An Analysis on the Phylogenetic Tree of Protists

An Honors Thesis
Submitted as partial fulfillment of the
Requirements for the Honors Scholar Program
Concentration in Molecular Biology and Biotechnology

by

Shahd Duzdar and Tamer Abuaita

University of Michigan – Flint
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Approved by:

Dr. Joe Sucic Ph.D.
Thesis Advisor
Molecular Biology/UM Flint

Dr. Joe Sucic
Reader
Molecular Biology/UM Flint

Maureen Thum Ph.D.
Honors Director Department of English

PREFACE

For six weeks during the summer, we had the opportunity to do research at the University of Wuppertal in Wuppertal Germany. This opportunity allowed us to do research in my chosen field of study of molecular biology. Both of us worked in the lab of Dr. Preisfeld, the head of the biology department, at the University of Wuppertal.

The goal of the six-week project was to create a phylogenetic tree of the eight protists that we were given. In order to make the phylogenetic tree, we were trying to compare the DNA that coded for all the rRNA and proteins of the ten protists. This included running a polymerase chain reaction (PCR) for each of the protists. The PCR included a specific primer for the DNA that encoded the rRNA in each protist. This stretch of DNA was then amplified for each of the protists. Once we had the amplified DNA region for the small ribosomal subunit, we had to clone the DNA of each protist into a vector. This vector was the pCR 2.1 plasmid for *E. coli*. Once the DNA was put into the vector, the TOP 10 strain of *E. coli* was made competent to take up the vector. The TOP 10 was then sent for DNA sequencing. Unfortunately, we did not get to fully complete the phylogenetic tree, yet, due to the fact that we had to leave before we received the sequencing results.

Research in Germany was a very rewarding experience. It taught us new techniques for procedures that we had previously performed such as those for DNA extraction and gel electrophoresis. It also showed us how to prepare many of the things that we used in the lab. In previous labs, gels and buffers had already been prepared for us beforehand. In the lab at Wuppertal, though, we learned how to prepare our own agarose gel, how to examine it with UV light and how to run a thermocycler. Along with the research in the lab, we also attended a weeklong seminar for Master's biology students at the University of Wuppertal. This opportunity

was very informative. It gave the both of us an opportunity to interact with other German students and to see how their university's labs are run.

ABSTRACT

For a long time, now, Kingdom Protista has been regarded as the end all, catch all kingdom. The properties that distinguish a protist from other kingdoms are so variable that one can only determine that a species is a protest due to the fact that it's not a plant, animal, fungi, eubacteria, or archaebacteria. With the aid of molecular biology techniques such as DNA sequencing and phylogenetics, the relationships of species within the Protista kingdom can be determined as well as their relationships to species in other kingdoms. The goal of this study was to create a phylogenetic tree of 8 species, both protists and some eucaryotes. This was done in order to find genetic relationships between these species to determine which ones are closely related and when they branched off from each other throughout the course of evolution.

ACKNOWLEDGMENTS

Throughout our four years at the University of Michigan-Flint, we have been provided with many opportunities that would have not been possible without the support and guidance of numerous people. From faculty to family members, we cannot thank these individuals enough. We would first like to thank our families and friends for their extended love and support throughout our entire lives, especially our undergraduate careers. Without their encouragements, many of our accomplishments would not have been possible.

We would also like to thank the Honors Program Director, Dr. Thum. The amount of time and effort she puts into the program is admirable and she genuinely cares for each and every student. Dr. Thum also assisted with creating an off-campus study that will last for many years so that future students can have the same wonderful experience in Wuppertal, Germany that we had. Finally, funding for this trip could not have been possible without the support of Dr. Thum and the honors program.

Next, we would like to thank Dr. Preisfeld and her entire research lab at the University of Wuppertal. Without her willingness to let us do research in her lab, this entire project would not have been possible. Her entire lab was extremely patient with us, especially with the language barrier. We learned so much from them and our techniques and overall understanding of molecular biology have greatly improved because of our experiences in her lab. We thank her so much for the experience and we will forever be grateful to her.

Finally, we would like to thank our thesis advisor, Dr. Sucic for his patience and guidance during the completion of our thesis paper. His willingness to read our paper and provided suggestions and improvements was greatly appreciated. Not only has he been a great

thesis advisor, but he has also been a great mentor for both of us throughout our entire undergraduate career.

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Early History and Classification of Protists

Before the 20th century, the classification of organisms into groups was based on whether they were a plant or an animal. The emergence of new scientific methods and microscopic organisms required scientists to produce a new system of classification. Classifications such as Protozoa, Protophyta, Phytozoa and Bacteria emerged as lower forms of organisms compared to plants and animals (Scamardella, 1999). Due to the variety of species within our living world, the study of taxonomy or grouping species emerged. Taxonomy created a way for species with similar qualities to be grouped together in order to make identifying and naming species easier. The highest level of taxonomy is the kingdom, in which there has been the most confusion over the years.

The Kingdom Protista was not identified until 1866 when German naturalist, Ernst Haeckel suggested this as the third kingdom in addition to plants and animals (Scamardella, 1999). **Figure 1** denotes the three original kingdoms that Haeckel proposed in the late 1800's. Protista means first of all or primordial, which is what Haeckel regarded the organisms within this kingdom to be. At the time, Haeckel recognized bacteria in the Monera category as members of Kingdom Protista as well. The emergence of this new kingdom allowed the kingdoms of plants and animals to be distinct in their characteristics (Scamardella, 1999). At this time, Kingdom Protista contained classifications of Protozoa, Protophyta, Phytozoa and Bacteria.

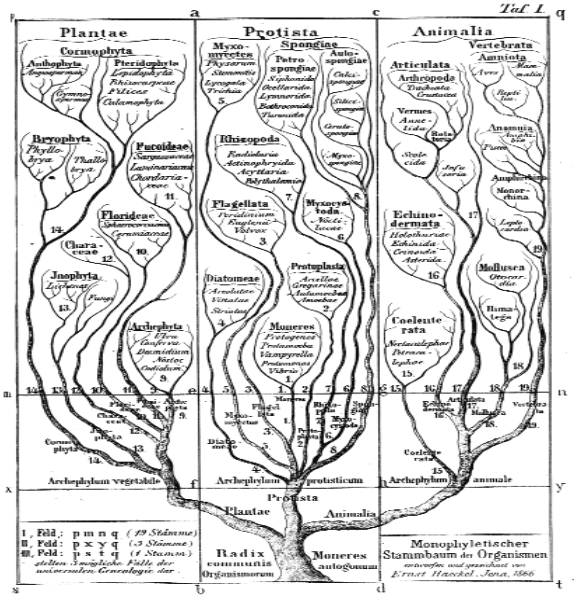

Figure 1: Haeckel's Three Kingdoms

Haeckel put these organisms into the following phyla: Monera, Protoplasta (amoebas), Diatomaceae, Flagellata, Myxomycetes, Noctilucae, Rhizopoda, and Spongiae (Scamardella, 1999). In doing so, Haeckel grouped nucleated (eukaryotic) organisms and organisms without nuclei (bacteria). Haeckel later took Sponges out of Kingdom Protista and incorporated Fungi into this kingdom. Later, scientists of the 20th century made further changes to this kingdom but as time went on, this kingdom came to be known as a makeshift classification. Scientists started to realize that they could not pinpoint a specific characteristic within Kingdom Protista that unites all protists.

In 1938, biologist Herbert F. Copeland built on other scientist's work by proposing four kingdoms. These kingdoms were Monera, Animalia, Plantae, and Protista. Because bacteria were so different from the other protists, Copeland believed that they needed to be classified in their own kingdom (Scamardella, 1999). Whittaker then modified this classification in 1969. As the concept of eukaryotic and prokaryotic organisms became more accepted, Whittaker used this to explain why Kingdom Monera should be in a kingdom by itself. In addition to this, Whittaker also created a kingdom for the Fungi instead of grouping them with Kingdom Protista (Scamardella, 1999). This five-kingdom model of classification is the one that is still used and taught in many schools today.

Throughout its history, Kingdom Protista seemed to be the end all, catch all kingdom. It once encompassed both bacteria and fungi, but as scientists began to discern between different species of the Protista kingdom, they realized that certain organisms needed to be put in their own kingdoms. Because bacteria were shown to lack nuclei and other organelles, they left Kingdom Protista and were put into their own Kingdom Monera. Based on their common mode of nutrition, Fungi were also taken away from Protista and put into their own kingdom. This

eventually left the organisms that could not be fully classified into one category or another in Kingdom Protista.

Kingdom Protista Today

Today, the Kingdom Protista is composed of many diverse organisms that have been classified as eukaryotes that are not plants, not fungi, and not animals. Protista is not considered an official kingdom but a term to group organisms that fall into the category of "other". This group has caused much confusion in trying to determine what makes organisms fit into the protist category and has even lead to new systems of classification that do not involve the traditional kingdoms that we are familiar with (Adl et al., 2007).

It is believed that protists evolved from prokaryotes 1.5-2 billion years ago through some type of endosymbiosis (Finlay, 2004). In this way they are regarded as simple eukaryotes. Though they are simple eukaryotes, protists are extremely important in regulating our environment. The unicellular algae found in oceanic systems are responsible for most of the carbon fixation in the ocean and the world's freshwaters (Finlay, 2004).

Morphology

Most protists are either unicellular or simple multicellular with most of them being small in size ranging from 20-20,000 micrometers. They are mostly classified into protozoa, algae or slime molds (Finlay, 2004; Adl et al., 2007). Because this group of organisms is so heterogeneous, it is difficult to pinpoint a specific morphology of protists. Some organisms exhibit bilateral symmetry, others exhibit radial symmetry and yet more organisms do not exhibit any type of symmetry (Corliss, 2002). In terms of movement, some protists such as the amoebas have been shown to move through pseudopodia while others have cilia or flagella for movement (Adl et al., 2007) Protists contain all kinds of protection. Protists like amoebas only have their

cell membrane as protection, while algae contain strong cell walls to protect them (Raven & Johnson, 2001).

Metabolism and Feeding

In regards to obtaining nutrition, protists have a wide range of diversity in their feeding habits. It has been shown that protists have every way of obtaining their nutrition except for being chemoautotrophic. The algae represent the photoautotrophs of the protist group. These photoautotrophs account for most of the carbon fixation in the world's freshwaters. Other protists exhibit heterotrophic modes of obtaining nutrition by phagocytosis (amoebas) or by osmotrophy (slime molds). Yet there are other types of protists that are parasitic, such as the smut fungi that are able to cause diseases in corn and sugar cane.

Reproduction

Many protists reproduce asexually but some are able to reproduce sexually in times of stress (Raven & Johnson, 2001). Asexual reproduction in most protists can be achieved by binary fission, multiple fission or by budding (Corliss, 2002). It is believed that asexual reproduction in protists is different from other eukaryotes in that the nuclear envelope remains intact during the process of mitosis (Raven & Johnson, 2001). Sexual reproduction within the protist group can differ as well. Ciliates and flagellates undergo gametic meiosis much like mammals yet protists like algae undergo intermediate meiosis. This is very similar to plants and will result in the organism living part of its life cycle as haploid and the other part as diploid. When conditions are bad, some protists are able to go into a dormant phase of the cell cycle and produce spores, a vital part of survival in harsh conditions.

Classification

For this study, classifications are based on Adl et al. (2012) "*The Revised Classification of Eukaryotes*." Adl et al. state six major groups of organization for eukaryotes. They created a comprehensive study to classify eukaryotic organisms and were able to get rid of the five kingdom system in favor of new groups of eukaryotes related in genetic aspects rather than physical attributes. These groups are shown in **Figure 2** and include Sar, Archaeplastida, Excavata, Amoebozoa, and Opisthokonta (Adl et al., 2012). Most of the protists used in our study fell into the Sar, Archaeplastida, and Excavata categories. We also used a fungus and a plant from the Opirsthokonta classification.

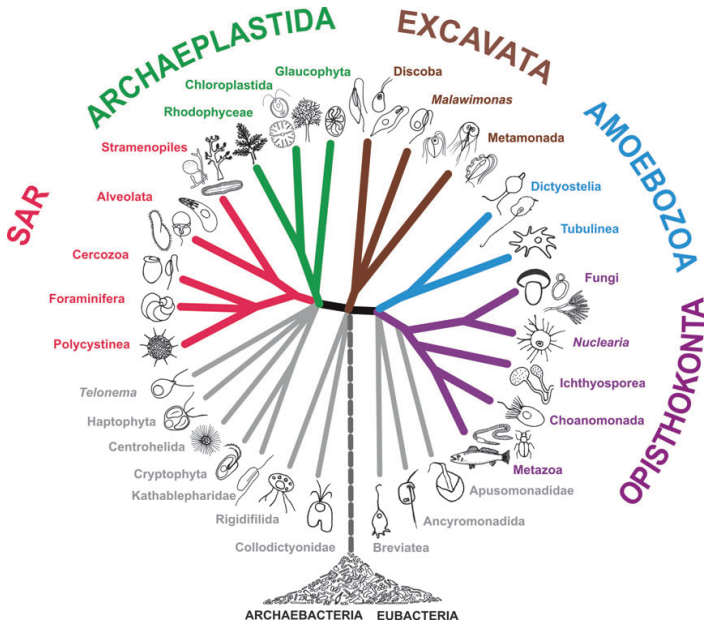

Figure 2: Classification of Organisms

Because protists are so diverse, scientists have begun to use molecular biology tools in order to study their relationships to each other and to determine what makes protists different from other kingdoms. In order to study familial relationships between species and when they diverged from one another, scientists use the study of phylogenetics.

Phylogenetics

Phylogenetics is the study of classifying organisms by grouping the living descendants of a certain ancestor in specific groups (Sleator, 2011). These groups are then able to provide insight into the shared characteristics of the members to the common ancestor of the group. The history of phylogenetics dates back to 1849 with Darwin's *The Origin of the Species*.

To study the familial relationships between protists, scientists use molecular biology techniques to create a phylogenetic tree. A phylogenetic tree or "gene tree" is the comparison of alleles for any specified stretch of DNA between organisms (Sleator, 2011). The emergence of phylogenetic trees is mostly new, and has coincided with the newly popular molecular biology techniques, such as polymerase chain reactions and the development of cloning vectors. Phylogenetics is dependent on data gathered from heritable variation that can be directly compared via homology statements (Wiley & Lieberman, 2011). Scientists have to be sure that each character that is being compared is homologous across all species within the data set. For example, one cannot compare the genes for cilia in *E. elegans* with the genes coding for flagella in *Gymnodinium* because they do not have a common origin even though both cilia and flagella are both used as a mechanism of transportation. Only homologous characters should be used within a phylogenetic analysis.

Traditionally, the most common type of data used for protists have either been mitochondrial DNA or ribosomal DNA (Wiley & Lieberman, 2011). Other types of information such as behavior, ecology, physiology and developmental characters can also be used to reconstruct phylogenetic relationships. These phylogenetic trees allow researchers to study the genetic relatedness of species, instead of the population genetics that have been used before this technology. Molecular surveys of the diversity in mitochondrial DNA were the first used

markers to study the phylogenies for different species and have provided us with extensive data for the estimation of these gene trees (Wiley & Lieberman, 2011). Mitochondrial DNA is the most commonly used DNA to compare different eukaryotic species since this DNA is always inherited from the mother. Because mitochondria are inherited from the mother, its genetic lineage can be traced and comparisons between the mitochondrial DNA between different species can be made. Other types of DNA such as ribosomal DNA can also be used. Ribosomal DNA is advantageous to use because the genes coding for ribosomal proteins or rRNA are highly conserved within both eukaryotes and prokaryotes (Wiley & Lieberman, 2011). These sequences are less prone to mutations, so studying them will give us a clearer idea of the relatedness of two or more species.

Once a DNA sequence is obtained, it is compared to other sequences. Scientists look for similarities and differences in the DNA sequences to compare relationships of organisms on a phylogenetic tree. Closely related species are more likely to have similar DNA sequences. Conversely, a species are distantly related to each other if their DNA sequences have more differences (Creating Phylogenetic Trees from DNA Sequences, 2014). Comparisons of DNA sequences across different species can be used in addition to computer software programs such as Geneious. These programs change the positions of the sequences relative to one another to try and maximize the number of matches in two sequences. In addition, many sequences can be compared to one another if working with multiple organisms (Creating Phylogenetic Trees from DNA Sequences, 2014).

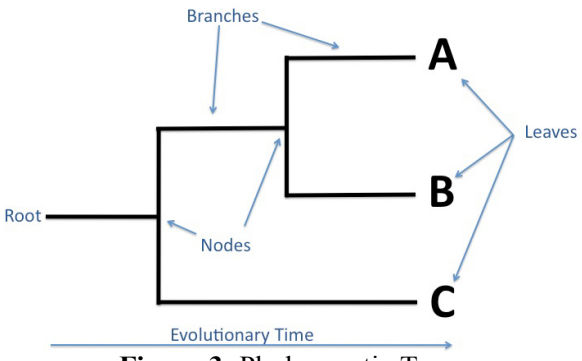

Figure 3: Phylogenetic Tree

There are many different methods for creating the phylogenetic tree. Within a tree, the end of each branch represents a species or sequence. A branch point in a tree is a place where two branches split apart. A branch point represents the most common ancestor of the species on those branches (Creating Phylogenetic Trees from DNA Sequences, 2014). The root of the tree is a single branch point where all the branches of the tree have originated. The node closest to the root of the tree is the most common ancestor for all the organisms in the tree.

On the other hand, trees can be unrooted. These trees only show relative relationships between organisms and they do not show a common ancestor among the group. Branches can be conveyed in different ways, but ultimately this does not change the information in the tree (Creating Phylogenetic Trees from DNA Sequences, 2014). The trees are used to predict traits of similar organisms and sequence comparisons can also clarify anatomical comparison.

Experiment

Overall, our project involved creating a phylogenetic tree of several species from the kingdom Protista and several other eukaryotes. Because so many protist groups and genera have been changed so much, the classification scheme of Kingdom Protista has become unclear (Adl et al., 2012). Protists must be compared to each other as well as other groups to determine if there are genetic relationships between. As members of Dr. Preisfeld's lab, we were presented with eight eukaryotic organisms to work with for the duration of our study. These organisms were Tetrahymena, *Euglena gracilis*, Salat, Koralle, and Amoeba.

Chapter 3-Materials

1. DNA Extraction
    a. Heating block set at 65 ℃ AE elution buffer, bowl of ice, liquid nitrogen, labeled microcentrifuge tubes, mortar and pestle, buffer AP1, RNase A, buffer AP2, centrifuge, pipettes, buffer AP3, spin column, collection tube, buffer AW, elution buffer.
2. PCR
    a. Template DNA, primers, Magnesium, dNTPs and Taq polymerase, Thermocycler
3. DNA extraction and purification from agarose gel
    a. Knife, agarose gel, microcentrifuge tubes, Gel solubilizer, binding optimizer, pipets, spin filter and receiver tube, centrifuge, washing solution.
4. Competent Cell Preparation using Rubidium Chloride
    a. LB plates, SOB medium, $MgSO_4$, 250 mL flask, centrifuge, TFB1 (30 m potassium acetate, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% glycerol) ice bath, pipets, tubes, flasks, TFB2 (10mM MOPS or PIPES, 74mM $CaCl_2$, 10mM RbCl, 15% glycerol).
5. Transformation
    a. Top10 *E. coli* strain, pCR2.1 plasmid, SOC medium, LB plates

Chapter 4-Methods

1. DNA Extraction

    a. We were presented with living samples of the eukaryotic organisms. *Gymnodinium*, *Melosira*, *Eudorina elegans*, *Tetrahymena*, and *Amoeba* were all presented in broth culture while Champignon, Koralle, and Salat were presented as solid living samples. In order to extract DNA we used the Qiagen Kit for DNA genome extraction. For liquid samples, we took 2mL of the sample and centrifuged at 10,000 rpm for 1 minute and discarded the supernatant. Liquid nitrogen was then used to freeze the samples, which were then broken apart using mortar and pestle. Once this was done, we added 400 microliters of buffer AP1 and 4 μL of RNase A to the sample. This was then vortexed and incubated for 10 min at 65 ℃, inverting 2-3 times. The AP1 buffer contains EDTA and SDS and is needed to lyse the cells, while RNase A degrades mRNA that might be present in the sample. Next, 130 μL of buffer AP2 were added, mixed and incubated on ice for 5 min. This buffer is mostly an acetic acid mixture that allows polysaccharides and detergent to precipitate. The lysate was put into a spin column and centrifuged for 2 min at 14,000 rpm to get rid of large molecules and organelles. Large molecules and organelles are going to be located in a pellet at the bottom of the tube, while lighter molecules such as DNA are going to be in the supernatant. Due to this, the supernatant was transferred into a new tube, while the pellet was discarded. Once this was done, 1.5 volumes of buffer AP3 was added and mixed into the flow-through by pipetting. Once this was done, 650 μL of the mixture was transferred into the spin column and centrifuged for 1 minute at 8,000 rpm. The liquid was then discarded and the centrifugation was repeated again. The spin column

was then placed in a new collection tube and 500 μL of buffer AW was added. This was centrifuged for 1 minute at 8,000 rpm and the supernatant was discarded afterwards. Another 500 μL of buffer AW was added, the sample was centrifuged for 2 minutes at 14,000 rpm and the supernatant was discarded. The spin column was then placed into a new tube and 100 microliters of elution buffer were added to each of the samples that we used. This was incubated for 5 minutes at room temperature and centrifuged for 1 minute at 8,000 rpm to elute the DNA off of the matrix. The final step was repeated again to elute any remaining DNA off of the matrix.

2. PCR

    a. In order to amplify our DNA sequence, we performed PCR on the DNA of each of the eukaryotic samples. PCR stands for polymerase chain reaction and is a technique that allows for the amplification of a sequence of DNA. PCR involves the use of different cycles of heating and cooling. In the thermocycler, the DNA was heated to 95°C so that the DNA strands can denature. Then the reaction was cooled to about 53°C in order for the primers to anneal. Next, the reaction was raised to 72°C so that the polymerase can elongate the DNA sequence from the primer. This was repeated for 35 more cycles in order to amplify the DNA. In order to perform the PCR reaction, the extracted DNA from each organism was added in addition to dNTPs, two primers, magnesium, and DNA polymerase. Because this reaction was performed in a thermocycler and goes to high temperatures we needed a DNA polymerase that is able to withstand high temperatures. The polymerase that was used in our PCR reactions was called *Taq* Polymerase. This polymerase was extracted from the

thermophilic bacteria *Thermus aquaticus,* which is why it can withstand high temperatures.

The DNA that we aimed to amplify in our reactions was part of the gene for the 18s rRNA of each species that we were presented with. Ribosomal DNA sequences are some of the most conserved stretches of DNA from species to species, which is why these were chosen for comparison. The primers for the PCR reactions were determined using the DNA extracted from *E. gracilis*. We were presented with primers 1&5 (forward and reverse) and 2&6. Primers 1&5 amplify a product 1.5kb in mass, while primers 2&6 amplify a product 1.6kb in mass. It is shown below that the primers differ in their sequences:

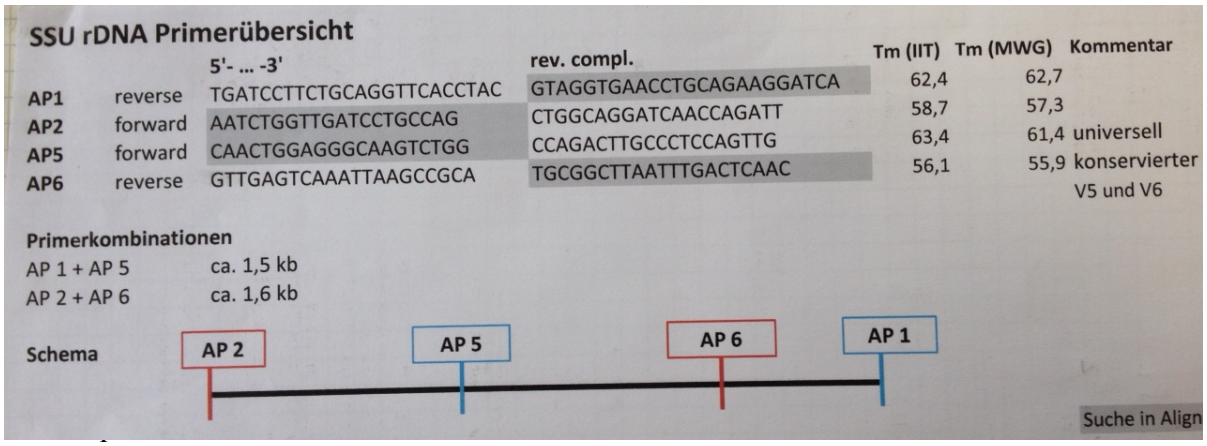

Figure 4: Primer Sequences

Primers 1&5 amplify a product slightly downstream of the product that primers 2&6 amplify. Annealing temperatures for the primers depend on the length and nucleotide composition of the primers. Longer strands of DNA have higher melting temperatures. The annealing temperature is usually optimal at plus or minus 7 from the actual annealing temperature. A gradient PCR was used in

multiple PCR reactions to determine the optimal annealing temperatures for primers 2&6 and are as follows: 48°C, 48.7°C, 49.6°C, 50.7°C, 52°C, and 53.4°C. Because primers 1&5 had a higher percentage of guanine and cytosine bases, their temperatures were slightly higher and the following temperatures were used to determine the optimum annealing temperature: 52°C, 53.4°C, 54.8°C, 56.1°C, 57.1°C, 58°C, 58.4°C. These temperatures were used to determine the best set of primers to amplify 18s rRNA as well as the optimum temperature for these primers to anneal. The optimum set of primers was then used in subsequent PCR reactions to amplify the small subunit ribosomal DNA from the other organisms that were used in this project.

3. DNA extraction and purification from agarose gel
    a. In order to find out if our DNA polymerase amplified the right bands, gel electrophoresis was used to examine this. In order to do this, we used a 1.5% agarose gel in TEA buffer and visualized it by staining with ethidium bromide. Once the correct DNA fragment was found, the band was cut out and purified using the Qiagen Gel Extraction Kit (Valencia, CA) for genomic DNA. This involved transferring the gel slice into a reaction tube and adding 650 μL of Gel solubilizer. This was then incubated for 10 min at 50 ℃ until the agarose gel slice was completely dissolved. Next, 50 μL of Binding Optimizer were added and mixed by vortexing to ensure DNA binding to the silica columns. This sample was then applied to the spin column, which contains a silica membrane for the DNA to bind to. This was then centrifuged at 12,000 rpm for 1 min and the filtrate was discarded. Once this occurred, 700 μL of washing solution LS was added and the solution was centrifuged at 12,000 rpm for 1

min. The filtrate was then discarded and the washing step was repeated again. The solution was then centrifuged at maximum speed for 2 minutes to remove all traces of ethanol and the filtrate was discarded. Finally, the spin filter was put into a 1.5 ml elution tube and 30-50 μL of Elution Buffer was added. This was incubated at room temperature for 1 minute and then centrifuged at 8,000 rpm for 1 minute. Another elution step was performed in order to increase the yield of DNA.

4. Ligation Reaction
    a. Once each sample of ribosomal DNA was amplified through PCR, the products had to be ligated into plasmids in order to produce enough DNA so that we can sequence it. The ribosomal DNA was ligated into a pCR 2.1 plasmid. The PCR product of the 18s rRNA was cut using restriction enzyme EcoR1 and was then ligated into the plasmid pCR 2.1.
5. Competent Cell Preparation using Rubidium Chloride
    a. Once the DNA was ligated into the plasmid, it had to be transformed into competent *E. coli* cells. To make the *E. coli* competent we took the TOP10 strain of *E. coli* and used the procedure from the Promega *Protocols and Applications Guide* (Madison, WI). We first inoculated a single colony from an LB plate in 2.5 mL of SOB medium. This was then incubated overnight at 37 ℃ with shaking (approximately 225 rpm). On the following day, the overnight culture was used to inoculate 50 milliliters of SOB medium containing 20 mM MgSO4 (this results in a 1:100 dilution). These cells were then grown in a 250 mL baffled flask until the A_{600} reaches 0.4-0.6 (typically 2-3 hours). A 250 mL flask is necessary for proper aeration during growth of these *E. coli*. The cells were pelleted from the media by centrifugation at 4,500 x g for 5

minutes at 4 ℃. These cells were then gently resuspend in 0.4 volume (based on the original culture volume) of ice-cold TFB1. For the remaining steps, the cells were kept on ice while all pipets, tubes and flasks were chilled. The resuspended cells were incubated on ice for 5 minutes and cells were then pelleted by centrifugation at 4,500 x g for 5 minutes at 4 ℃. The TOP10 *E. coli* cells were then resuspended in 1/25 of the original culture volume of ice-cold TFB2. Both solutions of TFB1 and TFB2 contain rubidium chloride but in differing concentrations, with TFB1 having a higher concentration of rubidium chloride. The rubidium chloride is used to alter the cell membrane so that these strains take up the cloning vector. The cells were then incubated on ice for 15-60 minutes and then aliquoted into pre-chilled tubes. The tubes were then frozen in liquid nitrogen and stored at -70 until they were ready to be used in transformation.

6. Transformation
    a. To perform the transformation of the pCR2.1 plasmid ligated with the ribosomal DNA into TOP10 competent *E. coli* cells, one shot of *E. coli* cells were thawed on ice (200 μL). 10 μL of each ligation reaction were pipetted into cells and stirred gently with the pipette tip to mix. These vials were then incubated on ice for 30 minutes. This was heat shocked for 1 minute at 42 ℃ without shaking and transferred to ice. Once this occurred, 500 μL of SOC medium were added to each vial. The vials were then incubated with shaking at 37 ℃ for 1 hour at 225 rpm. After the incubation, 300 μL from each transformation vial were plated on LB plates and incubated overnight.

Chapter 5:Results

1. DNA Extraction

    a. DNA was extracted from the following organisms:

	Name				
A	*Cyclotella sp.*	Bacillariophyceae	Kieselalge	SAR (Stramenopila)	A
B	*Closterium moniliferum*	Chlorophyta	Grünalge	Archaeplastida (Chloroplastida)	W
C	*Gymnodinium sp.*	Dinophyceae	Dinoflagellat	**SAR** (Alveolata)	W
D	*Melosira sp.*	Bacillariophyceae	Kieselalge	**SAR** (Stramenopila)	W
E	*Dinobryon divergens*	Chrysophyceae	Goldalge	**SAR** (Stramenopila)	W
F	*Spirogyra sp.*	Chlorophyta	Grünalge	Archaeplastida (Chloroplastida)	W
G	*Euglena gracilis*	Discoba	Euglenozoa	**Excavata**	
H	*Eudorina elegans*	Chlorophyta	Volvocales	**Archaeplastida** (Chloroplastida)	
I	*Tetrahymena thermophila*	Ciliophora		**SAR** (Alveolata)	
K	Champignon			**Opisthokonta** (Fungi)	
L	Koralle			**Opisthokonta** (Metazoa)	
M	*Amoeba proteus*	Tubulinea		**Amoebozoa**	
N	Salat ???				

Figure 5: List of Organisms and their Classification

It was shown that DNA was indeed extracted by running the DNA from each organism on an agarose gel. Some of the extractions proved to be difficult, so out of these 14 organisms, only eight of them were used for the transformation and ligation reactions. These organisms included *Gymnodinium*, *Melosira*, *Eudorina elegans*, *Tetrahymena*, Champignon, Koralle, *Amoeba*, and Salat.

2. PCR

In order to check for the right primers, a gradient PCR was performed on samples of *E. gracilis* DNA. The results of the PCR are shown in the following gel:

Legend:
Lanes 1-6: Products amplified by primers 1&5
Lanes 8-13: Products amplified by primers 2&6

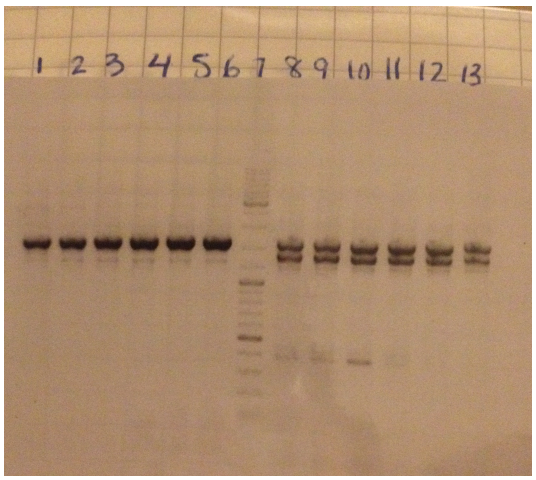

Figure 6: Gel of *E. gracilis* rRNA using primers 1&5 and 2&6

Primers 1&5 are in lanes 1-6 in order of increasing temperature in the gradient PCR, while primers 2&6 are in lanes 8-13. Because primers 2&6 showed two bands, this indicated that another DNA sequence was being amplified other than the ribosomal DNA sequence. Due to this, we picked primers 1 & 5 at an annealing temperature of 53.4. The PCR reactions of the other organisms were confirmed using gel-electrophoresis.

3. Purification from Gel

Before the transformation, a final gel of all of the PCR cleanups from each preparative gel electrophoresis was performed before ligations into the cloning vectors. This gel is shown below:

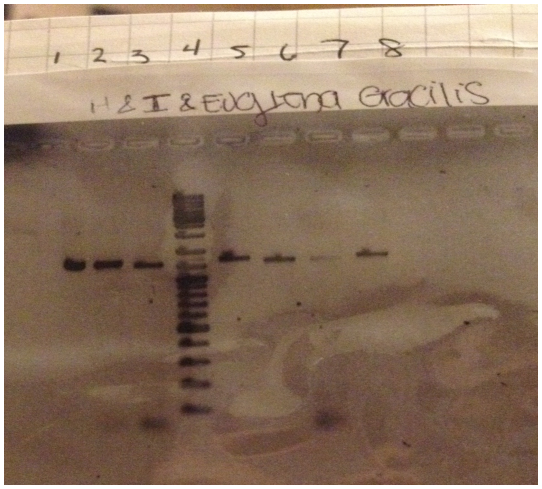

Figure 7: Final gel of all PCR cleanups

Legend
Lane1: *Melosira*
Lane 2: *Eudorina elegans*
Lane 3: *Tetrahymena*
Lane 4: DNA ladder
Lane 5: Champignon
Lane 6: Koralle
Lane 7: Gymnodinium
Lane 8: Salat

This shows that only seven of the organisms used showed bands. The only organism that did not show a band was *Gymnodinium*. All of the others were then determined to be sufficient to use in ligation within the cloning vector.

4. Ligation Reaction

The plasmid used for the ligation reaction is pCR 2.1. This plasmid contains the reporter gene lacZ so that we can check to see if the transformation was successful. In addition to this, it also has ampicillin and kanamycin resistance. These can also be markers to ensure that the plasmid has been taken up. The following shows the regions on the plasmid:

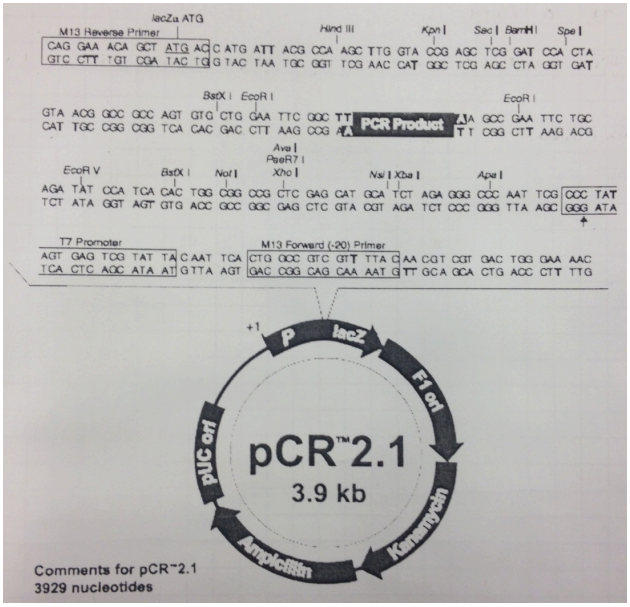

Figure 8: Plasmid Regions

5. Transformation

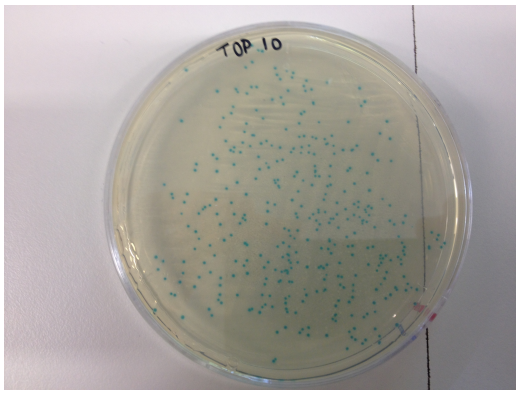

Figure 9: TOP 10 *E. coli* with transformed vector spread on X-Gal plates

In order to check if the transformation was successful, the cells were put on plates that contained X-Gal. The PCR product was supposed to go into the cloning region within the LacZ gene, thus disrupting the LacZ gene. A disrupted LacZ will not be able to produce B-galactosidase and the colonies will then stay white. A cell that has not taken up the plasmid will have a functional LacZ gene and will then be making B-galactosidase. This enzyme breaks down X-Gal to produce a blue precipitate, thus showing blue colonies. Our results for the transformation of *E. gracilis* showed that most of the colonies of TOP10 cells were blue, thus indicating that the plasmid was indeed taken up, but that the ligation did not work.

Chapter 6: Conclusion

Unfortunately, we were not able to complete the transformation for the other organisms that had the ribosomal (rRNA) gene amplified. Due to the time constraints, we were unable to fully finish our study. Further steps would have included transforming the DNA of the other organisms into the *E. coli*, lysing the *E. coli* cell and then sending the plasmid DNA to be sequenced. Sequencing of the DNA would allow us to compare the rRNA gene in all of the organisms studied. Using the DNA sequences, we would have then made a phylogenetic tree of how these organisms are related using the phylogenetic software that we were shown in our week long master's course at the University of Wuppertal.

Overall this project has provided both of us with a great wealth of knowledge and a sense of community in a university hundreds of miles away from home. Though we were not able to finish our project, the skills that we have learned throughout our six weeks in Dr. Preisfeld's lab have given us insight into the world of a research scientist and have prepared us for future lab work that we might encounter in our academic and professional careers.

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