Spring 5-2013

Intergenic Regions and Repeating Gene Sequences in the Dinoflagellate *Karenia brevis*

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by

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A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Biological Sciences

May 2013

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Introduction

Karenia brevis is a species of dinoflagellates that is responsible for "red tides" (more formally known as harmful algal blooms) found in the Gulf of Mexico. The blooms have adverse effects on humans and marine animals. *Karenia brevis* produces toxins that, in humans, can cause upper and lower respiratory problems as well as nausea and vomiting. For marine mammals, birds, fish, and other marine organisms, exposure to brevetoxins can result in morbidity and even death.

Even with the technology and resources we have today, scientist do not fully understand the mechanisms behind toxin production and bloom initiation. Because cellular processes may play a major role in *Karenia brevis* blooms, attempts have been made to understand *K. brevis* at the molecular level, but studies are still ongoing due to lack of understanding gene organization and expression. Thus, our goal was to help guide the reconstruction or sequencing of the genome. The purpose of my research was to find if there are multiple copies of certain gene sequences, and what variability exists between and within the genes. I attempted determine the copy number of a suspected multi-copy gene and if any sequence variability existed within the gene and the intergenic regions. Focus was placed on the gene for proliferating cell nuclear antigen (PCNA) whose known function is to aid in DNA replication and repair, because the PCNA is present in multiple copies and (Zhang et al. 2006) and the EST collection of *K. brevis* contained several PCNA single nucleotide polymorphisms (Lidie et al, 2005; McLean, unpublished data).

Techniques involved were DNA extraction, PCR (polymerase chain reaction) primer design, PCR amplification of genic and intergenic regions of the chromosomes, and PCR purification and ligation. Research stopped at this point due to time constraints. Southern blotting (to quantify the number of copies) was to be performed and then the PCRs products sent to another lab to be sequenced. Simple bioinformatic gene sequence analysis would have determine if there were base changes that could result in changes in amino acids. Intergenic sequence analysis would have determined if each copy of a gene was capable of being expressed or regulated. Results of further research could advance understanding of the genetics of *K. brevis* and dinoflagellates in general. Additionally, results could shape further predictions on mutation rates and variability in the dinoflagellate genome.

Literature Review

Dinoflagellate Ecology

Dinoflagellates are unicellular eukaryotes and some of the foremost primary producers in Earth's oceans and fresh waters (Hackett et al. 2004). Numerous species are armored to provide rigidity and protection; the armor is made of layer of flattened vesicle containing polysaccharides that prevent the cell from being distorted (Hackett et al. 2004). Other dinoflagellates exist as zooxanthellae, dinoflagellate symbionts within coral tissues that help corals construct coral reefs by providing up to 90% of the coral's energy (Berkelmans and Oppen 2006). However, over 60 species produce various toxins that can cause sickness and death in marine mammals, fish, sea birds, and humans (Lin 2008; Fleming et al. 2011). In recent years, interest in dinoflagellates has increased due to the media attention surrounding the discovery of a new suite of toxins, the azaspiracids, and of course, to Florida red tides that have caused/resulted in the deaths of Florida manatees and at least one major dolphin die-off (Fleming et al. 2011). Though research has increased within the past few decades, scientist still do not know much about factors responsible for blooms, or toxin productions, or the cellular processes involved.

Red Tide and Harmful Algal Blooms: Ecological and Economic Effects

A high biomass of algal cells can discolor the water to create a "red tide." (Figure 1). High biomass also disrupts marine ecosystem structure and function as it causes oxygen depletion, shading that blocks sunlight for submerged plants, and can be associated with concurrent toxin production and release (Anderson, Gilbert, and Burkholder 2002). These environmental problems often produce economic costs

(Anderson, Gilbert, Burkholder 2002; Hoagland et al. 2002). Economic hardships cost governments millions (Hoagland et al. 2002), because the governments must spend money monitoring waters and taking environmental samples along with administrating beach closures and clean ups. Businesses lose money when tourists choose other travel destinations and when fisheries close or fishermen move elsewhere to find their catch. Consuming contaminated fish or inhaling poisonous aerosols leads to illnesses such as diarrhetic shellfish poisoning (DSP), Ciguatera Fish Poisoning (CFP), paralytic shellfish poisoning (PSP), or neurotoxic shellfish poisoning (NSP). DNA damage, chromosomal aberrations, oxidative stress, and histamine release are only some of the suggested effects on the immune system. In numerous documented cases, brevetoxins irritate the throat and lead to respiratory problems such as difficulty breathing (Backer 2005). Fleming et al. (2011) believes that the histamine release could be a cause of respiratory symptoms. In cases of neurotoxic shellfish poisoning (NSP) trips to the emergency room or intensive care are required to prevent respiratory failure. Governments and individuals must spend money to cover medical cost (Hoagland et al. 2002).

Figure 1. Example of a red tide. (Woods Hole Oceanographic Institute 2007)

Factors that Contribute to Bloom Dynamics

Dinoflagellates are motile cells that can move up and down within the water column, and are sensitive to a variety of environmental stimuli including chemotaxis,

geotaxis and phototaxis (Hackett et al. 2004). Environmental conditions and diel vertical migration could play a role in bloom initiation and growth of *K. brevis* since cell division is phased to a photoperiod, and much of the population migrates to surface waters after cell division in order to maximize nutrient intake (Dolah et al. 2009). With motility, dinoflagellates are able to actively seek nutrients to maximize growth. In fact, Dolah et al (2009) reported that *K. brevis* would selectively swim through barriers based on nitrogen levels**.**

K. brevis generally blooms during upwelling events that occur where the seasonal thermocline and the edge of the continental shelf meet (Tester Steidinger 1997). Red tides caused by *K. brevis* are reported throughout the Gulf of Mexico from Florida all the way to the eastern coast of Mexico. Blooms usually begin offshore in the late summer and early fall and are then moved further inshore by winds and along-shore currents (Tester and Steidinger 1997). In the Gulf of Mexico, *K. brevis* is thought to be transported throughout its range by the Gulf Stream, the Florida Current, and the Gulf Loop current (Tester and Steidinger 1997). In addition, HABs of brevetoxin-producing organisms have been reported worldwide in places as far away as New Zealand and Scotland (Fleming et al. 2011) likely as a result of transport in the ballast water of commercial ships.

The major controversy surrounding red tides and harmful algal blooms is whether anthropogenic sources affect the frequency, range, and toxicity of these blooms. Nitrogen- and phosphorous-rich conditions increase toxin levels to three times the normal conditions (Dolah et al. 2009). Other factors suggested to affect blooms are the make-up of microbial communities. Certain proteobacteria are associated with presence of bloom

initiations and declines (Dolah et al. 2009) and could even be a factor in toxin production in some dinoflagellate species (Lin 2008).

Brevetoxins

The PKS genes are suspected to be the toxin producing genes (Fleming et al. 2011). They produce brevetoxins, which are lipid soluble, cyclic polyethers. (Figure 2) They cause the voltage gates in Na+ ion channels in the cell membrane to remain open so that there is an influx of sodium into the cell, causing cell shrinkage and eventually leading to cell death (Bortner and Cedlowski 2003). Brevetoxins are also tasteless, odorless and acid stable, rendering most other means of detection useless (Fleming et al. 2011).

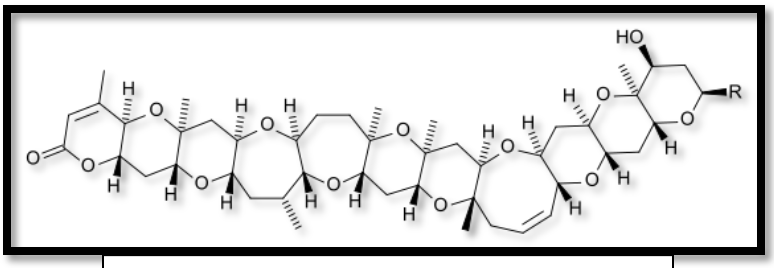

Figure 2. Bevetoxin. Toxin produced by species *Karenia brevis* (Matsuo et al. 2011)

Dinoflagellate Genome

Dinoflagellate chromosomes are uniform in size and have a banding pattern (birefringent) that indicates the DNA exists in a liquid crystalline state (Hackett et al. 2005). Though the chromosomes are also in a permanently condensed state (except during DNA replication), most dinoflagellates lack nucleosomes that are typically involved in DNA packaging in all other eukaryotes (Hackett et al. 2005; Bachvaroff and Place 2008; Dolah et al. 2009; Lin 2011). Until recently, most of the scientific community believed that dinoflagellates lacked histones. However, Lin (2011) reports that scientists have found that cells contain four core nucleosomal histones along with

histone-like proteins (HLPs). The protein to DNA ratio is 1:10; where as it is usually a 1:1 ratio in other eukaryotes (Hackett et al. 2005).

Low presence of histone proteins plus the lack of TATA boxes and other transcriptional regulators such a downstream polyadenylation sites can explain the fact that only 10 to 27% of mRNA is regulated transcriptionally (Dolah et al. 2009). In *K. brevis* 33% of genes studied were found to be involved in the post-transcriptional processing of RNA under light and dark conditions (Dolah et al. 2009). Bacharoff and Place (2009) believe a phenomenon called *trans*-splicing is a major regulating mechanism in mRNA translation and might be involved in gene expression in addition to transcription (Lin 2011). The splice leader (SL) is found in nuclear-encoded protein-coding genes and is a highly conserved sequence of 22 nucleotides that is added to the 5' end to form mature mRNA (Lin 2011). The splice leader's role is trans-splicing is to convert polycistronic primary mRNA transcripts into 5′-capped monocistronic individual strands of mRNA. Additionally, spliced leader t*rans*-splicing is believed in increase the efficiency of translation (Zhang and Lin 2009).

Dinoflagellates have a concentration of DNA that is much higher than most other eukaryotes and as much as 80 times higher than the human genome (Lin 2011). *Karenia brevis*, in particular has a genome 30 times larger the human genome (Van Dolah et al. 2009). The reason or purpose behind such high DNA content is unknown, but one hypothesis is that it may be related to the high copy number of some genes found in tandem repeat (Bachvaroff and Place 2008). This simply means that individual copies of the same gene are present numerous times. In a study done by Bachvaroff and Place (2008), it was noted that synonymous substitutions were found between different copies

of the same gene. With multiple copies of a gene present in the genome, it is reasonable to expect that the exact nucleotide sequence of the gene may vary among these copies. These variations, a base change here and there, are called single nucleotide polymorphism (SNPs). If each gene is subtly different in nucleotide sequence, researchers would expect that this would result in amino acid sequence differences in the coded proteins. The cells would produce multiple, slightly altered versions of the same protein, each version with an altered function in specificity, timing, or kinetics (Bachvaroff and Place 2008). For example, one protein might function during daylight and the other might function at night.

Despite high copy number, most of the genome is composed of non-coding DNA, or intergenic regions (Lin 2011). An intergenic region, also termed a "non-coding region" of DNA because it does not code for proteins, is basically a DNA sequence between genes. An intergenic region is not to be confused with an intragenic region, or intron, which is a non-coding region within a gene. Short intergenic spaces link genes in tandem repeats, but little is known about the function or sequence diversity of dinoflagellate intergenic regions (Bachvaroff and Place 2008). Some species contain repetitive sequences in these intergenic regions (Lin 2011). Repetitive coding and non-coding DNA and a large genome have made it difficult to sequence or analyze genomes of dinoflagellates; in fact, no one at the present time has sequenced an entire dinoflagellate genome (Lin 2011).

Summary

Not much is known about the mechanisms of gene regulation and how dinoflagellate genomes are organized, but curious features of dinoflagellates make it obvious that gene regulation in dinoflagellates differs from other eukaryotes (Bachvaroff and Place 2008; Lin 2011). Results of further experiments can aid scientists in understanding the genetic processes of *K. brevis* and dinoflagellates in general**.** Specifically, studying tandem repeats of a gene sequence and how many copies of the same gene sequence exists, and what variability exists between and within the genes, could advance our knowledge in the function of these regions and of repetitions within dinoflagellate genome. Understanding the dinoflagellate genome and genomic processes could lead scientist to finding contributing factors to toxin production or bloom formation.

Methodology

DNA Extraction

DNA extraction from *K. brevis* was accomplished following manufacturer's instructions in the DNeasy ® Blood and Tissue Handbook using spin-column protocol (2006). Briefly, cell samples were placed in a microcentrifuge tube with 200 µL of buffer ATL to lyse the cells. RNase A (4 µL) was added to ensure RNA-free genomic DNA is obtained. The mixture was vortexed for 15 seconds mixed with 200 µl Buffer AL and then mixed with 200 µL of ethanol to precipitate the DNA out of solution. Mixture was centrifuged in a tube with spin column for one minute and the flow-through discarded. Columns were washed with 500 µL of Buffer AW2 for 1 minute and then dried by spinning for an additional 3 minutes. Spin columns were removed and placed in a new tube where 200 µL Buffer AE will be added. After a one minute incubation, the columns were spun for one minute to capture the eluted DNA in the flow-through fraction.

A crude DNA extraction was also performed by simply collecting a *K. brevis* cells then spinning the cells down in a large centrifuge at 1,500 rpm using a Rotanta 460 Hettich Zentrifugen centrifuge. Pellet was collected then placed in PCR tube.

Primer Design and PCR Amplification

First MacVector was used to align existing DNA sequences and determine the best, i.e. the most conserved, regions against which to construct PCR primers for the intragenic and intergenic regions of PCNA. Such primers were designed using IDT (Integrated DNA Technologies, Inc.) online tools. At the IDT website, the sequence for

the proliferating cell nuclear antigen was entered along with relevant reaction conditions and parameters. The software calculates and lists a number of primer pairs that could be used. Primers were chosen based on G/C content, primer length, product size, the melting temperature of the primer, and the primer's ability to self complement (Bachvaroff and Place 2009). The primers used are listed in Table 1. The two master mix recipes are displayed in Table 2, and the mixture ratios of all PCR components are shown in Table 3. During PCR amplification, DNA was denatured at 94°C. Primers were annealed at a specific temperature for the designed primers which was 55°C, and extension of the primers occurred at 72°C. (Table 4) The number of cycles was determined empirically. Touchdown and Nested PCRs were also performed for DNA amplification. Touchdown PCR was used with aims to reduce non-specific background by starting at a high annealing temperature and then gradually lowering annealing temperature. (Table 4) The higher temperatures give greater specificity for primer binding, then moves to lower temperatures for more efficient amplifications from products formed in earlier cycles. Nested PCR was attempted to amplify the DNA in the case that an extremely low amount of DNA was present in previous reactions. (Table 4). All PCRs were performed using an Eppendorf thermocycler. *Taq* and buffers for PCR were prepared in the laboratory (Table 5) All dNTPs were prepared and shipped from New England Biolabs.

Primer set label	Forward primers	Reverse primers
P1	Intragenic right	Intragenic left
P2	Intergenic left	Intergenic right
P3	ADP ribo	ADB ribo
P4	F63mod	R635sq
P5	F635sq	R1630
P6	F635sq	R1411sq

P7	F635sq	R2077sq
P8	F1586	R2077sq
P9	F63mod	R1630
P10	F63mod	R1411sq

Table 1. Primer sets used for PCR.

	Master Mix 1 (M1)	Master Mix 2 (M2)
dH20	16.6 µL	19.05 µL
10x PCR buffer (Table 5)	2.5 µL	2.5 µL
25µM dNTPs	0.4 µL	0.2 µL
55 or 10 ng/µL DNA	2 µL	2 µL
Taq	0.5 µL	0.25 µL
10µM Primer 1	1 µL/tube	0.5 µL/tube
10µM Primer 2	1 µL/tube	0.5 µL/tube
Final volume	24 µL	24.2 µL

Table 2. Master mixes used for PCR.

DNA	2.5 µL
10µM Primer 1	1 µL
10µM Primer 2	1 µL
H20	10 µL
2x Master Mix	12.5 µL
Final volume	25 µL/tube

Table 3. Standard protocol master mix for nested PCR.

	C1	C2 (Touchdown)	C3 (Nested PCR standard protocol)
Denature	94°C	94°C	94°C
Anneal	55°C	64, 61, 58°C	46, 63°C
Extension	72°C	72°C	72°C

Table 4. PCR temperature conditions.

	for 10mL	for 100mL
500mM Tris-HCl, pH 9.2 (25°C)	5mL of 1M	50ml of 1M
160mM $(NH_4)_2SO_4$	0.2112g	2.112g
22.5mM $MgCl_2$	225ul of 1M	2.25mL of 1M
20% (v/v) DMSO	2mL	20mL
1% (v/v) Tween20	100ul	1mL

Table 5. PCR buffer

PCR Purification and Ligation and Transformation

After running the PCR products on a gel, the appropriate band representing amplification of the intragenic region needed to be cut from the gel and then the DNA extracted and purified. This was accomplished following manufacturer's instructions in the QIAquick ® Spin Handbook using QIAquick Gel Extraction Kit Protocol (2012). The gel slice was placed in a tube then weighed. Three volumes of Buffer QG to one volume of the gel was added to tube. Gel and buffer were incubated for 10 minutes at 50°C until the gel dissolved. During this time the tube was vortexed every 2-3 minutes. One gel volume of isopropanol was mixed into the sample, then placed into a 2 mL collection tube and centrifuged for one minute. Buffer PE (750 µL) was added to the collection tube and centrifuge for one minute to wash. Flow-through was discarded and centrifuged for another minute. Next, the spin column was placed in a new tube and 30 µL of Buffer EB for the elution step. After allowing the column to sit for one minute, it was centrifuged and elution was collected.

Before ligation, elute was mixed with equal parts isopropanol and allowed to sit overnight. The next day the collection tube was centrifuged for 30 minutes at 4°C. All liquid was removed from tube with pellet. One microliter of 70% ethanol was added to pellet and tube was centrifuged for another 30 minutes at 4°C. Collection tube was then allowed to air dry for 10 to 15 minutes.

Ligation was performed following the Technical Manual: pGEM®-T and PGEM®-T Easy Vector Systems protocol (2010). Mixed, in order, were 10 µL 2x rapid ligation buffer, 8µL PCR product, 1 µL T4 DNA ligase (3 Weiss units/µL), and 1µL

vector (50ng). Reaction was mixed by gently pipetting and was allowed to sit at room temperature for at least one hour.

Five microliters of the plasmid DNA was added to a tube of thawed competent cells (100μL) on ice, and then mixed gently. DNA and cells were allowed to incubate for 5 minutes and then 50 μL was spread on each of two LB agar plates. Plates were incubated at 37°C for two days.

Results

Two sets of primers (Intragenic primers [P1] and intergenic primers [P2]) along with master mix 1 under C1 temperature conditions were used to amplify the PCNA gene and a surrounding intergenic region. (Table 1, Table 2, Table 4). After running a gel for both reactions, the first results showed nothing except for one "long blur". (Figure 3)

Note: the DNA ladder is the same for this and all subsequent gels.

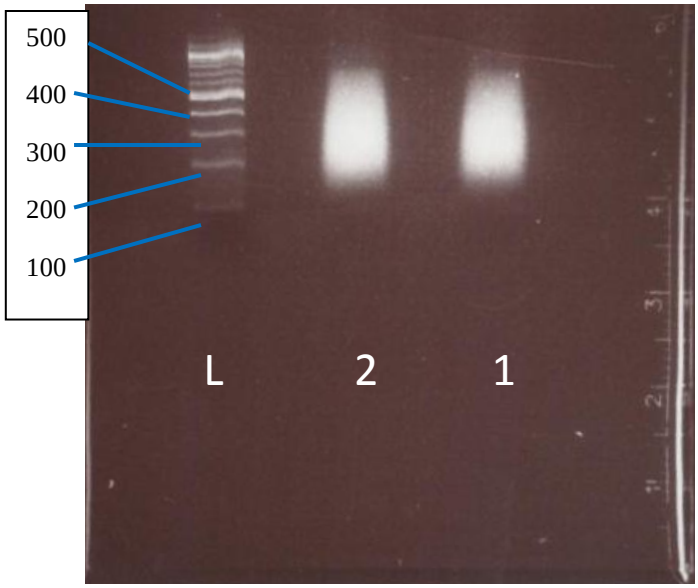

Figure 3. PCR to amplify intra- and intergenic region of PCNA.
L is ladder control, 2 is a PCR using primer set 2, and 1 is a PCR using primer set 1.

A second PCR and gel yielded the same results.

Next touchdown PCR reactions with master mix 1were used. (Table 2). Denature and extension temperatures remained the same, at 94°C and 72°C respectively. The melting temperatures started at 64°C, then lowered to 61°C, and had a final melting temperature of 58°C. (Table 4) The results were similar to the first two PCRs (negative results). (Figure 4)

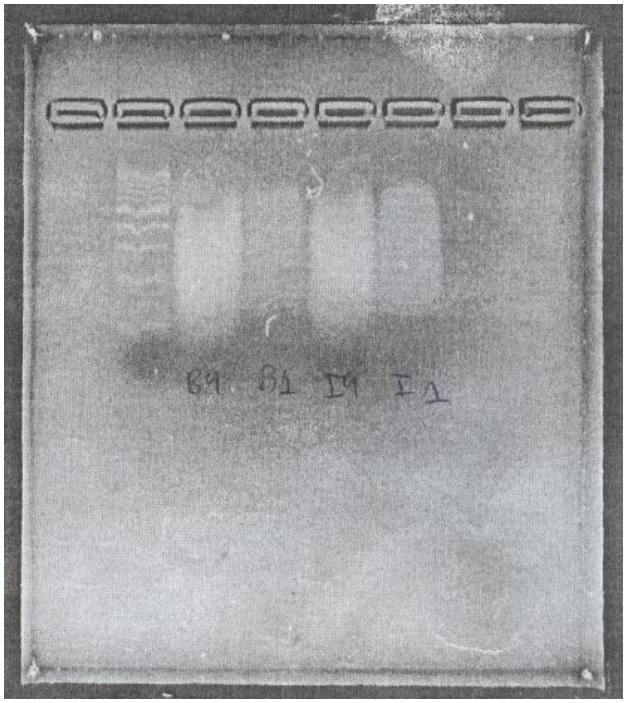

Figure 4. Touch-down PCR to amplify intra- and intergenic region of PCNA.
B refers to amplicons of the intergenic region, and I refers to amplicons of the intragenic region. 9 refers to 9 µL of PCR product mixed with 1 µL gel star, and 1 refers to 1 µL of PCR product mixed with 1 µL gel star.)

A fourth attempt and fifth attempt was made to obtain results using ADP ribo forward and reverse primers as a control with *K. brevis* DNA, but ended with identical results. (Figure 5) Though, a band was present for the one set of the ADP ribo primers (primer set 3)

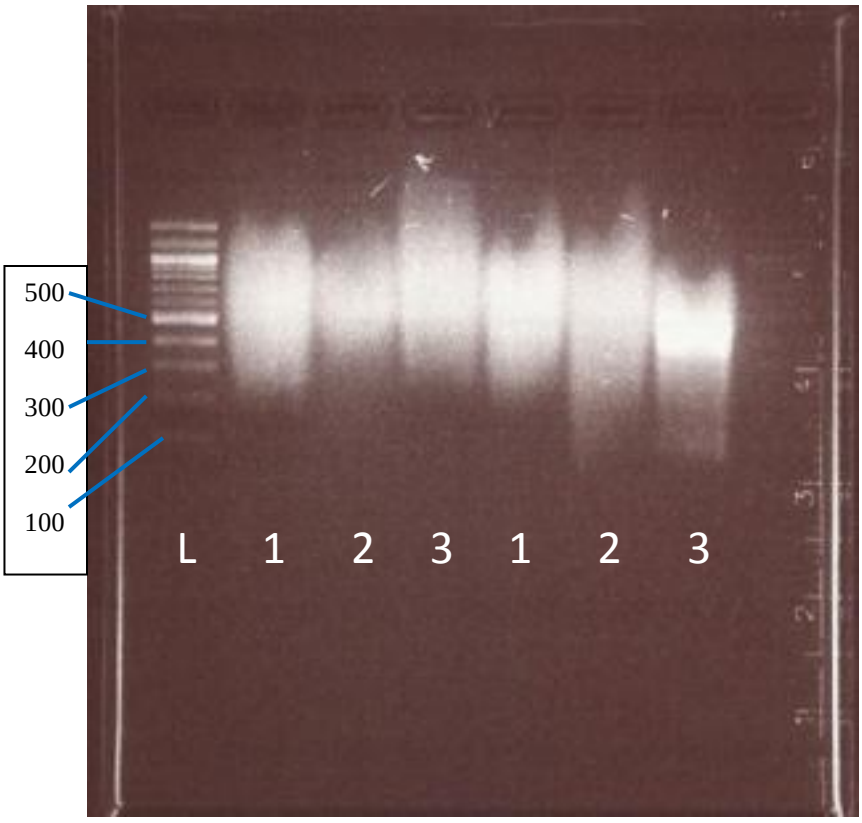

Figure 5. Fifth attempt with touchdown PCR products with intra-, inter-, and ADP ribo primers. L is the ladder. The numbers refer to the primer set used (Table 1 in the respective PCR.

For my sixth endeavor, I performed a crude DNA extraction of *K. brevis*. Four different amounts of DNA from extraction were used in PCRs (0.5 µL, 1 µL, 2 µL, and 2.5 µL) for the intragenic, intergenic, and the control ADP ribo primers with master mix 1. (Table 1, Table 2) Each PCR product yielded only "one long blur" after running the gel, as happened for previous attempts at amplifying the PCNA gene. (Figure 6)

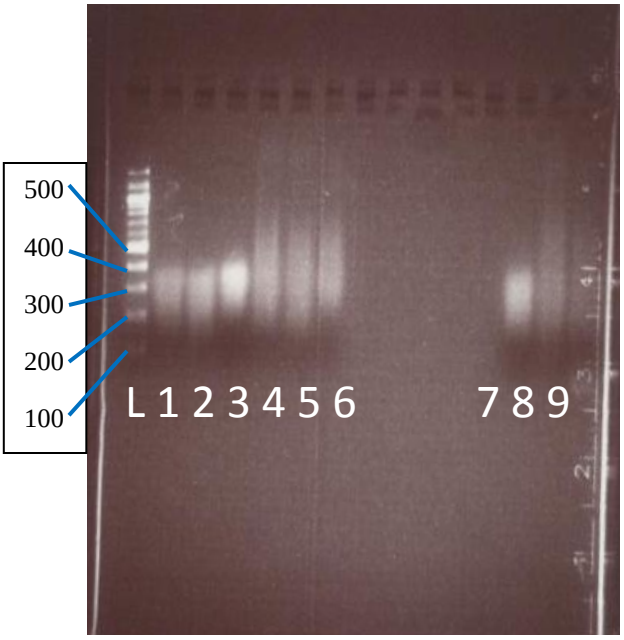

Figure 6. Sixth attempt with PCR comparing crude DNA extractions to purified DNA.
L is a ladder control. Lanes 1-3 are the results of PCRs using purified DNA. Lanes 4-9 are the results of PCRs using crudely extracted DNA. Primer set 1 was used for reactions in lanes 1, 5, and 8. Primer set 2 was used for reactions in lanes 2, 6, and 9. Primer set 3 was used for reactions in lanes 3, 4, and 7. Lanes 1-3 used 2 µL of DNA per reaction. In lanes 7-9, increasing amounts of DNA were used in the reactions: lane 7-0.5 µL, lane 8 - 1 µL, and lane 9 - 2.5 µL.

Several trials were run with primers that had previously shown positive results. Two sets of seven pairs of large subunit (LSU) ribosomal RNA *K. brevis* primers that have successfully amplified products in previous reactions were utilized for positive controls with master mix 2. (Table 2) Both sets used the same primers and DNA, but each set used different dNTPs, *Taq*, water and buffer for PCRs. All seven primers pairs had been known to work in other experiments. Only one of the seven primer sets showed any bands in Set 1, and only three of the seven showed any bands in Set 2. (Figure 7)

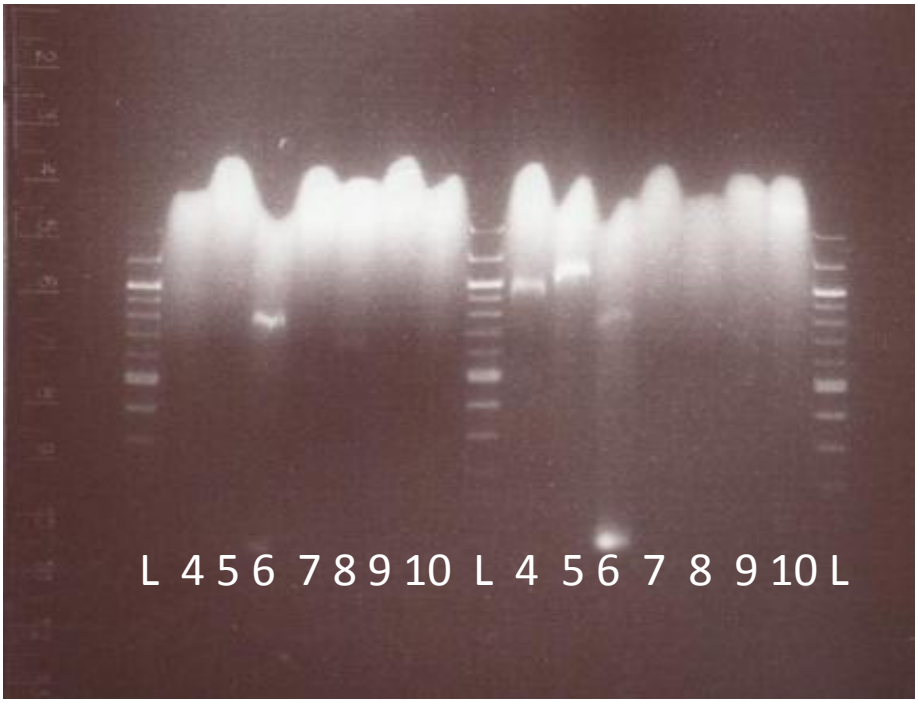

Figure 7. Seventh attempt using various LSU primers for control PCRs.
L is a ladder control. The numbers refer to the primer set used (Table 1) in the respective PCR.

On the eighth try, the *Taq* and buffer were changed to find if results would improve. Results did not improve, and once again only three primer pairs had positive results.

The first four primer sets (P4 through P7) with master mix 1 were utilized for a ninth PCR, this time with newly extracted DNA (10ng/µL). (Table 1, Table 2) For a third time only the first three primer pairs (P4-P6) revealed any bands.

A standard protocol using nested PCR with tick DNA and primers was tried in a tenth PCR set. Positive control shows a band above the 500 ladder mark along with primer dimers in wells 5, and 9-11. (Figure 8)

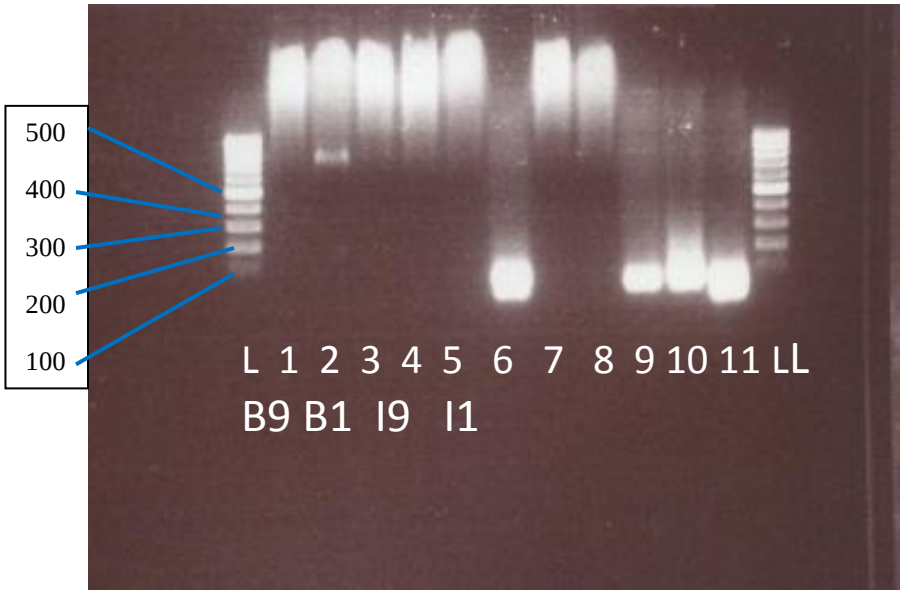

Figure 8. Gel using nested PCR products.
L is a ladder control. Lane 1 is a PCR control that only contained water. Lanes 2, 9-11 used tick positive control DNA. Lane 3 used sample 3 tick DNA. Lanes 4 and 6 used sample 1 tick DNA. Lane 5 used sample 2 tick DNA. Lanes 7 and 8 used *K. brevis* DNA. Lanes 1-5 and 7 used protocol primers. Lanes 6 and 11 used primer set 6 (Table 1). Lanes 9 and 10 used primer sets 4 and 5, respectively (Table 1).

For the eleventh attempt only wells that used PCR products that did not contain the standard protocol master mix had any bands. Sample 1 DNA with standard protocol primers, Sample 1DNA with P5, #1 DNA with P7, and #3 DNA with standard protocol primers had band above the 500 ladder mark.

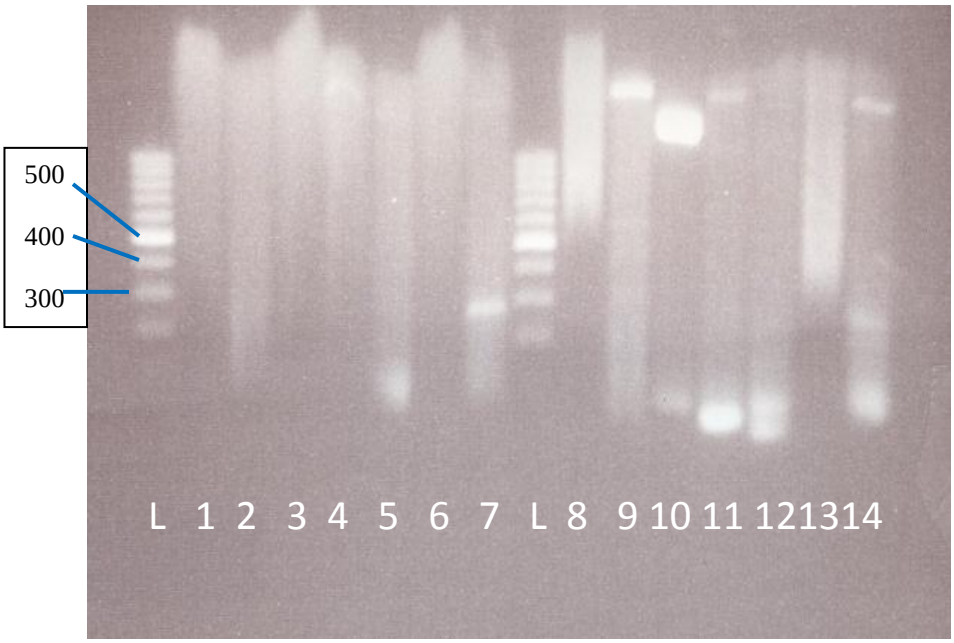

Figure 9. PCR products using two different master mixes.
L is a ladder control. The first seven wells contain PCR products using the standard protocol master mix. The second set of seven wells contain master mix 2. Lanes 1-7 and 8-14, each respectively contain in order: Sample 1 tick DNA with standard protocol primers, Sample 1 tick DNA with P5 primers, Sample 1 tick DNA with P7 primers, *K. brevis* DNA with P5 primers, *K. brevis* DNA with P7 primers, Sample 2 tick DNA with standard protocol primers, and Sample 3 tick DNA with standard protocol primers.

For the final attempt in obtaining band for the intragenic and intergenic regions of *K. brevis* original master mix was used. *Karenia brevis* with intra- and intergenic primers used touchdown PCR, while *K. brevis* with P5 and P7 primers used PCR conditions set forth by the standard protocol for nested PCR. (Table 4) A definite band for intragenic region of PCNA appeared on the gel. Next I ran the same PCR products from lane 3 on a low melt gel and cut out band for cloning.

Figure 10. PCR final products and master mix 2.
L is a ladder control. All lanes contain *K. brevis* DNA with the following primer sets: 5, 7, 1, or 2 primers.

The final PCRs involved the intergenic primers. Figure 9 below shows PCR products after four different final annealing temperatures for touchdown PCR conditions.

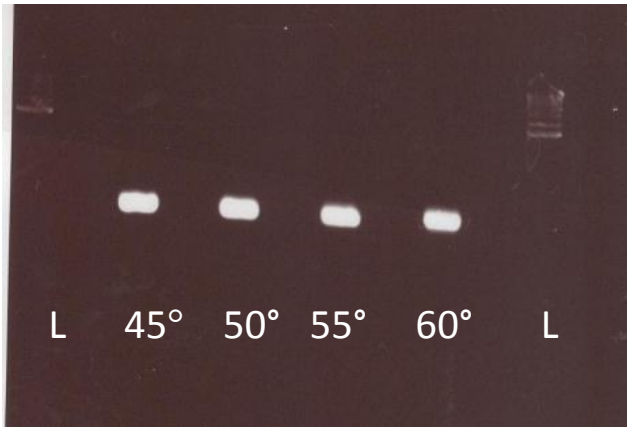

Figure 11. PCR using intergenic primer set 2 under different annealing temperatures.
L is a ladder control. The respective annealing temperature (in degrees Celsius) for each reaction is shown under the appropriate lane.

The plug of agarose containing the isolated PCR product (intragenic PCNA sequences) was melted, and the liberated amplicons were captured on special spin columns designed to purify the amplicons. After elution from the columns, a Nanodrop was used to confirm the presence of the DNA and to quantitate it. DNA concentration was determined to be 1.2 ng/μL. Ligation and transformation was then performed with plasmid DNA and competent cells, but no colonies appeared on LB agar plates after a total of 48 hours of incubation at 37°C.

Discussion and Conclusion

A large genome size, repeating DNA sequences, and permanently condensed chromosomes that lack nucleosomes are but a few of features of dinoflagellates that are likely to make gene regulation in dinoflagellates different from other eukaryotes (Bachvaroff and Place 2008; Lin 2011). EST sequencing has shown that the exact nucleotide sequence of a gene may vary indicating the presence of multiple copies of a single gene (Bacharoff and Place 2008; Lidie et al, 2005; McLean, unpublished data). Attempts were made to determine variability and copy number of the intragenic and intergenic regions of the proliferating cell nuclear antigen (PCNA) in *Karenia brevis*, a red-tide producing dinoflagellate.

As stated in the previous section, the results of the first PCR yielded nothing but one "long blur" per PCR product. This indicated that the intergenic region could have many fragments of many different sizes, but it did not explain why there were no positive results for the intragenic region. Touchdown PCR (Figure 4) was attempted for following reactions with the aims of reducing non-specific background by starting at a high temperature and then gradually lowering the annealing temperature. The high temperature would, theoretically, give greater specificity for primer binding then move to lower temperatures for more efficient amplification from products formed in earlier cycles (Korbie and Mattick 2008).

Following more negative results, I used ADP ribo primers for a positive control in fourth and fifth PCRs in an attempt to provide a baseline result for comparison against previous results. Once again, negative results followed. Only one of the two wells with

ADP ribo primers revealed a band, thus not providing an entirely reliable baseline. (Figure 5)

Because no positive results were being attained a crude, or dirty, extraction method was performed in attempt to quickly and efficiently extract DNA. In PCR with crude DNA it is important to understand that omitting the extra extraction steps in purifying the DNA could weaken the sensitivity for the target DNA sequence (Zhang, Kermekchiev, and Barnes 2010). In either case, the dirty extraction method did not yield positive results. (Figure 6)

As only negative results were being obtained, several trials were done to rule out contaminated or nonfunctional "ingredients" in the PCRs. Primers (that had previously been successful), dNTPs, *Taq*, water, buffer, and DNA were all changed throughout the sixth to the ninth attempts at PCR, but no changes were seen after running PCR products on a gel.

Nested PCR with similar goals as trying touchdown PCR, i.e. increase specificity by reducing a nonspecific background, was also attempted. (Figure 8) With a nested PCR protocol, two primer sets are used. The first pair of primers amplify similarly to standard PCR, but the second set of primers is designed to bind and amplify within the first PCR product, producing a smaller amplicon. The idea is that a too-large or wrong product was amplified the first time, the second set of primers would corrects this mistake (Puig et al. 1994).

For the eleventh attempt only wells that used PCR products that did not contain the standard protocol master mix had any bands. (Figure 9) This is most likely due to

the fact that the standard protocol master mix was not immediately put on ice; rather it was removed from the freezer for an hour, possible inactivating the *Taq*.

After so many attempts with negative results, even using a control, a standard protocol, and other primers that previously had been successful, the integrity of the thermocycler and gel box was put into question (excluding results from 11th trial with possibly compromised *Taq*). However, attempts to PCR and detect a band in the gel was performed one more time. This time, a definite band appeared on gel. (Figure 10) The sample was rerun on a low melting agarose gel, the band was cut out of the gel, and the liberated amplicon was purified on a spin column. During the elution process a mistake was made. Buffer EB for the elution step was accidentally added prematurely to the spin column and at a greater amount than intended (750μL v 30μL). However, the Nanodrop confirmed that this mistake did not destroy or dilute product beyond recovery; and DNA concentration was determined to be 1.2ng/μL. Ligation and transformation was then performed with plasmid DNA and competent cells, but after two days of incubation the agar plates showed no sign of cell growth. The project was terminated due to time constraint.

Though this experiment (to verify variability and copy number of the intragenic and intergenic regions of the proliferating cell nuclear antigen (PCNA)) did yield many conclusive results, this area of research is worth continuing. The research of Zhang et al. (2006) has shown that PCNA is present in multiple copies and there is confirmation in EST collections that *K. brevis* contains several PCNA SNP's (McLean unpublished data, Lidie et al, 2005). Other scientists have found synonymous substitutions between different copies of the same gene in tandem repeats. If each gene is subtly different in

nucleotide sequence, it is possible that the amino acid sequence would differ in the coded proteins. Thus, dinoflagellate cells could produce multiple, slightly altered versions of the same protein, each version with an altered function in specificity, timing, or kinetics (Bachvaroff and Place 2008).

Additional focus on the cellular processes of the dinoflagellate, such as gene regulation, evolution, toxin production, and bloom dynamics could lead to better understanding of harmful algal blooms and their detrimental effects on terrestrial and marine organisms.

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Acknowledgments

This thesis would not have been possible without the support of many people. I would like to express my deepest gratitude to Dr. McLean; without his patience and support and guidance, I could have never have accomplished this project. Special thanks also to fellow students and researchers: David Jayroe, Scott Anglin, Helen Namataka, and Joseph Keim for giving their invaluable assistance. I would also like to convey my thanks to the University of Southern Mississippi's College of Science and Technology for providing research facilities and to the Honors College for making undergraduate research like this possible.