ATTEMPT TO IDENTIFY NOVEL IFT MUTANT THROUGH PCR SEQUENCING AND ANALYSIS OF CHLAMYDOMONAS REINHARDTII FLAGELLAR MUTANTS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Attempt to Identify Novel IFT Mutant through PCR Sequencing and Analysis of *Chlamydomonas reinhardtii* Flagellar Mutants. (May 2013)

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The flagella organelle is crucial to many different cells and organisms because it allows for sensation and movement throughout a matrix. The Intraflagellar Transport system (IFT) is of particular interest since it helps build and maintain the flagella through anterograde and retrograde transport. *Chlamydomonas reinhardtii* is a commonly used model to study the mechanisms of how the flagella organelle functions because its flagella organelle is highly conserved and similar to human cilia and flagella. One route in which to study the flagella organelle is through the creation and analysis of flagellar mutants. Six possible *C. reinhardtii* IFT mutants (created from a previous transformation process and confirmed through a phototaxis test) were chosen to perform PCR on. These regions of disrupted DNA used for PCR were hypothesized to be related to IFT. The DNA was sent to be sequenced in order to determine what sections of the *C. reinhardtii* genome were disrupted by the transformation process. Conclusive results are currently being determined. The consequences of improper functioning flagella can be devastating to a cell. Through this research, the flagella organelle and its IFT processes can be

better understood, and the findings may even aid in the discovery of cures for flagellar diseases (such as polycystic disease, retinitis pigmentosa, etc.).

DEDICATION

I would like to dedicate this thesis to my grandfather, who has taught me that education and the pursuit of knowledge is the most important task to accomplish in life because no one can ever take that away from you. I would also like to dedicate this thesis to my parents, who have given me all that I need and much more, and have provided me with the means to pursue a great education.

ACKNOWLEDGEMENTS

I would like to thank Professor Qin for believing in my abilities to work in the lab, for letting me use the materials in the lab, and for allowing me to have this wonderful undergraduate research experience. I would also like to thank the Technician, Graduate, and Undergraduate students in the lab, who were very helpful and welcoming to me. I would also like to thank The Louis Stokes Alliance for Minority Participation; without this organization, I probably would not have been involved with research in Professor Qin's lab in the first place. Finally, I want to thank the Undergraduate Research Scholars Program for allowing me to participate in their program, for giving me funding for my project, and for guiding me as I write my Thesis.

NOMENCLATURE

Wt	Wild type *Chlamydomonas reinhardtii* cells, *cc*125 (mt +)
mt	Mating type
Hygr	Hygromycin B antibiotic
TAP	Tris-acetate-phosphate media
HEPA	High-efficiency particulate air filter
ddH_2O	Double distilled water
PCR	Polymerase chain reaction
TAP-N	Nitrogen-deficient TAP media
IFT	Intraflagellar transport
bp	Base pair
DMSO	Dimethyl sulfoxide

CHAPTER I

INTRODUCTION

There are many instances where the flagella organelle has proven to be crucial to the longevity of cells (Seravin, 1992). One example is seen in the ability of different cells and organisms to move "towards food or away from toxins" through opposing rotations of the flagella (Meadows, 2011). IFT is a critical component of flagellar function since its role is to build and maintain the flagella. This is achieved through the anterograde and retrograde transport of motor-like proteins along the microtubules of the flagella. If IFT or other important components of flagellar machinery are mutated, basic cellular functions would be compromised. These flagellar mutations may lead to human diseases such as Kartagener syndrome (Afzelius *et al.,* 2001), polycystic kidney disease (Pazour *et al.,* 2000), retinitis pigmentosa (Pazour *et al.,* 2002), and male infertility due to immobile sperm (Ibanez-Tallon *et al.,* 2003). Further study and analysis of the genetic makeup of the flagella will bring the scientific community one step closer to discovering the genetic culprit of these diseases and, quite possibly, their cures.

Chlamydomonas reinhardtii is a photosynthetic, unicellular, green alga that depends on the flagella organelle for movement and sensation throughout a matrix. Considering this organelle's functions, the flagellum is very important and crucial for *C. reinhardtii*'s wellbeing. This is especially true since this organism is photosynthetic. Without a proper functioning flagellum, the cells would have trouble moving towards a light source that is important for obtaining energy for the cell (as with vascular plants) and even aids to the growth of the cell (Rochaix, 2001*). C.*

*reinhardt*ii is not only is an excellent model that demonstrates why the flagellum is important, but also provides a means for in depth study of this organelle.

The flagella organelle can be studied further by the creation and analysis of *C. reinhardtii* flagellar mutants. Mutants are created through a transformation process which is illustrated in Figure 1.

Figure 1. Transformation Process of *Chlamydomonas reinhardtii*.

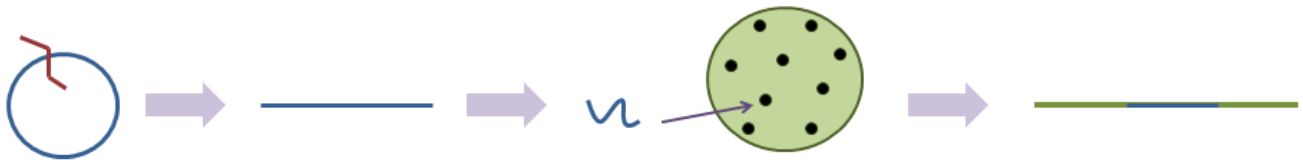


Figure 1. The pHyg3 plasmid was linearized at *Sca*I and inserted into the membranes of Wt *Chlamydomonas reinhardtii* cells that were punched with holes. The linearized plasmid entered the genome of the cells, disrupting various sites in the DNA. These disruptions led to the creation of different *C. reinhardtii* mutants.

The first step in this process was the linearization of the pHyg3 plasmid at the *Sca*I restriction enzyme site. This plasmid is of known sequence and contains a Hygromycin B resistance gene called Streptomyces hygroscopicus aminoglycoside phosphotransferase. The linearized plasmid was then inserted into the C. reinhardtii cells through holes that were punched into the membrane of the cells. Once in the cell, the plasmid entered the genome of the *C. reinhardtii* Wt cells

(Manshouri *et al.*, 2012). These plasmids, when in the genome of *C. reinhardtii* cells, caused various mutations by disrupting important genes in *C. reinhardtii* (Qin, 2011-2012).

A phototaxis test was then performed on the newly synthesized mutants in order to differentiate *C. reinhardtii* flagellar mutants from other *C. reinhardtii* mutants (Manshouri *et al.*, 2012). An illustration of this test can be seen in Figure 2.

Figure 2. Phototaxis Test.

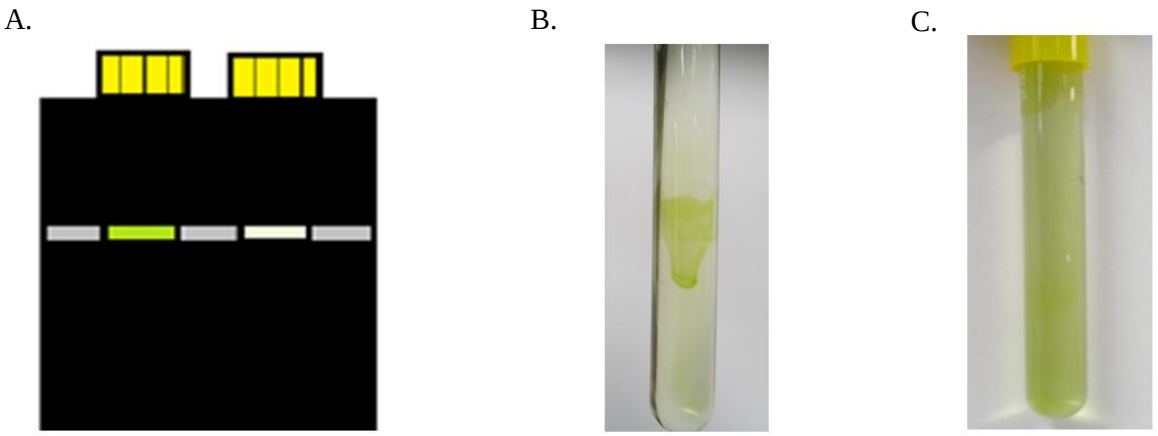


Figure 2. Possible *C. reinhardtii* flagellar mutants were isolated from other mutants using a phototaxis test. Figure 2A. Phototaxis box. This test consisted of placing tubes of inoculated mutants in a black box that had a slit. The box was then placed near a light source. Figure 2B. Non-flagellar mutants. If the cells were able to move towards the light, they were assumed to be non-flagellar mutants. Figure 2C. Flagellar mutants. If the cells could not move towards the light, then they were assumed to be possible flagellar mutants.

This phototaxis screening test is similar to what Wallace and his lab presented in their paper "The Mother Centriole Plays an Instructive Role in Defining Cell Geometry." (Wallace *et al*., 2007). Its first step consisted of placing test tubes inoculated with mutant strains into a black box. A slit that runs across the box allowed the only light that is exposed to the cultures to shine through. The tubes were left in the box for approximately ten minutes. After the inoculated tubes are removed, if the cells have migrated towards the light (which will be known if they are condensed into a line where the slit exposed the test tubes to the light), this indicated that the test was positive and they were probably not flagellar mutants. If the inoculated tubes were removed with the cells still evenly dispersed throughout the tube, then the test was negative and the strains can be assumed to be flagellar mutants (Manshouri *et al.*, 2012).

After the flagellar mutants were distinguished from the other *C. reinhardtii* mutants, each strain was inoculated into TAP media, cultured, and observed under the microscope. Its phenotypic characteristics were identified and recorded, and the strains were streaked onto media. Single colony isolation was also performed in order to assure that the cells are only of one particular strain. These strains were then plated on slants and stored for later use (Manshouri *et al.,* 2012).

The research that will be performed on these mutants will help identify what regions of the *C. reinhardtii* genome are vital to flagellar function through PCR sequencing. Procedures such as backcross and rescue can then be performed to verify that only one gene is associated with the corresponding mutant phenotype, and therefore necessary for proper flagella function. After, further study can be conducted to determine why the particular gene is vital to flagellar function

and methods to restore the flagellar function of the mutant can be determined. This research is important because it may help in understanding IFT better and may even provide clues as to what regions of DNA are damaged in flagellar diseases (polycystic kidney disease, retinitis pigmentosa, etc.).

CHAPTER II

MATERIALS AND METHODS

Culturing mutant strains

The strains utilized include six flagellar mutants from a previous transformation using a plasmid cut at the *Sca*I site (Manshouri *et al.*, 2012): 2P40, 6P1, 6P7, 6P9, 6P10, and 6P11. The strains were streaked from slants onto 1.2% (of agar) TAP plates containing Hygr (10 µg/mL). This was done under sterile conditions (under a HEPA system using a sterile inoculating loop). The instructions for making TAP media can be found in Appendix A. The plates were then placed under light for several days (to allow for growth) and stored for later use.

DNA extraction

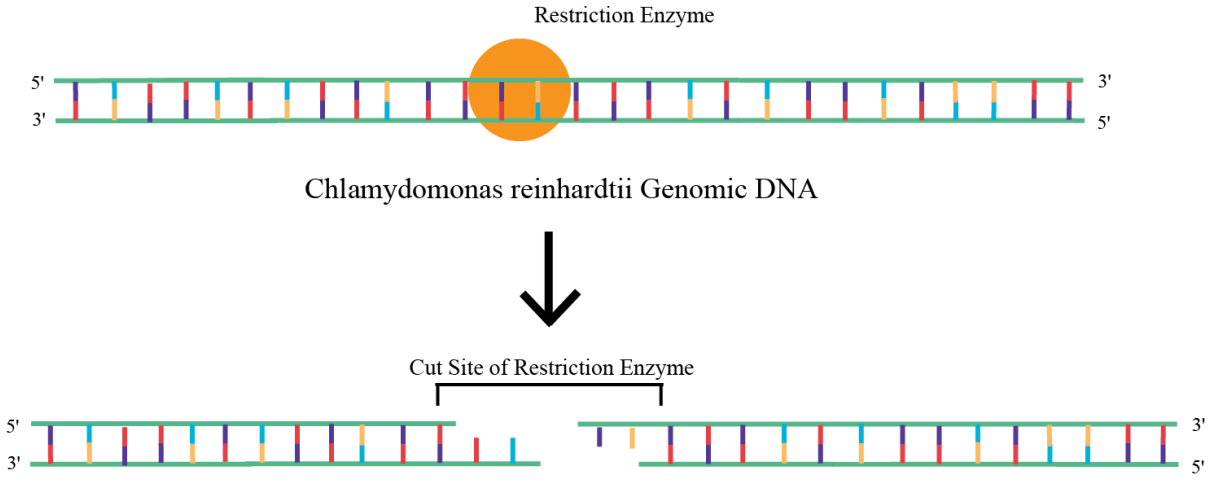
Fresh cells (about a week old) were transferred from the Hygr TAP plates and inoculated in a 250 mL Erlenmeyer flask containing approximately 200 mL of liquid TAP. This was done under sterile conditions. The cells were cultured using a Kimble serological pipette attached to an aeration system under light for two days. The cells should be a light, deep green color throughout the media. The cells were then spun down using a 5810 R Eppendorf centrifuge. The supernatant was discarded and the cells were resuspended in about 20 µL of ddH_2O. This solution was transferred to a 1.5 mL microcentrifuge tube. The DNA was then extracted using a Genomic DNA Purification Kit from Fermentas Life Sciences. The DNA was stored in -20˚ C for later use.

PCR reaction and sequencing

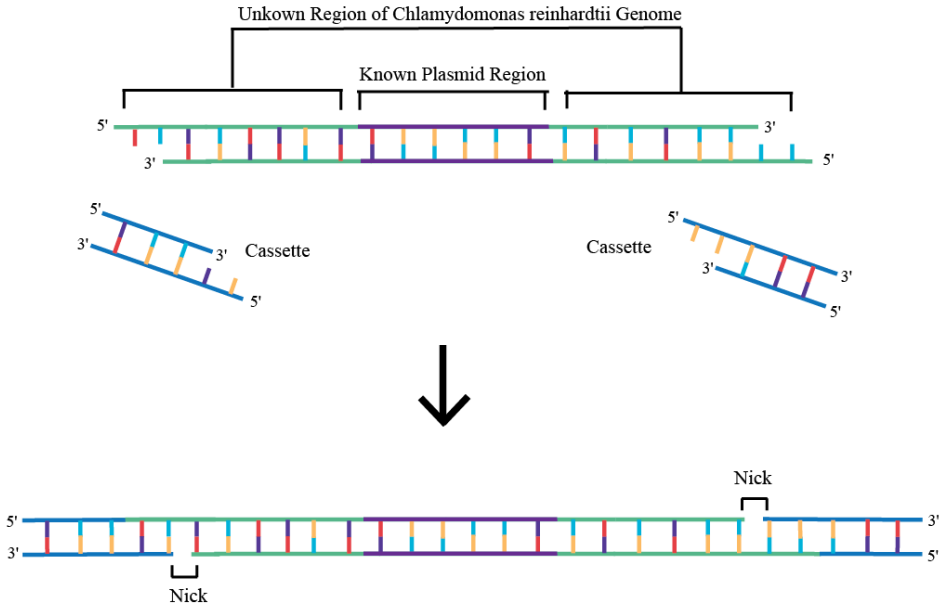
Figure 3 illustrates the principle of how PCR was used to amplify specific regions of *C. reinhardtii* DNA.

Figure 3. Principle of PCR.

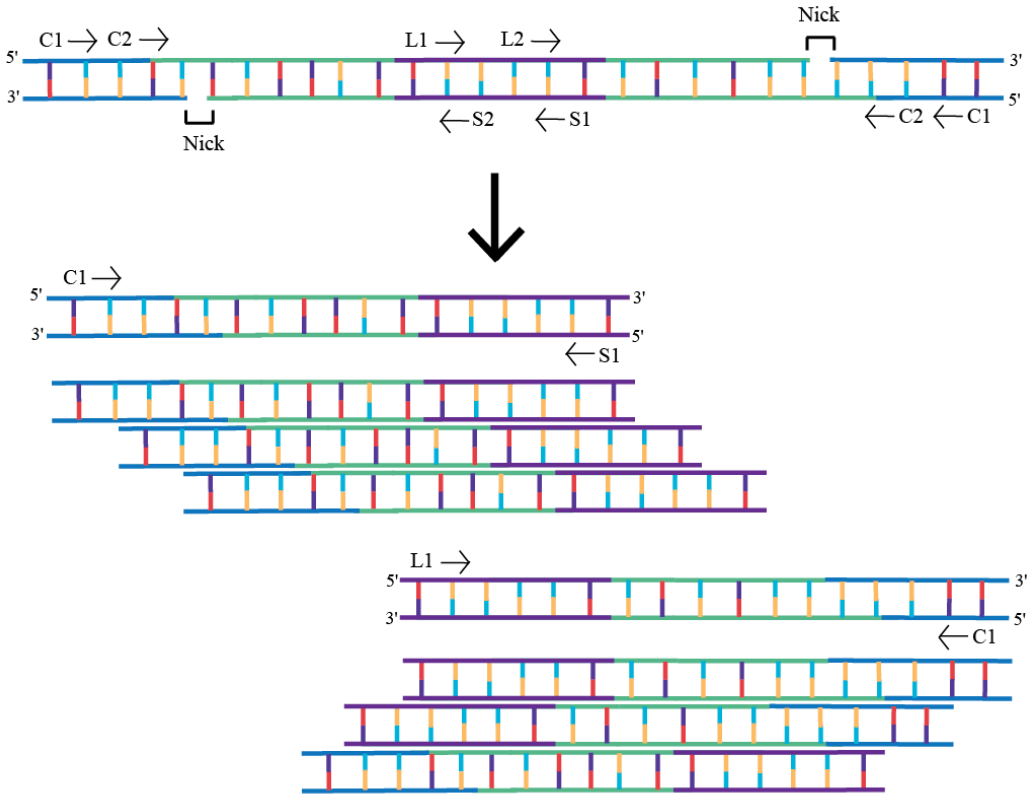
A.



B.


Unkown Region of Chlamydomonas reinhardtii Genome
Known Plasmid Region
5'
3'
3'
5'
5'
3'
3'
5'
Cassette
5'
3'
3'
5'
Cassette
Nick
5'
3'
3'
5'
Nick

C.


C1
C2
L1
L2
Nick
5'
3'
3'
5'
Nick
S2
S1
C2
C1
C1
5'
3'
3'
5'
S1
L1
5'
3'
3'
5'
C1

D.

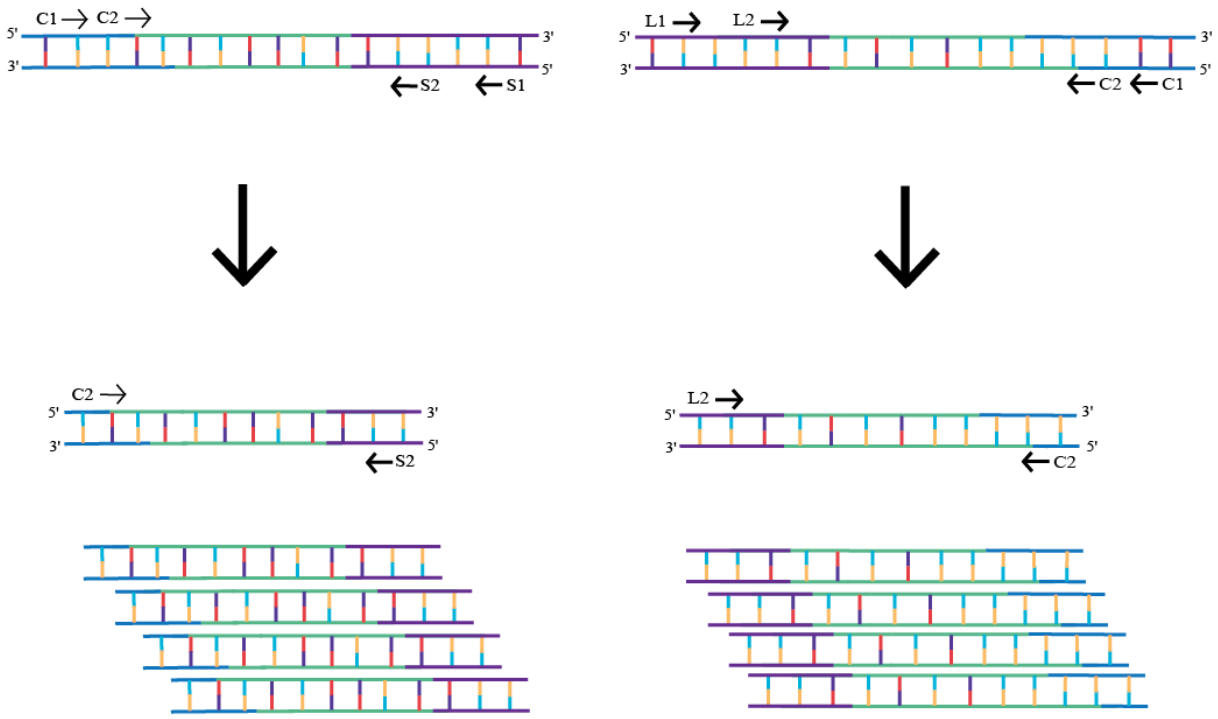


Figure 3. The route in which PCR is able to amplify specific regions of the *C. reinhardtii* genome. Figure 3A. Restriction Enzyme Digestion. DNA is cut by a restriction enzyme at a specific cut site creating restriction fragments of DNA. Figure 3B. Ligation. Cassettes are ligated to the ends of the restriction fragments, but two nicks are left in the DNA. Figure 3C. First PCR. The genomic DNA containing the plasmid is amplified. The nicks allow for this specific amplification since the C1 primer will stop at the nick and S1/L1 is needed to complete amplification. Figure 3D. Second PCR. The amplified DNA is further specified by using C2 and S2/L2 primers which are closer to the genomic DNA. Further runs are similar to the Second PCR concept. This figure was adapted from Takara's LA PCR *in vitro* Cloning Kit.

Restriction enzyme digestion of DNA

Four reactions for each mutant were prepared in 1.5 mL microcentrifuge tubes containing 5 µL of Fermentas Restriction enzyme (either *Hind*III, *Pst*I, *Sac*II, or *Apa*I, 10 u/ µL), 5 µL of their

corresponding Fermentas Restriction enzyme buffer (10X), 40 µL maximum amount of extracted DNA, and enough ddH_2O to bring the final volume up to 50 µL. This mixture was incubated at 37˚ C for three to five hours. Ethanol precipitation was then used to produce a DNA pellet that was dissolved in 5 µL of ddH_2O. This was stored at -20˚ C until further use.

Ligation

Cassettes were prepared by adding 10 µL of the first Cassette and 10 µL of the second Cassette (0.1 µmol/mL) for each particular restriction enzyme in a PCR tube. These tubes were then heated at 94˚ C for ten minutes using a PCR machine. Reactions were then setup in PCR tubes using 5 µL of the DNA solution from the Restriction Enzyme Digestion, 3 µL of T4 ligase (3 u/ µL), 3 µL of T4 ligation buffer (10X), 2.5 µL of each reaction's corresponding prepared cassette, and enough ddH_2O to bring the final reaction volume to 30 µL. The cassettes were synthesized by Integrated DNA Technologies and their sequences can be seen in Table 1 in Appendix B. These reactions were then run on the PCR machine for approximately eight hours at a constant temperature of 16˚ C. The DNA was collected using ethanol precipitation and the DNA pellet was dissolved in 5 µL of ddH_2O. The tubes were stored at -20˚ C until further use.

First run

1 µL of the ligation product was added to 13.5 µL of ddH_2O in a PCR tube and heated at 94˚ C for ten minutes using the PCR machine. Reactions were then setup using the 14.5 µL of the ligation solution, 0.5 µL of C1 primer (1×10^{-5} mMol/mL), 0.5 µL of S1or L1 primer (1×10^{-5} mMol/mL), 5 µL buffer (5X), 5 µL GC melt buffer (5X), 0.25 µL of Polymerase mix (5X), and

0.5 µL dNTP (10X). The final reaction volume was 26.25 µL. All primers were synthesized by Integrated DNA Technologies and their sequences can be seen in Table 2 in Appendix B. The PCR reactions were then run in a PCR machine using the program seen in Figure 4. After the reactions were run in the PCR program, the PCR products were stored in -20˚C until further use.

Figure 4. First Run PCR Program.

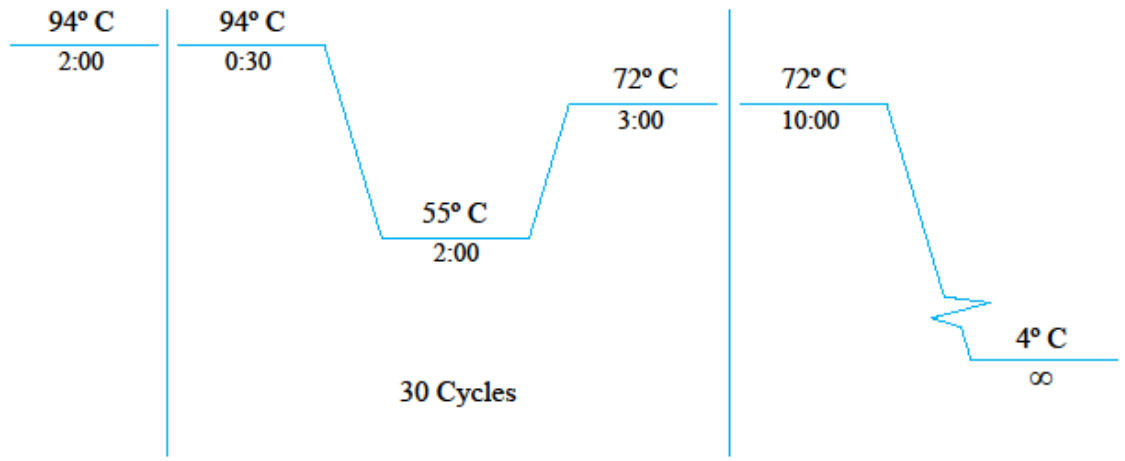


Figure 4. Program utilized for first run PCR products. Time is displayed in minutes:seconds. For the second run, annealing time is reduced to 1:30 and extension time is reduced to 2:30. Annealing time/temperature, extension time/temperature, number of cycles, and the template dilution vary for the third run and further runs based on the assessment of the quality of bands in the agarose gel.

Second run

PCR products from the first run were diluted 200X with ddH_2O. Reactions were prepared by adding 1µL of the diluted first run template, 1 µL of C2 primer (1×10^{-5} mMol/mL), 1 µL of S2 or L2 primer (1×10^{-5} mMol/mL), 5 µL GoTaq buffer (5X), 0.25 µL of GoTaq enzyme (5 u/

µL), 0.4 µL dNTP (10X), 0.75 µL of DMSO, 1 µL of $MgCl_2$, and 14.5 µL of ddH_2O. The annealing time of the PCR program was shortened to 1.5 minutes and the extension time was shortened to 2.5 minutes. After the program was completed, the PCR tubes were stored in -20˚C until further use.

Gel electrophoresis

5 µL of the PCR products were loaded and ran on a 1 % (of agarose) agarose gel with a 1 kb DNA ladder using electrophoresis at a rate of 96 volts. If the bands were bright and sharp (indicating a good concentration and specificity of the DNA), the second run reactions were prepared again using a 50 µL final volume and run in the second run PCR program. When the program finished, the total volume of the reactions were loaded onto a 1% agarose gel and ran at a slower rate of 80 volts. Those bands could then be used for the next step of the procedure. If the bands needed to be brighter or less smeared after the initial second run, further runs were performed.

Further runs

The procedure for the runs following the first two runs was basically the same as the procedure for the second run. The only differences were that the right and left primers (S and L) were changed to S3 and L3 for the third run and S4 and L4 for a fourth run if it was needed. The annealing time/temperature, extension time/temperature, number of cycles, and template dilution could also differ from the first two runs depending on the quality of the bands.

DNA gel extraction

Once the bands on the agarose gel were bright and sharp, the bands were cut precisely from the gel using a sterile knife and UV light. The DNA was then extracted from the gel using the Fermentas Life Sciences GeneJET Gel Extraction Kit. The DNA was stored in -20˚C until further use.

DNA sequencing and analysis

The DNA was added to a 1.5 mL microcentrifuge tube containing a reaction mixture adhering to the guidelines that the sequencing company stipulated. The company that sequenced the target DNA was Eurofins MWG Operon (located in Huntsville, Alabama). The results were then analyzed using an online database called the National Center for Biotechnology Information's Blast Server and the Sequencher 4.8 computer program.

Back-cross

Mutant strain preparation

The mutant strains were streaked onto large 1.2 % TAP plates containing Hygr in a concentrated area of approximately 1 cm in width. The *cc*124 (mt -) strain was streaked so that cells were covering the entire TAP plate. Both of these steps were done under sterile conditions. The plates were placed under light until a thick layer of cells were present. The cells were then transferred to TAP-N plates (instructions for preparation in Appendix A) and kept under light for three days (Jiang *et al.,* 2009).

Mating

The strains were transferred from the TAP-N plates to a 50 mL Erlenmeyer flask containing 2.5 mL of sterile water. Each strain and its corresponding Wt mating culture had about the same cell concentration. These steps were performed under sterile conditions. The cells were then placed under bright light and agitated (placed on a shaker) for two hours. After two hours, the cells become extremely motile (motility was checked under the microscope using 20 µL of cells and 40X objective). After motility was checked, *cc*124 strain and mutant strains were combined together into a 50 mL Erlenmeyer flask. This step was also performed under sterile conditions. The combination of cells were left under bright light without agitation. The amount of time that it took for the cells to mate varied from thirty minutes to four hours depending on the mutant strain. 300 µL of the mating mixture was then plated on 4% TAP plates in four separate spots. The mating mixture was not agitated during this process. The plates were then dried under the HEPA system until the liquid on the plates was absorbed. The plates were left in light for eighteen hours and after, wrapped in foil and stored for five to seven days (Jiang *et al.,* 2009*).*

Random spore evaluation

The vegetative cells were gently scraped off with a blade under sterile conditions using a dissection microscope. Zygotes were present in the yellow-orange edges of the drops. A small piece of TAP media was cut out that contained approximately 30 zygotes. This piece was then dragged over a 1.5% TAP plate to spread out the zygotes. The newly streaked plate was held 5 cm over chloroform for 1.5 minutes in order to the kill the remaining vegetative cells. The plate was wrapped in paper towels and grown under light for 16 to 20 hours. 300 µL of sterile water was added to the plate. The cells were able to swim at this point (cells were checked by using the

dissection microscope) and the water and cells were spread over the plate in order to make sure the spores were separated. The plates were grown under light until single colonies formed. 96 colonies were picked and inoculated into a 96 well plate containing liquid TAP media. The phenotypes were checked using the dissection microscope in order to screen only flagellar mutants. Backcross was repeated on selected colonies four more times (mated to both + and – strains to determine mating type) (Jiang *et al.,* 2009).

CHAPTER III

RESULTS

DNA extraction did not work on mutants 6P9, 6P1, or 6P11. The inoculated mutants may have been cultured for too long. Rounds of PCR were performed on 2P40, 6P7, and 6P10 mutants. The results for the second round of PCR can be seen in Figure 5.

Figure 5. Second round of PCR on 2P40, 6P7, and 6P10.

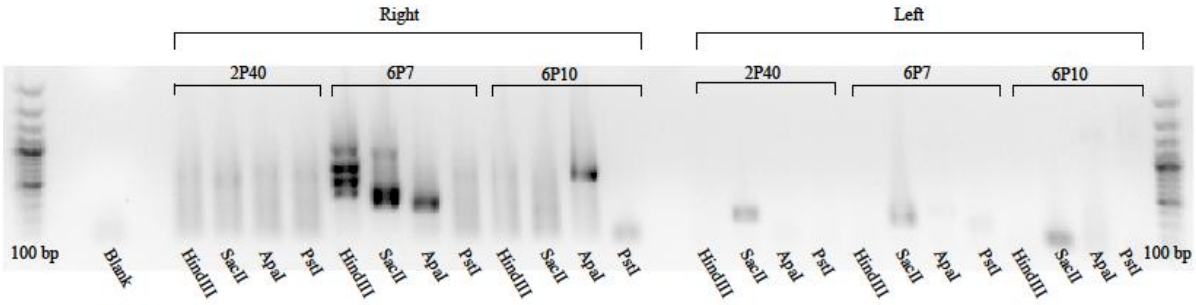


Figure 5. Agarose gel (1%) after second round of PCR, running at about 96 volts. 12 µL was loaded into each well. 100 bp ladder was used on either side of the lanes. Left side of PCR did not get any results for any of the three mutants. The right side got results for only 6P7 and 6P10 mutants.

The *Hind*III bands, about 1000 bp and 500 bp, were chosen for a third round of PCR for the 6P7 mutant. The reason for this is because the *Sac*II and *Apa*I bands are too short, having about 200-300 bp. Shortened bands are not favorable, since their sequences may only contain the insertional plasmid itself, or may not have enough genomic DNA to specify the exact location of the insert in the genomic DNA of *Chlamydomonas reinhardtii*. 6P10 only had a band for *Apa*I; this band

was also long enough for sequencing, having around 650 bp. This band was also chosen for a third round of PCR. The results for the third round of PCR can be seen in Figure 6.

Figure 6. Third round of PCR on 6P7 and 6P10.

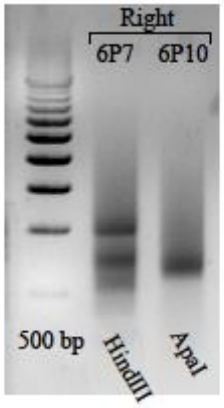


Figure 6. Agarose gel (1%) after second round of PCR, running at about 83 volts. 6 µL was loaded into each well and a 500 bp ladder was used. Bands were obtained for each sample. 1 µL of the template for the third round of PCR was diluted in 200 µL of ddH_2O. The program used for the third round of PCR was the same as the second round of PCR except that the annealing temperature was increased to 56.5˚C.

Both mutants got the same bands for the third round of PCR as they did in the second round of PCR. This is a good result since this means that these bands are specific. However, the concentrations do not look as good for either sample as they did in the second round of PCR. Therefore, the second round of PCR was performed again in the exact same manner as before, except that a 50 µL reaction was prepared for each mutant instead of a 25 µL reaction. A greater

volume of DNA is preferred when sending a sample for sequencing. The results from the second attempt of second PCR can be seen in Figure 7.

Figure 7. Second round of PCR on 6P7 and 6P10 (50 µL).

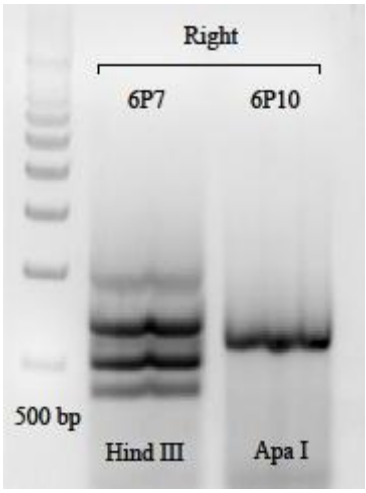


Figure 7. Agarose gel (1%) after second attempt of second round of PCR, running at about 83 volts. 6 µL of 500 bp ladder was loaded into first well. 50 µL of 6P7 DNA sample was loaded into second well and 50 µL of 6P10 DNA sample was loaded into third well. Bands were obtained for each sample.

The bands from this second attempt at a second run were of good concentration. Therefore, the two darker bands from 6P7 were cut out and sent for sequencing and the band from 6P10 was cut out and sent for sequencing. The sequencing results for each sample can be seen in Figure 8, Figure 9, and Figure 10.

Figure 8. Sequencing Results for 6P7, 500 bp.

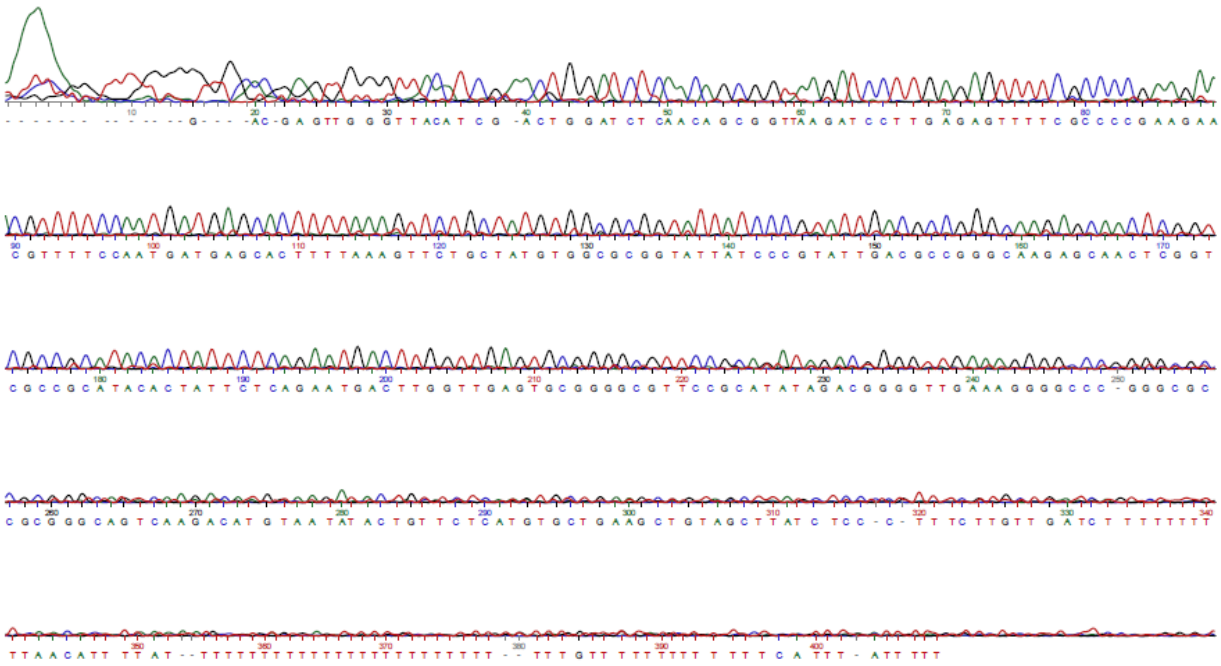


Figure 8. The sequencing results sent by Operon for the 500 bp band from the 6P7 mutant. There were 410 total bases. The sequencing for each bp was specific, but not well amplified. This means that the DNA was probably not of good quality.

Figure 9. Sequencing Results for 6P7, 1000 bp.

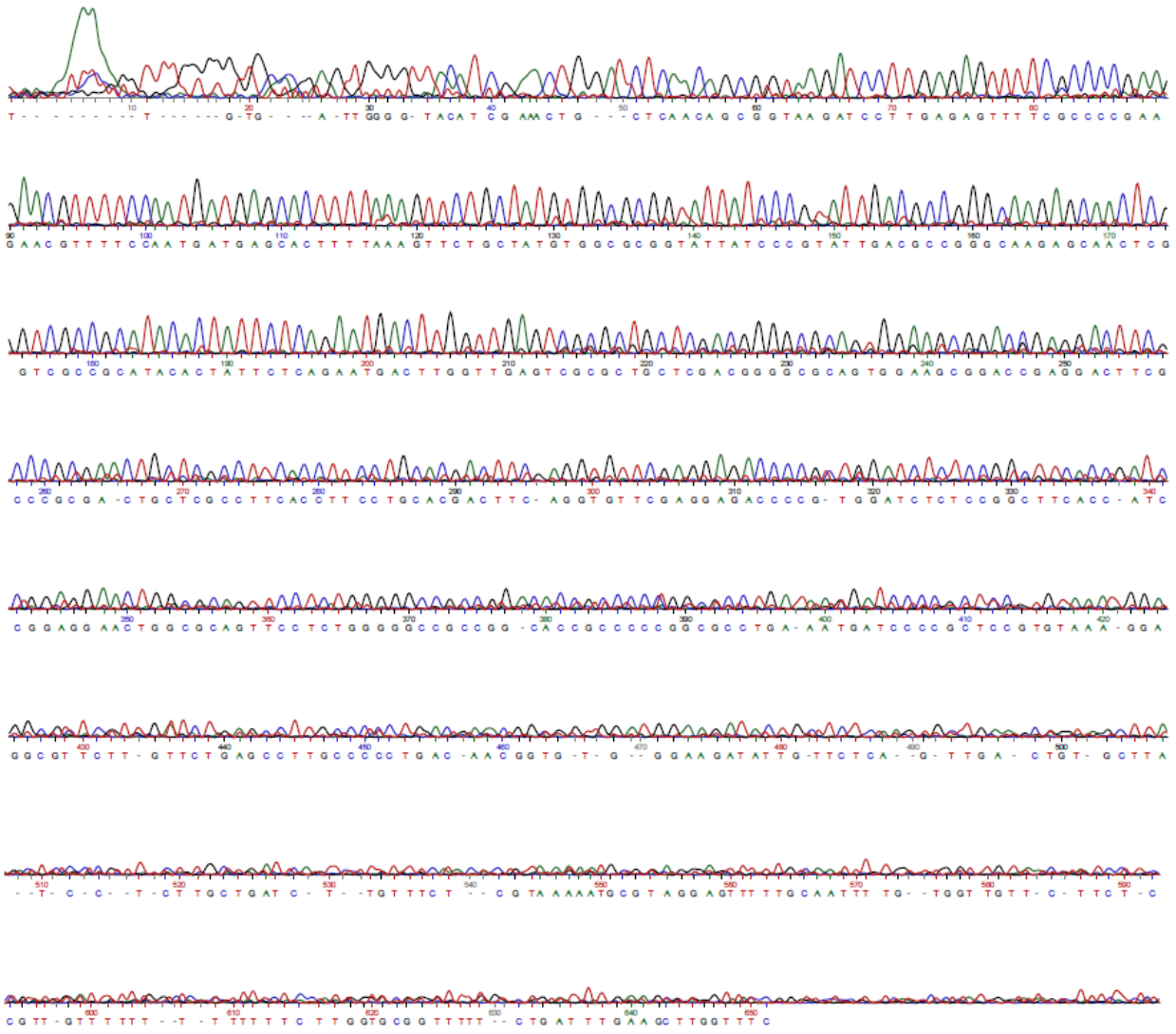


Figure 9. The sequencing results sent by Operon for the 1000 bp band from the 6P7 mutant. There were 652 total bases. The sequencing for the bp between 50-250 was specific and well amplified. However, this only gives 200 bp sequenced well. This band was supposed to be 1000 bp long. The fact that the sequencing did not sequence 800 of the bp well means that the DNA was not of good quality.

Figure 10. Sequencing Results for 6P10, 650 bp.

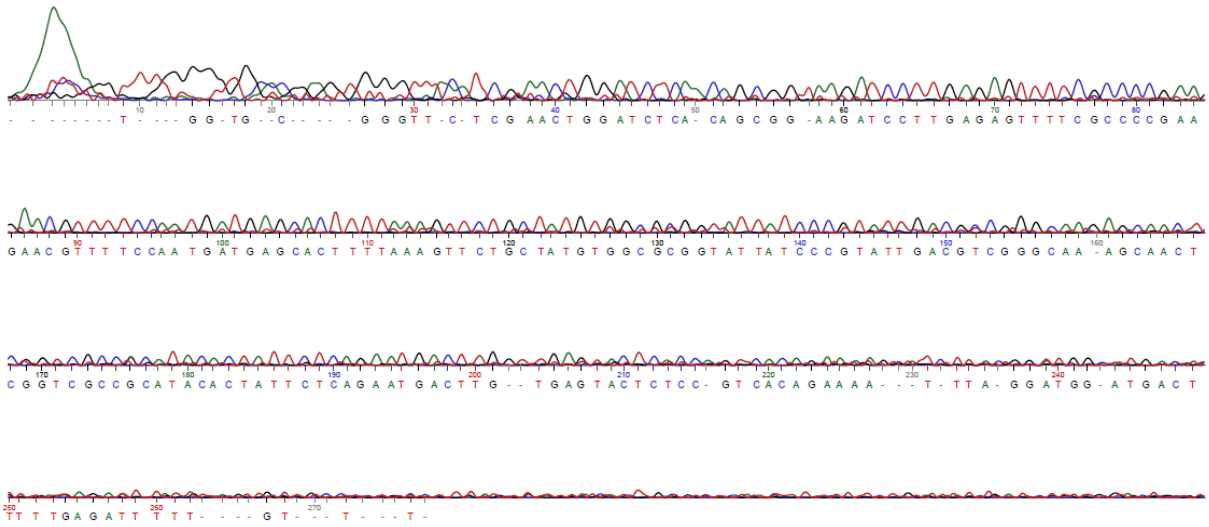


Figure 10. The sequencing results sent by Operon on the 650 bp band from 6P10 mutant. There were 278 total bases. The sequencing for each bp was specific, but not very amplified. This means that the quality of the DNA was not very good.

After analyzing the sequences of the mutant DNA using the Sequencher program 4.8, it was found that the sequences contained only the sequence of the insertional plasmid. This gives us no information about the location of the plasmid insert in the genomes of the two different mutants.

CHAPTER IV

DISUCUSSION

As stated before, if the bands that are sent for sequencing have too few bp, then the sequence may contain only the insertional plasmid. During this project, that is exactly what happened. Even though it was determined that the 1000 bp, 650 bp, and 500 bp bands would be long enough, this was not the case. The sequences of all the bands were only the sequence of the insertional plasmid. Unfortunately, these results do not give the information about where in the mutants' genomes the plasmid inserted. Therefore, the genes responsible for the mutants' phenotypes are still unknown.

One reason why this may have occurred is that the extracted mutant DNA was not of good quality. If the DNA was not of good quality, then the primers during the PCR process may have had trouble adhering to the genomic DNA. The primers instead may have adhered only to the insertional plasmid. Therefore, only the plasmid DNA was amplified and sequenced. One of the major steps in obtaining good DNA is that the cells which you extract the DNA from must be of good quality. The cells that were cultured before extracting the DNA from them must have been bad quality cells. This means that during the next trial, the cells should be cultured for two days at most.

Although conclusive results have not been achieved yet, this method does work. This is known from previous research conducted last summer (the region of the genome where the plasmid

inserted was successfully determined in one mutant). There are also promising aspects of the research conducted this year. The band quality that has been obtained following consecutive rounds of PCR during this project has been better than achieved last summer. This means that this PCR technique is being performed better than before. If this research is continued, while paying close attention to the quality of cells from which DNA will be extracted, conclusive results will be achieved. Hopefully, one of these six particular mutants is a novel IFT mutant. If there is a novel IFT mutant, then further research on that mutant can be conducted and the mechanisms of IFT can be better understood. This may lead to the better understanding of the cause of flagellar diseases, and may someday even aid in the discovery of their cures.

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APPENDIX A

TAP Media Preparation (500 mL)

1. Add 7.5 g of Agar and about approximately 200 mL of purified water to a 1L flask.

2. Wash the Agar three times with purified water. After washed, add purified water so the resulting volume is about 300 mL.

3. Add the following solutions in order to the 1L flask:

    5 mL Tris (100X, 4˚C)

    5 mL TAP salts (100X, 4˚C)

    500 µL Phosphate (4˚C)

    500 µL Trace metals (Hunter trace element, 4˚C)

    500 µL Glacial acetic acid

4. Use purified water to bring the final volume to 500 mL.

5. Autoclave the media for 30 minutes and cool in a 50˚C water bath for 30 minutes.

6. If wanted, add antibiotic (10 µg/mL) and pour the media into plates. Allow to media to harden and collect plates in a bag. This step should be done under sterile conditions.

7. Store plates at 4˚C.

TAP-N Media Preparation (500 mL)

All the steps from TAP Media Preparation should be followed except TAP-N (nitrogen deficient) salts are used in place of TAP salts.

APPENDIX B

Table 1. Cassettes synthesized for PCR reaction.

Cassette Name	Sequence	Length (bp)
*Hind*III Cassette-1	5´-GTA CAT ATT GTC GTT AGA ACG CGT AAT ACG ACT CAC TAT AGG GAG A-3´	46
*Hind*III Cassette-2	5´-AGC TTC TCC CTA TAG TGA GTC GTA TTA CGC GTT CTA ACG ACA ATA TGT AC-3´	50
*Pst*I Cassette-1	5´-GTA CAT ATT GTC GTT AGA ACG CGT AAT ACG ACT CAC TAT AGG GAG ACT GCA-3´	51
*Pst*I Cassette-2	5´-GTC TCC CTA TAG TGA GTC GTA TTA CGC GTT CTA ACG ACA ATA TGT AC-3´	47
*Sac*II Cassette-1	5´-GTA CAT ATT GTC GTT AGA ACG CGT AAT ACG ACT CAC TAT AGG GAG ACC GC-3´	50
*Sac*II Cassette-2	5´-GGT CTC CCT ATA GTG AGT CGT ATT ACG CGT TCT AAC GAC AAT ATG TAC-3´	48
*Apa*I Cassette-1	5´-GTA CAT ATT GTC GTT AGA ACG CGT AAT ACG ACT CAC TAT AGG GAG AGG GCC-3´	51
*Apa*I Cassette-2	5´-CTC TCC CTA TAG TGA GTC GTA TTA CGC GTT CTA ACG ACA ATA TGT AC-3´	47

Table 2. Primers synthesized for PCR reaction.

Primer Name	Sequence	Length (bp)
Cassette Primer C1	5´-GTA CAT ATT GTC GTT AGA ACG CGT AAT ACG ACT CA-3´	35
Cassette Primer C2	5´-CGT TAG AAC GCG TAA TAC GAC TCA CTA TAG GGA GA-3´	35
Right Primer S1	5´-AAG AGT ATG AGT ATT CAA CAT TTC CGT GTC GCC-3´	33
Right Primer S2	5´-AGA AAC GCT GGT GAA AGT AAA AGA TGC TGA AG-3´	32
Right Primer S3	5´-AAG ATG CTG AAG ATC AGT TGG GTG C-3´	25
Right Primer S4	5´-CGT TTT CCA ATG ATG AGC ACT-3´	21
Left Primer L1	5´-GCA ACT TTA TCC GCC TCC ATC CAG TCT AT-3´	29
Left Primer L2	5´-CGC TCG TCG TTT GGT ATG GCT TCA-3´	24
Left Primer L3	5´-GGC GAG TTA CAT GAT CCC CCA TGT T-3´	25
Left Primer L4	5´-GAT CGT TGT CAG AAG TAA GTT GGC-3´	24
Left Primer L5	5´-TTC TCT TAC TGT CAT GCC ATC-3´	21