

Spring 5-2015

Tracking Plastid Gene Migration in *Karenia brevis*

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The University of Southern Mississippi

Tracking Plastid Gene Migration in *Karenia brevis*

by

Kelly Scott

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 2015

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Abstract

Karenia brevis is a marine dinoflagellate responsible for the harmful algal blooms (also known as red tides) in the Gulf of Mexico. *K. brevis* expresses antisense (AS) RNAs, each of which has a complementary region to the messenger RNA (mRNA) of a variety of genes. In dinoflagellates, many plastid (and mitochondrial) genes have migrated to the nuclear genome. It is unknown whether chloroplast genes, such as photosystem – D2, have migrated in *K. brevis*. It is also unknown where the gene that expresses the AS RNA for photosystem D2 resides. The protein-coding gene and the AS RNA-expressing gene could both reside in the chloroplast, both in the nucleus, or in some split combination between the two genomes. Primers designed from photosystem D2 ESTs were used in a series of RACE reactions to capture the unique regions of both photosystem – D2 AS RNA and mRNA. Gel imaging showed a distinct band for the unique 5' end of the mRNA. Sequencing of this band will allow for the design of a probe to determine which genome houses the photosystem – D2 mRNA. This work can be furthered to compile known locations for both the mRNA and AS RNA of both chloroplast and mitochondrial genes of *K. brevis*.

Key terms: *Karenia brevis*, harmful algal blooms, AS RNA, chloroplast genes, photosystem D2, RACE, sequencing

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List of Abbreviations

Harmful algal bloom (HAB)

Antisense RNA (AS RNA)

Messenger RNA (mRNA)

Complementary DNA (cDNA)

Expressed Sequence Tag (EST)

Universal Primer Mix (UPM)

Gene Specific Primer (GSP)

Chapter 1: Problem Statement

Karenia brevis is a marine dinoflagellate responsible for the harmful algal blooms (also known as red tides) in the Gulf of Mexico. These blooms have negative impacts on the ecosystem, the economy, and human health. Investigation of the molecular mechanisms involved in gene regulation of *K. brevis* may lead to further understanding of harmful algal bloom (HAB) dynamics. *K. brevis* expresses antisense (AS) RNAs, each of which has a complementary region to the messenger RNA (mRNA) of a variety of genes. These AS RNAs may play a role in the post-transcriptional regulation of gene expression in *K. brevis* cells. Before studying the effects of a potential interaction between the AS RNA and its complimentary mRNA, it would be beneficial to determine the genomic location of the separate genes coding for both strands. In dinoflagellates, many plastid (and mitochondrial) genes have migrated to the nuclear genome, but the extent of migration varies between species. It is unknown whether chloroplast genes have migrated in *K. brevis*. It is also unknown where the gene that expresses the corresponding AS RNA for the gene resides. It is believed that separate genes code for the mRNA and the AS RNA (McLean, unpublished results), and it may be the case that the two genes do not reside in the same genome. The protein-coding gene and the AS RNA-expressing gene could both be in the chloroplast, both in the nucleus, or in some split combination between the two genomes.

This study aimed to sequence the unique ends of both mRNA and AS RNA strands corresponding to the Photosystem D2 gene of *K. brevis*. Isolating and amplifying the 3' and 5' ends of the complementary DNA (cDNA) obtained from reverse transcription of both the mRNA and the AS RNA provided the template from which

probes shall be designed. Based on the fact that the majority of the sequence in the middle of the mRNA (Figure 1 – yellow) and the AS RNA (Figure 1 – green) are complementary, a probe complementary to any sequence in the overlapping region would have two potential targets in the DNA. Non-specific binding would confound an interpretation of the probing experiment if the two genes were in different genomes. To target only the mRNA- or AS RNA-producing genes, it is necessary to find unique sequence that is not complementary to the other RNA molecules, i.e. the ends. Generation of probes based on the unique ends will ensure that the mRNA-specific probe only binds to the mRNA-producing gene and the AS-specific probe to the AS RNA-producing gene and vice versa. Probing of both cytoplasmic and whole cell extracts will reveal in which genome each RNA type resides. RNA types present in only whole cell extracts probably reside in the nucleus, while RNA types present in both extracts likely reside in the chloroplast genome.

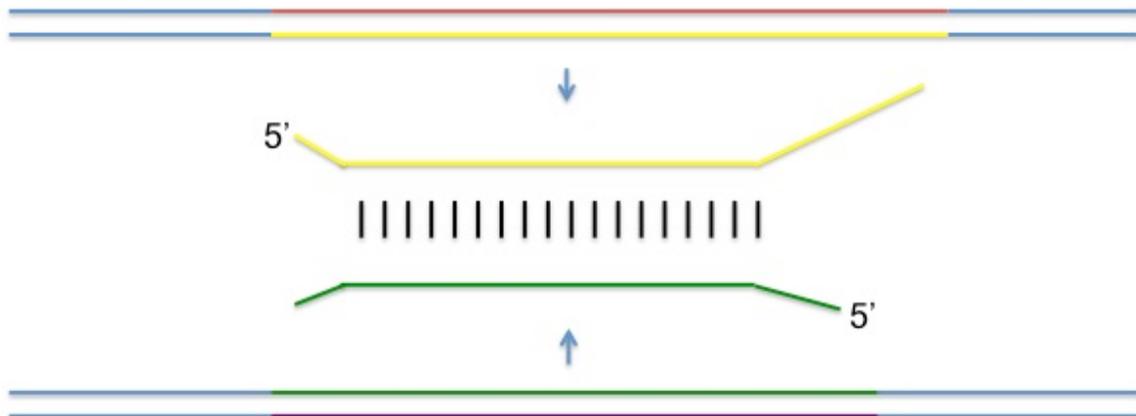


Figure 1: Complementarity of mRNA to AS RNA. Red/Yellow – gene that produces mRNA (red: coding strand; yellow: non-coding strand). Green/purple – gene that produces AS RNA (green: non-coding strand; purple: coding strand).

Chapter 2: Literature Review

Harmful algal blooms, an ecological problem commonly known as “red tides,” are caused by dinoflagellates. According to Lin (2011), over 60 of these bloom forming dinoflagellate species produce toxins. These toxins have detrimental effects on the local marine life and present a hazard to the health of exposed humans. Out of the six types of classical seafood poisoning, there is only one type not caused by dinoflagellate-produced toxins (Lin 2011). Resulting beach closures and massive fish kills have negative impacts on the tourism and fishing industries respectively, which in turn negatively impact the economy.

One of these 60 toxin producing dinoflagellate species is *Karenia brevis*, the marine dinoflagellate most responsible for the red tides in the Gulf of Mexico. Like all dinoflagellates, *K. brevis* is a flagellated, unicellular eukaryote. *K. brevis* is a mixotrophic organism, meaning that it is capable of photosynthetic energy production as well as heterotrophic energy consumption. As shown in the following image of a *K. brevis* cell as seen under an electron microscope (Figure 2), *K. brevis* possesses a heart-like morphology.

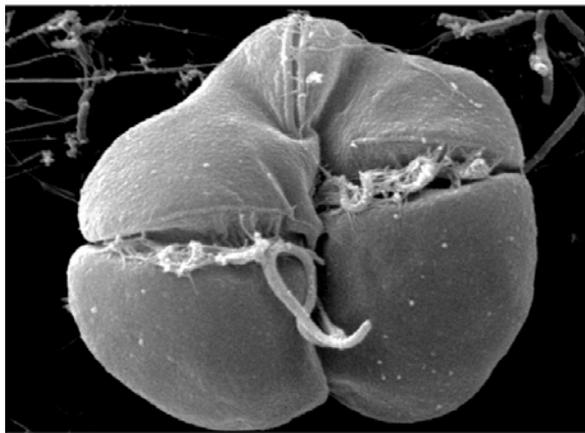


Figure 2: Electron micrograph of *K. brevis* cell provided by the Florida Fish and Wildlife Conservation Commission.

The toxins produced by *K. brevis* are potent neurotoxins known as brevetoxins. Brevetoxins, though only released upon death of a *K. brevis* cell, accumulate in high concentrations during blooms. A study done by Kristen M. Lester and her colleagues in 2008 corroborates the negative effect of *K. brevis* blooms on the health of nearby marine life. Their study specifically shows that an over abundance of *Karenia brevis* greatly hinders the growth and reproduction of some species of zooplankton (Lester, 2008). In addition to harming marine life, brevetoxins also negatively impact human health. Routes of human exposure to brevetoxins include both inhalation and ingestion. The inhalation of aerosolized sea spray from an area affected by a *K. brevis* bloom results in irritation to the respiratory system. Ingestion of shellfish that have been exposed to the high levels of brevetoxins encountered during blooms leads to neurotoxic shellfish poisoning (Errera, 2011). These negative impacts on both the environment and the economy cannot be avoided without a means of controlling or preventing either the bloom or its toxic effects.

Unfortunately, no mechanism for control or prevention of *Karenia brevis* blooms has yet to be found. Sengco (2009) compiles many potential but unsuccessful mechanisms for controlling blooms. As stated by Sengco (2009), the reduction of *K. brevis* cell counts is not the only factor that determines the success of potential controlling mechanisms. The cost of the application of the proposed mechanism, as well as its impact on the environment must also be taken into account. For example, the use of copper sulfate as a chemical control may successfully kill the *K. brevis* population, but it may also negatively affect the health of other organisms. Strong oxidants are another

potential mechanism for chemical control of *K. brevis* blooms. The use of ozone was specifically tested, but the concentration of ozone needed was too high to be cost effective. Other potential control mechanisms, such as the use of algicidal bacteria and clay flocculation, require more testing before a definitive conclusion can be made regarding their effectiveness (Sengco 2009).

Each of these studies approached the control of harmful algal blooms via the manipulation of *K. brevis*' external environment following the formation of a bloom. An alternative method may be to further understand the genetic mechanisms within the dinoflagellate that lead to the formation of these blooms. The genomic structure and function in *K. brevis*, and even dinoflagellates in general, differs greatly from other eukaryotic organisms. First, the nuclear genome of a dinoflagellate is notably large, and their chromosomes always remain condensed (Lin, 2011). The permanently condensed state of dinoflagellate chromosomes contrasts with the chromosomes of most other eukaryotes, which are only condensed during the actively dividing stages of the cell cycle. In addition to being perpetually condensed, dinoflagellate chromosomes lack nucleosomes and possess very few, if any, histone proteins (Moreno Díaz de la Espina, 2005). In most eukaryotes, these histones play a key role in transcriptional gene regulation. The lack of histones in the chromosomes of dinoflagellates indicates the use of an alternative method of gene regulation. Studies have indicated that the majority of *K. brevis* genes are regulated via a post-transcriptional mechanism (Van Dolah, 2009).

One recognized method of post-transcriptional gene regulation involves the use of non-coding strands of RNA (Eddy, 2001). One such RNA type present in *K. brevis* is antisense (AS) RNA. These AS RNA's have a portion of sequence that is

complementary to a region on a corresponding mRNA strand. AS RNAs can be derived from the opposite strand of DNA as the coding sequence of a gene (referred to as *cis*-antisense), or they can be synthesized at a separate genetic locus (referred to as *trans*-antisense). Currently, the McLean research laboratory has evidence to suggest that the AS RNAs in *K. brevis* are *trans*-AS RNAs (unpublished data). Since two separate genes code for the two types of RNA, their genomic location within the *K. brevis* cell is not necessarily linked. The genes for the corresponding mRNA and AS RNA may not even reside in the same organelle due to the occurrence of gene migration (Hackett, 2004). Analysis of EST datasets for *K. brevis* indicates that many chloroplast genes are actually housed within the nuclear genome (Lin, 2011). Determination of the genomic location of both genes will be beneficial for a further understanding of the potential interaction between the AS RNA and its corresponding mRNA.

In this exploratory study involving both the plastid and nuclear genome of the toxic dinoflagellate, *K. brevis*, there are multiple possible outcomes. A protein coding chloroplast gene may still reside in the chloroplast, or it may have migrated to the nuclear genome. The corresponding AS RNA expressing gene may also be found in either location. For example, both genes may be found in the same genome, or they may be found in some split combination between the two genomes. The results of this study may contribute in part to a further understanding of gene regulation within the *K. brevis* cell, which can be applied to the efforts to control the harmful algal blooms formed by this dinoflagellate.

Chapter 3: Methodology

The procedure for determining the genomic location of both the genes coding for the mRNA and AS RNA of Photosystem-D2 is described in the following 10 steps. Steps 1 through 7 should be repeated until the entire sequence of the unique ends of each target RNA strand is captured. Steps 8 through 10 are to be conducted in future works once the sequence of each unique end has been captured.

1. RNA extraction

The Qiagen Total RNA extraction kit was used for the extraction of RNA, with the protocol optimized for *K. brevis*. Under the hood, 700 μ L of RLT buffer was mixed via vortexing with 7 μ L of B-Me per sample. 200mL of *K. brevis* culture was spun at 1500 rpm for 5 minutes in a Hettich Zentrifugen Rotanta 460 centrifuge. The supernatant was discarded in the proper waste container and the pellet was allowed to air dry for 5 – 10 minutes. 350 μ L of B-Me/RLT buffer was added to the large centrifuge tube and mixed via pipette. The mixture was transferred to a fresh 1.5mL microfuge tube. 200 μ L of 100% EtOH was added to the microfuge tube, and mixed with a pipette. Another 350 μ L of B-Me/RLT buffer was added to the microfuge tube, mixed via pipette. 250 μ L of 100% EtOH was added to the microfuge tube, and the solution was homogenized via pipette. 700 μ L of sample was added to the spin column and spun at 8,000 rcf for 1 minute. The flow through was discarded into the Qiagen waste container. The remaining sample was added to the spin column, spun again at 8,000 rcf for 1 minute, and the flow through was discarded. 700 μ L of buffer RW1 was added to the spin column and spun at 8,000 rcf for 1 minute. The flow

through was discarded into the Qiagen waste container. 500 μ l of Buffer RPE was added to the spin column and spun at 8,000 rcf for 1 minute and the flow through discarded into a Qiagen waste container. An additional 500 μ L of Buffer RPE was added to the spin column, this time spun for 2 minutes at 8,000 rcf and the flow through discarded into the Qiagen waste container. The spin column was transferred to a fresh collection tube and spun at full speed for 1 minute. The collection tube was discarded and the spin column was placed into a fresh microfuge tube. 50 μ L of RNase-free water was added to the spin column and it was allowed to sit for 1 minute then spun for an additional minute at 8,000 rcf. Another 50 μ L of RNase-free water was added, allowed to sit for 1 minute, and then spun at 8,000 rcf for 1 minute. The elution, the volume of which should have been 100 μ L, was kept. 10 μ L, or 1/10 sample volume, of 3M sodium acetate was added. 200 μ L, or 2 times the sample volume, of 100% ethanol was added. The sample was stored in -20°C freezer for 1 hour – overnight.

2. RNA Preparation

The now precipitated RNA sample was spun at top speed for 35 minutes at 4°C. The supernatant was pulled off and discarded. 500 μ L of 70% ethanol was added to the pellet and mixed via inversion. The sample was spun at top speed for 25 minutes at 4°C. The supernatant was pulled off. The pellet was air dried under the hood for approximately 15 minutes. The pellet was resuspended in 7 μ L DEPC-H₂O. 1 μ L of 10x DNase buffer and 2 μ L RQ1 DNase were added (Promega). The sample was incubated at 37°C for 40 minutes to an hour. 1 μ L of stop solution was added and the

sample was heated to 65°C for 10 minutes. The volume of the sample was brought up to 100µL with DEPC-H₂O. 10µL of 3M sodium acetate and 200µL of 100% ethanol were added. The sample was stored in the -20°C freezer for 1hr – overnight. The sample was spun at top speed for 35 minutes at 4°C. The supernatant was pulled off and 500µL of 70% ethanol was added to the pellet and mixed via inversion. The sample was spun at top speed for 25 minutes at 4°C. The supernatant was pulled off and the pellet was allowed to air dry under the hood. The RNA was quantified using a Nanodrop.

3. Reverse Transcription

Following manufacturer's instructions for the Clontech RACE kit:

A buffer mix was prepared for both a 5' and a 3' reaction containing 5µL of 5x First-strand buffer and 2.5µL of both 20 mM DTT and 10 mM dNTP mix. All reagents were kept on ice. The RNA and primers were mixed in PCR tubes. In the 5' reaction tube, the volume of RNA sample could not exceed 2.75 µL. DEPC-H₂O was used if dilution was necessary to result in 500ng RNA in the reaction tube. 1µL of 5'-CDS Primer A was added, which brought the total volume up the 3.75µL. The 3' reaction tube was prepared in the same manner as the 5' tube, except the RNA volume could not exceed 3.75µL, which made the total volume 4.75µL after the addition of 1µL 3'-CDS Primer A. Both the 5' and 3' reaction tubes were incubated in PRESMArT. The tubes were pulsed to bring the solutions down to the bottom of the tube. 1µL SMARTer IIA oligo was added to the 5' reaction tube. Into both the 5' and 3' reaction tubes, 4.0µL of the buffer mix, 0.25µL RNase Inhibitor and 1µL

SMARTScribe RT were added. The reactions were mixed gently by pipetting and both tubes were pulsed. The reactions were incubated under SMT. 20 μ L of Tricine-EDTA buffer was added. The cDNA products were stored at -20°C or progressed immediately to the PCR step.

4. PCR of cDNA

A PCR master mix was prepared with 172.5 μ L of PCR-grade H₂O, 25 μ L of 10x Advantage 2 PCR buffer, and 5 μ L each of dNTP mix and 50x Advantage 2 Poly Mix (Clontech). As detailed in table 1, there was a total of 4 separate PCR samples, one each for the 5' AS RNA, 5' mRNA, 3' AS RNA, and the 3' mRNA corresponding to the Photosystem D2 gene. To both of the 5' reaction tubes, 2.5 μ L of the 5' cDNA, 5 μ L of the 10x Universal Primer Mix (UPM) were added in addition to 41.5 μ L of the master mix described above. 1 μ L of the gene specific primer (GSP), PhotosystemD2FS – left, was added to the 5' AS RNA reaction tube and 1 μ L of the GSP, D2m5RACE, to the 5' mRNA reaction tube. To both of the 3' reaction tubes 2.5 μ L of 3' cDNA, 5 μ L of the 10x UPM and 41.5 μ L of the master mix were added. 1 μ L of the GSP, qPCR reverse D2, was added to the 3' AS RNA reaction tube and 1 μ L of the GSP, qPCR forward D2, to the 3' mRNA reaction tube.

Each reaction tube was subjected to a PCR involving 25 cycles of a 30 second denaturation step at 95°C, a 4:00 annealing step at a temperature 2°C below the melting point of the GSP, and a 30 second elongation step at 72°C.

Sample	RNA	GSP
1	5' AS RNA	Photo D2FS - left
2	5' mRNA	D2m5 RACE
3	3' AS RNA	qPCR – reverse D2
4	3' mRNA	qPCR – forward D2

Table 1: RNA type and GSP per PCR reaction.

5. Gel Electrophoresis

A 1.5% agarose gel was prepared. To make a regular sized gel, 180mL of TAE buffer was combined with 2.7g of agarose. The solution was microwaved for 2 minutes, until completely homogenized. The agar was allowed to cool before being poured into the gel mold and the comb was inserted. Once the gel had completely hardened, it was positioned into the apparatus and covered with TAE buffer. Using a pipette, 1 μ L of gel loading dye was mixed with 5 μ L of each DNA sample. 5 μ L of each sample/dye mixture was loaded into its respective well. The electrodes were plugged in and the gel was run at 160V until the bands were adequately separated.

6. Low Melt Gel Electrophoresis

The procedure for this step was identical to that outlined in step 5 for gel electrophoresis with the exception of the agarose being replaced with low melt agarose. Additionally, the gel was run at 80 V to aid in a clean separation of the bands. After running the gel, the bands of interest were extracted from the gel using razor blades. Each band was stored in its own microfuge tube. The gel slices were

incubated at 55°C to melt the low melt agar without denaturing the DNA. These DNA samples were sent off for sequencing.

7. Sequencing

The DNA samples acquired from the gel slices were combined with premixed primers and sent to Eurofins Genomics for sequencing. Two sequencing reactions were prepared for each unique end: one used a gene specific primer, and the second used a universal primer mix. For the 3' end of the Photosystem D2 mRNA, qPCR forward D2 5-3 was used as the gene specific primer, and 3-UPM as the universal primer. For the 5' end of the mRNA, D2m5RACE 3-5 was used as the gene specific primer, and 5-UPM as the universal primer.

8. Primer Design

Based on the sequences obtained from each sample, a unique primer can be designed to uniquely hybridize with the AS RNA or the mRNA.

9. Extract Prep

To obtain the cytoplasmic extract, a 200mL sample of *K. brevis* culture was spun at 1500rpm for 5 minutes to obtain a cell pellet. The pellet was resuspended in 200µL of CE buffer (Table 2) and left on ice for 10 minutes. The solution was dounced for 25 strokes using an ice-cold mortar and pestle, and then spun for 6 minutes at 2200 ref. The supernatant is the cytoplasmic extract.

To obtain the whole cell extract, another 200mL sample of *K. brevis* culture was spun at 1500rpm for 5 minutes. The pellet was resuspended in 200µL of NE (Table 2)

buffer and left on ice for 10 minutes with occasional vortexing. The solution was then dounced for 25 strokes. This is the whole cell extract.

CE Buffer	NE Buffer
10mM Hepes pH 7.7	10mM Hepes pH 7.6
1mM EDTA	1mM EDTA
60mM KCl	60mM KCl
1mM DTT	1mM DTT
0.1% NP40	-
Protease Inhibitors	Protease Inhibitors
DEPC – H ₂ O	DEPC H ₂ O

Table 2: Extraction buffer constituents.

10. Probe

The detailed procedure of this step will depend of the nature of the probes designed from the sequence of each RNA.

Chapter 4: Results

The total RNA extraction from the *K. brevis* cells was subjected to reverse transcription. The 5' reverse transcription reaction resulted in cDNA complementary to the 5' ends of the total RNA (5'cDNA), while the 3' reverse transcription reaction resulted in cDNA complementary to the 3' ends of the total RNA (3'cDNA). The cDNA samples were then subjected to PCR. PCR of the 5'cDNA using the gene specific primer, D2m5RACE, amplified the cDNA of the 5' end of the Photosystem D2 mRNA, denoted simply as 5'mRNA. Likewise, PCR of the 5'cDNA using the gene specific primer, Photosystem D2FS-left yielded the 5' AS RNA. PCR reactions of the 3'cDNA

amplified the 3'mRNA and 3'AS RNA when using the gene specific primers qPCR forward D2 and qPCR reverse D2 respectively.

Gel electrophoresis of all four PCR samples revealed that only the 3'mRNA and 5' mRNA ends had been successfully amplified. Two samples of each end were sent to Eurofins Genomics for sequencing. One of each sample was premixed with UPMs, and neither reaction resulted in successful sequencing.

The sequencing reaction of the 3' end of the Photosystem D2 mRNA using the gene specific primer qPCR forward D2 5-3 generated a readable sequence (Figure 3). However, BLAST results indicated that this sequence is not Photosystem D2. The closest matches are hypothetical proteins from *Emiliana huxleyi* (51% coverage, E-value of 10e-76, and 72% identity – accession number XP 005789954.1) and other microalgal species, (e.g. *Aureococcus anophagefferens*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*).

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5'ACACGCCTTNNAAAGATGCGAGTGTATTCCGAGGATGGGTTGACCCGACCCTTCAGT
GCTGTCATCGCAACCCCTTATGGTGGAGAGAACACAGTTGCCGTCGTAACGTTGGG
CAGATCGAGTTCCCCCTGGCTGCACACGTGACTGCCAATGAGGTTGATGAGCCATC
TCCGACTTCCGTGACACCTCCATCATAGTGCAGGGAGGAGCCCTCCGAACCTTCAAC
TTTGACNNCTCTGTTGAGAGTGTGAGATCATGTTGAAGACTGACGGACGTCCGCTT
AATGCCAGAATTGAGCTGCTCCAGGGTCTAACAATAACAAGCAAGTTATTGAGCTT
TACACTGAGGATGGACTTGAGCGTCCCTTCTTCGCCGTTGTTGATACCATGGGAAGT
GGAAATGTTGTCCGTGTTGTGAACACTGCTCCCATGGAGTTCCCCATGACTGCCAGT
GTACAGCCTCACAGTATCAACGAGAAGATGTCACTCACTGATCCTGTTATTGGAGGT
GGCAGGAGGTTCTGATTGAAGTACTAATTTAGGCTTGCAAGTCCAGCCATAGAGTAA
GTGCTGGAATTGAGGCCACTGTGGTACATTTTCGCACACAGCCTTTTGCTTGCACACA
TTTGCAC TTGTACAGGCATAATTAAGAGTATGGAAAATGATTTTGTGTTGTTCCGCA
GGGCCAAGTTGATGAGCTAATTACGATCATTTACTGGCCGCTGCGGTGAAGCAGCAC
ATGAACCGAACCAACAATTCATGCTGCCATGTTTAAACGGGCGGCGTCCAATTTTTT
TGTTCCGTTGCCGTTTCCCGGCTCATTCTGCATGTCNACTGCTGCACCGATGTGCTG
GTCGTANTTGTAACAGTCGTTTCTTGACGGTCTTGGACCTCACNTTCGACTTGTATGA
CNNNAATGTANTCCATTGCCAGAGCTGNNTTCAGCCNTGGNAATAAAAAAANNA
AAAAAAAAAAAAA3'

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Figure 3: 3'mRNA generated sequence.

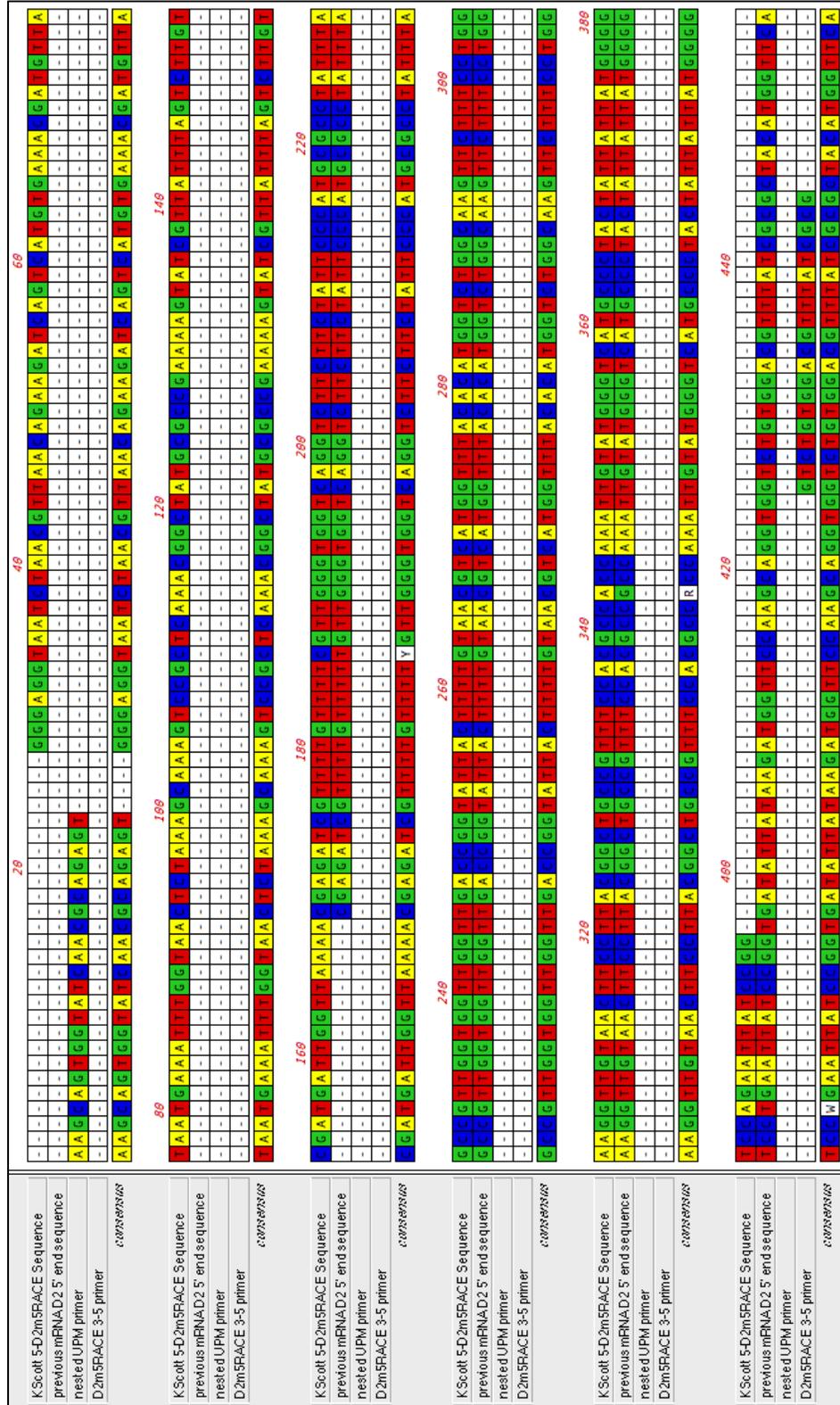
The sequencing reaction of the 5' end of the Photosystem D2 mRNA using the gene specific primer D2m5RACE 3-5 generated a readable sequence (Figure 4).

```
5'GGGAGGTAATCTAACGTTAACAGAAGATCAGTCATGTGAAACGATGTTATAATGA  
AATTTGGTAACTCTAAAGCAAAGTCCGCTCAAACGGCTATGCGCCGAAAAGTATCGT  
TATTTAGTCTTGTTCGATGATTGGTTAAAACGAGATCGTTTTGTTTTCGTTGGGTGGTC  
AGGTCTTCTTCTATTCCCATGCGCCTATTTAGCCGTTGGTGGTTGGTTGACCGGTATT  
ACTTTTGTAACGTCATGGTTTACACATGGTCTGGCAAGTTCTTTCCTGGAAGGTTGTA  
ACTTCCTTACGGCTGCCGTTTCCACGCCACCAAATTGTATGGGTCATGCCCTACTATT  
ATTATGGGGTCCAGAATTATCCGG3'
```

Figure 4: 5' mRNA captured sequence.

Alignment with previously captured sequence for the 5' mRNA (Figure 5) revealed the successful addition of 140 nucleotides to the end of the known 5' sequence. Unreadable sequence in the electropherogram (Figure 6) that does not appear to match the UPM primer sequence indicated that the 5' end of the Photosystem D2 mRNA has yet to be fully sequenced.

Figure 5: 5' mRNA Sequence alignment.



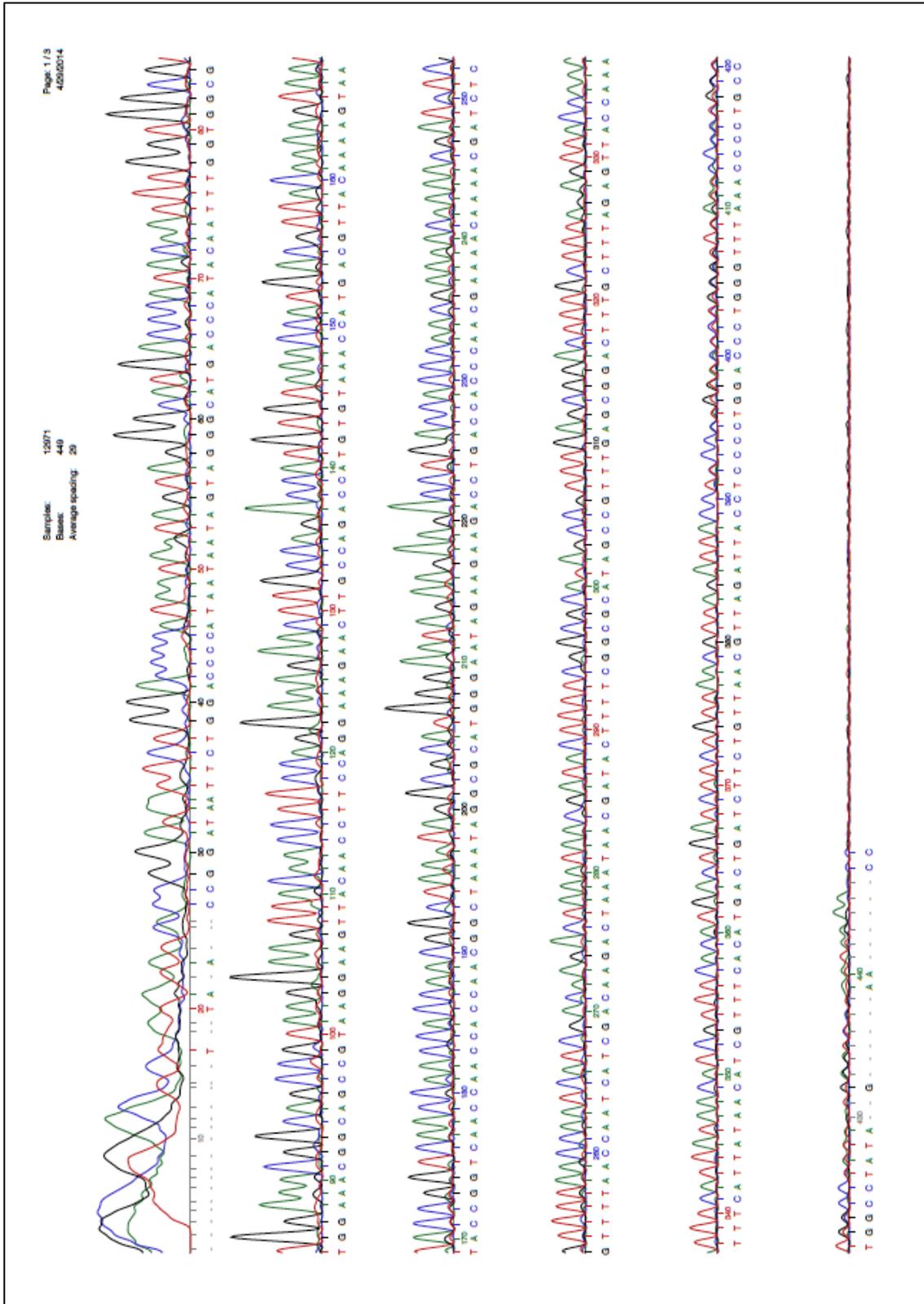


Figure 6: 5' mRNA electropherogram.

Chapter 5: Discussion

The overall purpose of this study was to detect the presence of Photosystem D2 mRNA and AS RNA coding sequences in either the nuclear genome or the plastid genome of *K. brevis* cells. The presence of each coding sequence in either the nuclear or plastid genome may reveal useful information toward understanding the regulatory mechanisms utilized by *K. brevis* in the control of gene expression. Knowledge regarding *K. brevis*' regulation of gene expression, in turn, may contribute to our understanding of the formation, maintenance, and termination of harmful algal blooms.

The specific purpose of this study was to capture additional sequence of the unique 5' and 3' ends of both the mRNA and AS RNA coding sequences. With the capture of each additional portion of unique sequence, more specific primers can be designed to aid in the isolation and amplification of the cDNA from the ends of each RNA type. While this study set out to capture four unique regions of coding sequence, previously unknown sequence was added solely to the 5' end of the mRNA coding sequence. Neither of the AS RNA ends was visualized via gel imaging. Hence these samples were not progressed to the sequencing stage. The 3' mRNA product was visualized via gel imaging; however, the sequence obtained did not align with Photosystem D2. On the other hand, the 5' mRNA generated a readable sequence that aligned with Photosystem D2 and extended the known sequence beyond the 5' end of the mRNA coding sequence of ~140 nucleotides. The capture of an additional sequence data beyond the 5' end of the mRNA coding sequence presents the opportunity for the development of a new primer to continue sequencing farther into the 5' direction.

As evidenced by these results, obtaining the full sequence of each unique end may require multiple cycles of amplification, sequencing, and primer design. First, the amplification step may not always yield an adequate concentration of cDNA for visualization via gel imaging as was seen in the 3' and 5' ends of the AS RNA. In these cases, it may be necessary to subject the DNA sample to additional rounds of PCR. Secondly, the sequencing reaction may not capture the sequence of the entire end. As shown in figure 5, there is still more unknown sequence to the 5'mRNA, and additional work with updated primers is necessary to capture the remaining sequence. Lastly, the designed primer may not be specific enough for a pure amplification of the target coding sequence. The generated sequence from the 3'mRNA sample was probably a result of non-specific primer binding during the PCR stage. In this instance, the problem is two-fold. Firstly, the target DNA was not amplified to an adequate concentration for visualization via gel imaging. Additionally, non-specific binding of the primer to a portion of non-target DNA occurred. If a more specific primer design is not possible, a potential solution is to subject the same DNA sample to further rounds of PCR. This may produce two bands of DNA after subsequent gel electrophoresis. Both bands may be sent for sequencing and compared to the known sequence of Photosystem D2. The target DNA should align with previously captured sequence and ideally provide additional sequence of the unique end.

More work is clearly necessary to fully sequence the unique ends of the respective RNA's. To achieve comprehensive sequencing, there should be an updated primer design corresponding to each addition to the known sequence. Once each unique sequence has been captured, future research objectives should include extraction

preparation and probing. While the specific nature of the primer and probe will depend on the obtained sequences, the extraction method – as detailed in the methodology chapter – has already been optimized for *K. brevis* cells. Each of the four unique ends must be fully sequenced before probe design can commence. Prematurely proceeding into the probing step could result in the non-specific binding of the probe to non-target sequences of DNA. Incorrect binding may yield misleading results regarding the genomic housing of the respective coding sequences for the Photosystem D2 mRNA and AS RNA.

This study was a first step toward the goal of fully sequencing the unique ends of both the messenger and antisense RNAs corresponding to the *K. brevis* chloroplast gene, Photosystem D2. Utilizing these unique sequences to indicate the genomic housing of the genes coding for the mRNA and AS RNA will provide information regarding interactions between the RNA types and their potential impact on the regulation of *K. brevis* gene expression. Hopefully the results of this study will contribute to the development of genomic-based strategies that prevent or control *K. brevis* induced harmful algal blooms.

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