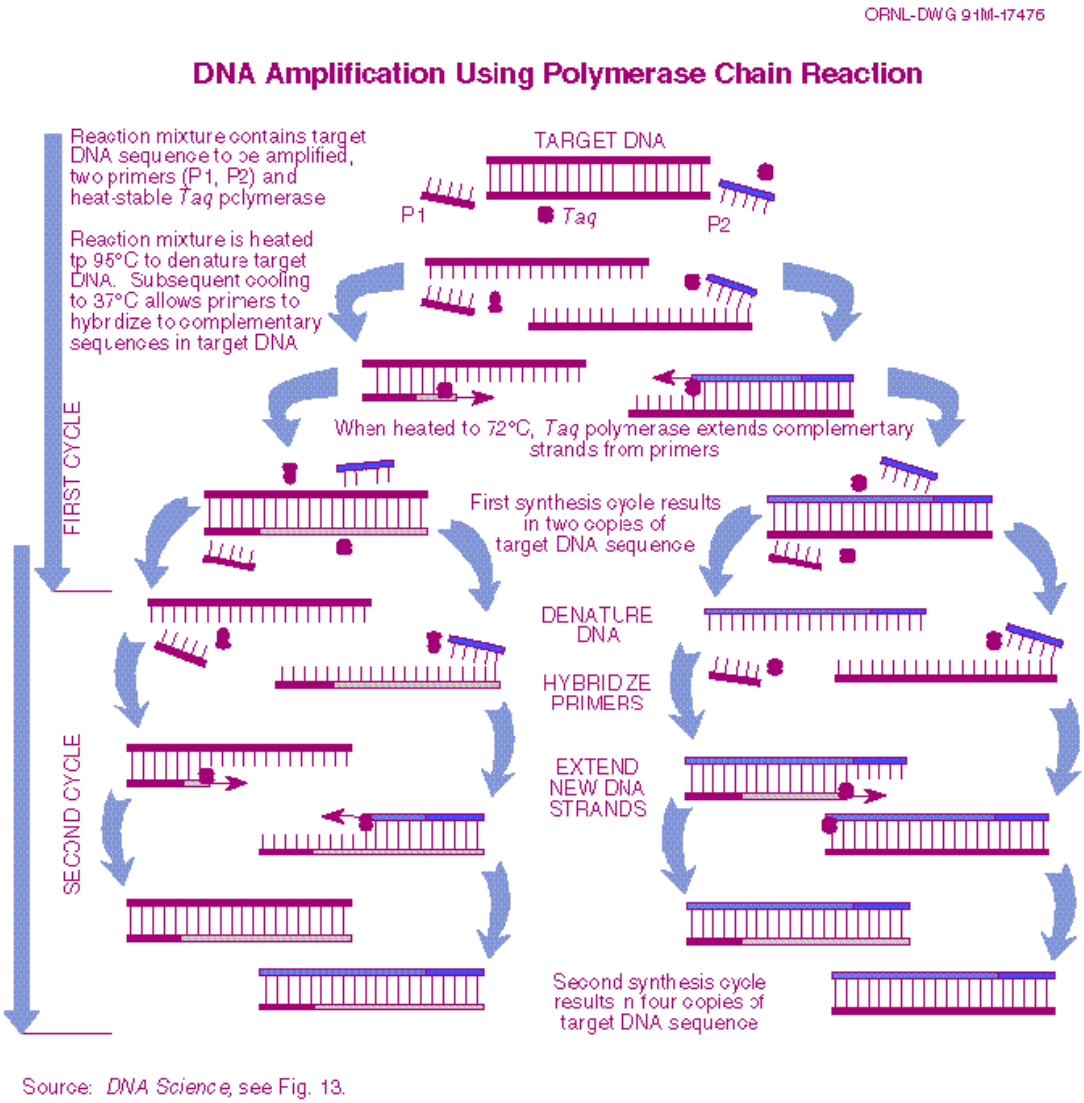
	Maritime Academy					
81	Cape Cod, MA Massachusetts Maritime Academy	-	7.3	1.5	0.90	Frozen
82	Cape Cod, MA Massachusetts Maritime Academy	A-D	9.7	7.7	4.70	Frozen
83	Cape Cod, MA Massachusetts Maritime Academy	-	5.7	2.5	1.50	Frozen
84	Cape Cod, MA Massachusetts Maritime Academy	A-B	9.2	4.2	1.90	Frozen
85	Cape Cod, MA Massachusetts Maritime Academy	-	15.0	3.2	1.00	Frozen
86	Cape Cod, MA Massachusetts Maritime Academy	A-H	18.2	5.8	8.82	Frozen
87	Cape Cod, MA Massachusetts Maritime Academy	A-F	22.2	9.4	6.70	Frozen
88	Cape Cod, MA Massachusetts Maritime Academy	A-K	31.5	6.1	11.11	Frozen
89	Cape Cod, MA Massachusetts Maritime Academy	A-J	16.2	3.6	9.00	Frozen
90	Cape Cod, MA Massachusetts Maritime Academy	A-F	29.1	5.0	7.10	Frozen
91	Cape Cod, MA Massachusetts Maritime Academy	A-D	21.8	6.0	3.90	Frozen
92	Cape Cod, MA Massachusetts Maritime Academy	A-B	15.4	4.8	2.52	Frozen
93	Cape Cod, MA Massachusetts Maritime Academy	A-C	23.0	4.7	3.04	Frozen
94	Cape Cod, MA Massachusetts Maritime Academy	-	14.9	3.6	1.40	Frozen
95	Cape Cod, MA Massachusetts Maritime Academy	A-B	9.2	4.7	2.20	Frozen
96	Cape Cod, MA Massachusetts Maritime Academy	-	10.7	4.7	1.45	Frozen
97	Cape Cod, MA Massachusetts Maritime Academy	-	9.5	4.4	1.30	Frozen
98	Cape Cod, MA Massachusetts Maritime Academy	-	14.1	4.1	1.50	Frozen

99	Cape Cod, MA Massachusetts Maritime Academy	-	10.5	3.5	0.89	Frozen
100	Cape Cod, MA Massachusetts Maritime Academy	A-B	21.0	3.0	2.03	Frozen
101	Cape Cod, MA Massachusetts Maritime Academy	A-B	19.0	4.5	2.27	Frozen
102	Cape Cod, MA Massachusetts Maritime Academy	-	2.0	7.0	0.58	Frozen
103	Cape Cod, MA Massachusetts Maritime Academy	A-D	23.0	5.5	4.03	Frozen
104	Cape Cod, MA Massachusetts Maritime Academy	-	12.3	2.5	0.69	Frozen
105	Cape Cod, MA Massachusetts Maritime Academy	A-E	13.0	9.0	5.22	Frozen
106	Cape Cod, MA Massachusetts Maritime Academy	A-I	25.0	8.5	9.45	Frozen

**Beaver Tail refers to Beaver Tail State Park, Jamestown, RI.*
**Fort Getty refers to Fort Getty State Park, Jamestown, RI.*

Table 2. Comparison of published protocols for isolating red algae genomic DNA.

DNA Isolation Protocols	Wattier et. al (2000).	Hong et. al. (1997)	Hu et. al. (2004)
Amount of Tissue	10 mg	100 mg	120 mg
Tissue Preservation Method	Silica Dried Tissue	Fresh Frozen Tissue	Freeze Dried Tissue
Extraction Method	Tris HCl, EDTA, NaCl	EDTA, LiCl, Sarcosyl, PVP, βME	Tris HCl, EDTA, NaCl, SDS, PVPP, βME
Precipitation Method	Isopropanol EtOH	3 M NaOAc 100% EtOH	Isopropanol
Color of Final Solution	Pink, Red, Green	Pink	Clear, Pink



http://www.bio.davidson.edu/Courses/Molbio/MolStudents/01jeklotz/pcr2.gif

Figure 1. Schematic diagram of the polymerase chain reaction.

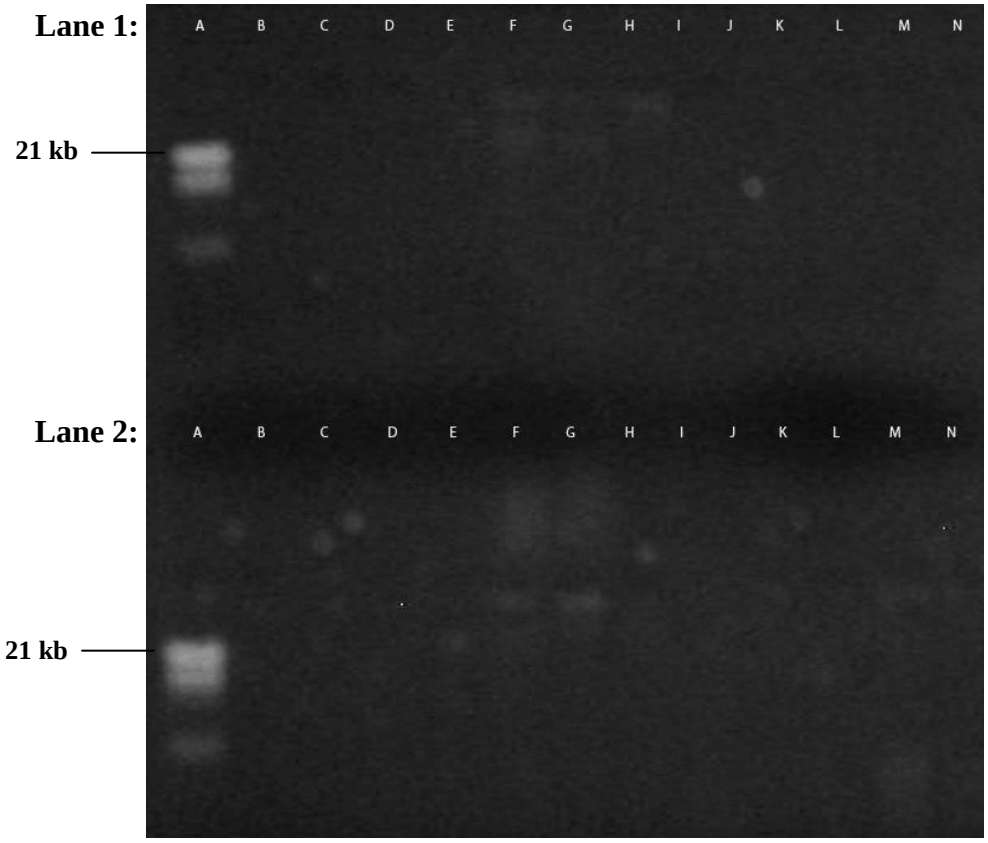


Figure 2. 0.6 % Agarose Gel Electrophoresis of isolated *G. turuturu* genomic DNA following published protocols and stained with ethidium bromide. Lane 1: 1 µl DNA (A) Commercial 21 kB Ladder (B) – (G) *G. turuturu* genomic DNA isolated via Wattier et. al (2000). (H) – (I) *G. turuturu* genomic DNA isolated via Qiagen DNeasy Plant Mini Kit. (J) – (N) Hong et. al (1997). Lane 2: 3 µl DNA (A) Commercial 21 kB Ladder (B) – (G) *G. turuturu* genomic DNA isolated via Wattier et. al (2000). (H) – (I) *G. turuturu* genomic DNA isolated via Qiagen DNeasy Plant Mini Kit. (J) – (N) Hong et. al (1997).

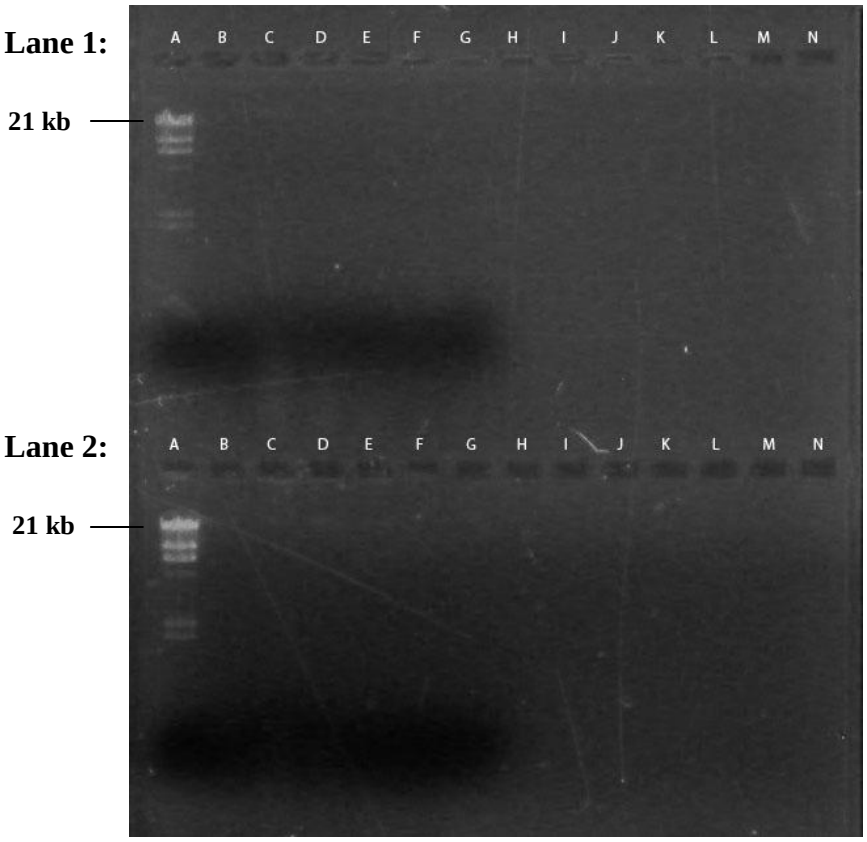


Figure 3. Repeated 0.6 % Agarose Gel Electrophoresis using isolated *G. turuturu* genomic DNA following published protocols from Figure 1, stained with ethidium bromide. Lane 1: 1 µl DNA (A) Commercial 21 kB Ladder (B) – (G) *G. turuturu* genomic DNA isolated via Wattier et. al (2000). (H) – (I) *G. turuturu* genomic DNA isolated via Qiagen DNeasy Plant Mini Kit. (J) – (N) Hong et. al (1997). Lane 2: 3 µl DNA (A) Commercial 21 kB Ladder (B) – (G) *G. turuturu* genomic DNA isolated via Wattier et. al (2000) . (H) – (I) *G. turuturu* genomic DNA isolated via Qiagen DNeasy Plant Mini Kit. (J) – (N) Hong et. al (1997).

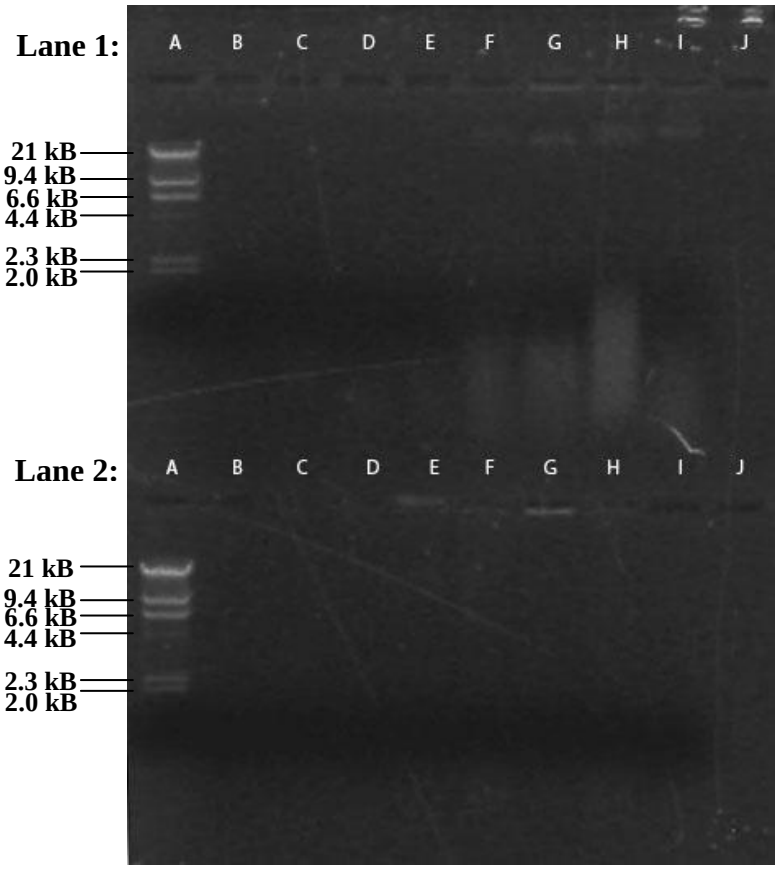


Figure 4. 0.6 % Agarose Gel Electrophoresis using isolated *G. turuturu* genomic DNA following repeat of published protocols and stained with ethidium bromide. Lane 1: 1 µl DNA (A) λ *Hind*III. (B) – (E) Qiagen DNeasy Plant Mini Kit. (F) – (I) *G. turuturu* genomic DNA isolated via Wattier et. al (2000). (J) Empty lane. Lane 2: 1 µl DNA. (A) λ *Hind*III. (B) – (I) *G. turuturu* genomic DNA isolated via Hu et. al (2004). (J) Empty Lane.

Table 3. Primer Sequences

Primer	Gene/Region of Amplification	Oligonucleotide Sequence (5' – 3')
F-7	RbcL	AACTCTGTAGAACGNACAAG
R-753	RbcL	GCTCTTTCATACATATCTTCC
F-577	RbcL	GTATATGAAGGTCTAAAAGGTGG
R-1371	RbcL	ATCTTTCCATAGATCTAAAGC

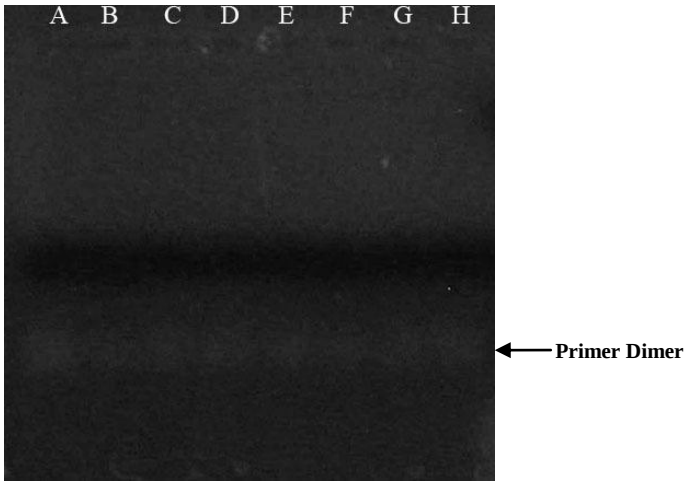


Figure 5. 1.2 % Agarose Gel Electrophoresis of PCR products and stained with ethidium bromide. (A) - (H) PCR products following Fredericq et. al. (2002) using genomic DNA isolated via Wattier et. al (2000) protocol as a template.

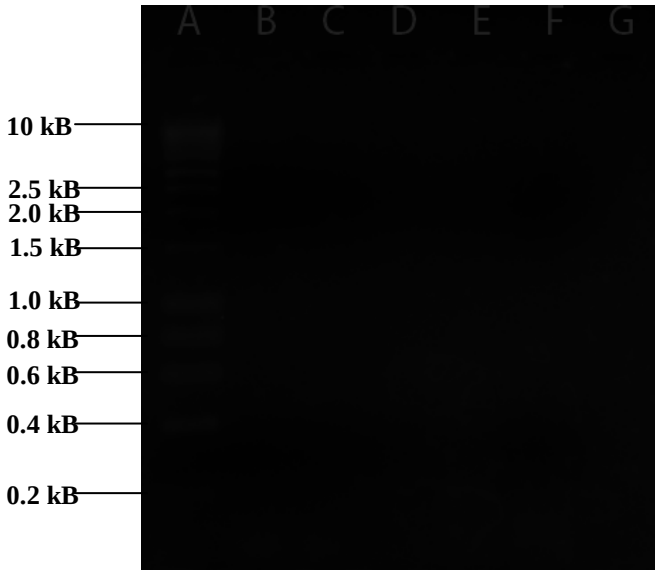


Figure 6. 1.2 % Agarose Gel Electrophoresis of PCR products varying the concentration of Genomic DNA template Isolated via Wattier et. al (2000) using conditions by Fredericq et. al. (2002). (A) Commercial 10 kB ladder (B) – (C) 1µl DNA template. (D)-(E) 3µl DNA template. (F)-(G) 1µl of 1:10 Dilution of DNA template.

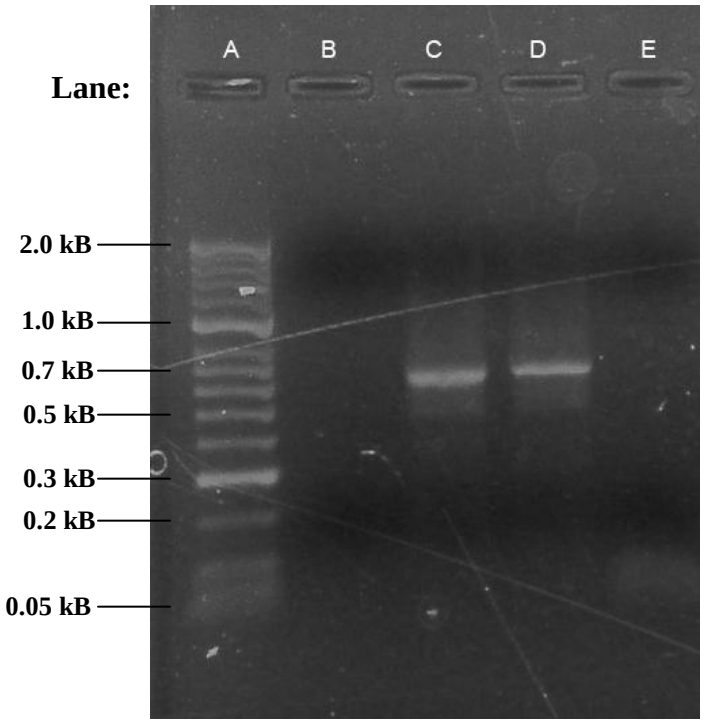


Figure 7. 1.2 % Agarose Gel Electrophoresis of positive control PCR using *Ulva* DNA and *Ulva* specific primers stained with ethidium bromide. (A) Commercial 2 kB ladder. (B) *G. turuturu* Genomic DNA isolated via Wattier et. al (2000) protocol and *Ulva* Genomic DNA. (C) Positive Control: *Ulva* Genomic DNA. (D) Positive Control: *Ulva* Genomic DNA (E) Negative Control.