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Sequencing the Mitochondrial DNA of Lagenidium juracyae

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Undergraduate Honors Thesis of

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Preface

After completing the courses Genetics, Cellular and Molecular Biology, and Genomics with Dr. Aurélien Tartar as my professor, I decided to supplement my coursework with research. As a pre-medical student, I realized the importance of conducting research which is often known to be a cornerstone of medicine. Thus, after developing a close relationship with Dr. Tartar and becoming fascinated with many of his current research projects, I decided that I wanted to tackle my own project alongside Dr. Tartar. Due to the pandemic, the initial stage of the research took place virtually as I finalized the project that I was interested in pursuing. After returning to campus, I met with Dr. Tartar where I began working to sequence the Lagenidium juracyae species beginning with DNA extraction.

My journey over the past 2 years has been extremely smooth. Working alongside Dr. Tartar has allowed me to learn a great deal of information about working in a lab. Although I had previous experience in a lab, it was this experience that taught me many of the basics including how to make an agarose gel and how to properly execute a PCR reaction. I feel as if Dr. Tartar allowed me to have independence in the lab but at the same time remained present just in case there was anything that I needed. During one specific experience, I was having trouble figuring out where in the PCR reaction I was going wrong. Dr. Tartar worked alongside me to find errors in my technique. After adjusting the methods, my results improved drastically and highlight just how much I was able to learn from this whole experience. In addition, through this research, I was able to understand more about mosquito infecting agents and the importance of sequencing these species in the field of pesticides. I am also someone who is interested in medicine and

thus hearing about the effects that the Lagenidium species have on mammals and their skin is something that was particularly important and interesting.

Towards the end of the research, I learned about how to write a proposal and a honors thesis which is something that has improved not only my scientific writing skills but has also greatly increased my ability to draw conclusions from the results.

Abstract

Lagenidium oomycetes are fungus-like eukaryotic microorganisms that are closely related to diatoms and brown algae. One defining characteristic of *Lagenidium* oomycetes is their ability to act as a parasite during the larval stage of mosquitoes. Thus, over the past few decades, mosquito infecting *Lagenidium* oomycetes have been considered to serve as pesticides in various industries. However, some *Lagenidium* oomycetes have been linked to new mammalian skin conditions, particularly in dogs. As more research has been conducted about these skin conditions, there has been new data showing the presence of new species of *Lagenidium* oomycetes aside from the most popular *Lagenidium giganteum*. My project set course to differentiate and sequence the mitochondrial DNA of one of these new emerging species known as *Lagenidium juracyae*. More specifically, my goal was to sequence the mitochondrial cytochrome c oxidase 1 & 2 genes and later compare these sequences to that of existing *Lagenidium* oomycetes. By doing this, more can be understood about the differences between the species and whether *Lagenidium juracyae* can be used instead of *Lagenidium giganteum* in future pesticides. After completing the necessary methods and procedures, various segments of the mitochondrial DNA of Lagenidium juracyae have been sequenced. This is important for future research projects which can look at the differences between the different Lagenidium species and their sequences, thus, allowing them to better understand which species may be a better alternative for future pesticides.

Acknowledgements

This Halmos College of Arts and Sciences Research Project and Farquar Honors College Thesis was made possible with the help of various individuals and organizations. I want to take the time to thank the Farquar Honors College for providing me with the resources necessary to take on an honors thesis. Specifically, I would like to thank Dr. Don Rosenblum who was an instrumental figure in helping me get started with my honors thesis and guided me as I worked to initially find an advisor for the project. I would also like to thank Dr. Andrea Shaw Nevins who was extremely accommodating with the timing of the thesis and was the person who made the defense of the thesis possible. I am most grateful for the help and mentorship provided by Dr. Aurélien Tartar who was there at every step of the process and ensured that not only was the thesis on track but made sure that I was continuously learning and enjoying the entire process.

Two years is a long sum of time and without Dr. Tartar's expertise, patience, and passion, none of this would have been possible. Finally, I wish to thank Nova Southeastern University for providing me access to research labs which was critical for this project to be completed.

Table of Contents

List of Figures

Sequencing the Mitochondrial DNA of Lagenidium juracyae

I. Introduction

a. Literature Review

In order to understand the relevance of why sequencing the mitochondrial DNA of *Lagenidium juracyae* is so pivotal, it is important to look back at previous research that has led to this point. Back in 2008, a journal called Trends in Microbiology released a study called *New insights into an*imal pathogenic oomycetes (1). This study focused on the devastating effects that different species of oomycetes can have particularly on the environment and on plants. The study elaborates on the well-researched and understood plant pathogenic species named *Phytophthora infestans* which is known for causing potato blight. However, more importantly, the article introduces the lesser-known idea of animal pathogenic species and emphasizes a few main species which have had major effects in the past. The article mentions how *S. parasitica* infects freshwater fish and how *A. astaci* has increased the mortality rates for freshwater crayfish. Relevant for the study of *Lagenidium* oomycetes, the article dives into the fact that the genus *Lagenidium* has been seen to affect dogs and other mammals. It is this portion of the study that builds the foundations for my project as I seek to sequence a specific species of *Lagenidium* oomycetes in hopes of finding an alternative to the disease-causing species that has already been used in commercial pesticides.

More about the impact that *Lagenidium* oomycetes have had on animals can be found in an article called "Transcriptome Analysis of the Entomopathogenic Oomycete *Lagenidium giganteum* Reveals Putative Virulence Factors." Specifically, this article served to characterize the transcriptome of the most common species of *Lagenidium* oomcyetes called *Lagenidium giganteum* (2). Through methods including 454

pyrosequencing and Sanger sequencing, the article reveals more about the ancestry of *Lagenidium giganteum* and the specific proteins that make it parasitic. Such an article provides me with information related to sequencing the *Lagenidium* species and thus serves as a useful source for my current research. Understanding more about the evolution of *Lagenidium* oomycetes is crucial as finding the link between different species is one of the main goals of the project. In regard to the species *Lagenidium juracyae*, the article "Phylogenetic and physiological traits of oomycetes originally identified as *Lagenidium giganteum* from fly and mosquito larvae" introduces new species of *Lagenidium* oomycetes which is found after the study investigated different isolates of mosquito larvae (3). Taking a closer look at the study that was conducted, it was found that the phylogenetic analysis of the United States Department of Agriculture Agricultural Research Service (USDA-ARS) Collection of Entomopathogenic Fungal Cultures found multiple clusters from ARSEF isolates that were expected to be *L. giganteum*. This discovery is one that is of major importance in the case of my project as it revealed a new species of *Lagenidium* oomycetes that were found in the mosquito larvae but not mammals. Other species of Lagenidium may have also been detected in bromeliads (4)

After reading about the discovery of *Lagenidium juracyae*, more information about its pathogenic activity and relationship to existing *Lagenidium* oomycetes is needed in order to determine whether it would serve as an effective pesticide. But how can this information be gathered? The answer to this question can be found in an article called "Comparative mitochondrial genome analysis of *Pythium insidiosum* and related oomycete species provides new insights into genetic variation and phylogenetic

relationships." The article highlights the importance of sequencing mitochondrial DNA when looking at the evolutionary history of a eukaryote (5). In addition, the article talks about the process of how to sequence as well as the details which can be collected after finding out the placement of an organism's genes. As the title suggests, the authors of the article look at *P. insidiosum* which is an oomycete found in standing water. They found that *P. insidiosum* was more closely related to *Pythium ultimum* than other oomycetes. Given that research has been conducted to show that *Lagenidium* oomycetes are phylogenetically similar to the plant pathogens *Phytophthora* and *Pythium* spp, it can be deduced that using similar lab methods will also allow us to learn more about the relationship between the different *Lagenidium* species.

II. Materials and Methods

The main objective is to sequence the mitochondrial DNA of *L. juracyae*. This involves first conducting DNA extraction in order to get the template DNA. This extraction only needs to be completed once for the duration of the project.

Performing polymerase chain reaction: Once the template DNA is extracted, the next step is to use PCR to amplify the DNA between various primers. Over the course of the semester, multiple attempts of PCR will be made to find out as much of the mitochondrial DNA as possible. For PCR to work, 2 primers will be used. One of these primers will be the forward primer and the other primer will be the reverse primer. In addition to the primers, template DNA will be added to the reaction as well as PCR buffer and dNTPs. Once these components are added, Taq polymerase will be used to build new DNA strands while the sample is in the thermocycler. The DNA extracted from Lagenidium Juracyae was amplified with various primer sets, including mitochondrial cytochrome oxidase subunit 1 and 2 primers, as well as other primers located in the mitochondrial DNA of Lagenidium Juracyae. The Taq polymerase used for all PCR reactions originated from the Taq PCR core kit (Qiagen). To prepare a 25 microliter reaction one microliter of DNA, one microliter of forward primer, one microliter of reverse primer, 2.5 microliters 9 of buffer, 0.5 microliters of dNTPs, 0.2 microliter of Taq polymerase, and 19 microliters of distilled water was added and mixed together. A master mix is prepared with these volumes to facilitate pipetting and divided according to the number of reactions needed. The master mix is also important for consistency in the results so that errors can be more readily be found instead of running each PCR reaction separately. For the 25 microliter reaction, all quantities were

multiplied by four and then divided by four in their respective tubes to add the different primers since there were four different reactions. Once the different reactions were prepared they are placed in the PCR machine to a particular program. PCR conditions were set to the following pattern repeated for a total of 30 cycles: 95 C for 30 seconds, 50 C for 30 seconds, and 72 C for 1 minute. However, over the course of the semester, many different variations to this program were used including the changing temperatures and increasing the number of cycles. This allows for the DNA to have more chances for amplification especially when the bands in the gel may not be bright enough or present.

After PCR is complete, gel electrophoresis was used to check to see if the PCR was successful. In order to complete gel electrophoresis, the first step was to set up the gel. This involved using agarose and TAE buffer to create the gel and a comb that will be used to make the wells. For this project, all gels were prepared using 50 mL of Tae buffer, 0.500 grams of agarose and 5 microliters of SYBR Safe. Then the flask was heated in a microwave to help the agarose dissolve in the flask and set for cooling for around 15 minutes after being poured into the gel plate. Once the gel solidified, the PCR products were then stained with 1 microliter of a purple loading dye and then placed in the gel. The gel ran for around 40 minutes to give time for the DNA fragments to separate due to an electrical current that was applied to the buffer. After the gel ran for the indicated time, it was then read using UV Light. At this point, the success of the PCR reaction was seen as the size of the band was compared to the expected band size.

For the last step, the band of DNA was cut out from the gel and purified using a DNA purification kit. Using a centrifuge and elution buffers, the DNA was washed and

purified. At this point, the final DNA sample as well as samples of the primers were sent in for commercial sequencing by Macrogen USA.

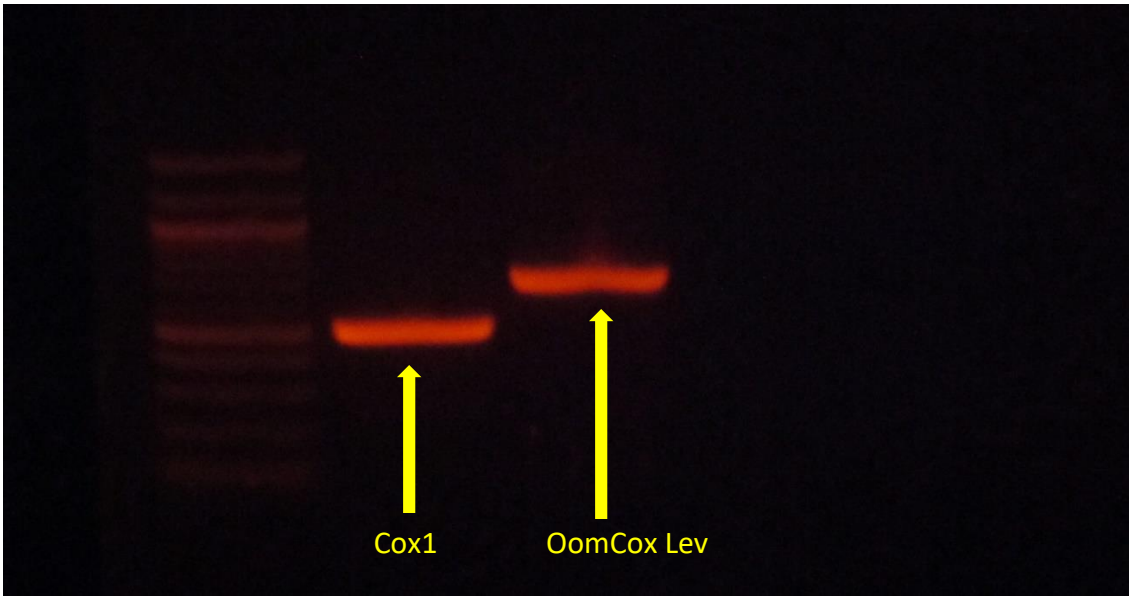
III. Results

The results of the research are broken down into different stages- with each stage aimed at using different primers to sequence different portions of the mitochondrial DNA of *Lagenidium juracyae.*

a. Part I: Sequencing using Cox1 and OomcoxLev Primers

The first primer that was targeted was Cytochrome c oxidase subunit I or Cox1. To sequence these this portion of the mtDNA, PCR reactions were used to amplify the DNA strands and gel electrophoresis was used to determine whether the PCR reaction as successful. A bright band in the gel signifies the presence of DNA which can then be purified and later sequenced. Below are images of the different gel results which reveal the successful PCR reactions of various genes in the mitochondrial DNA.

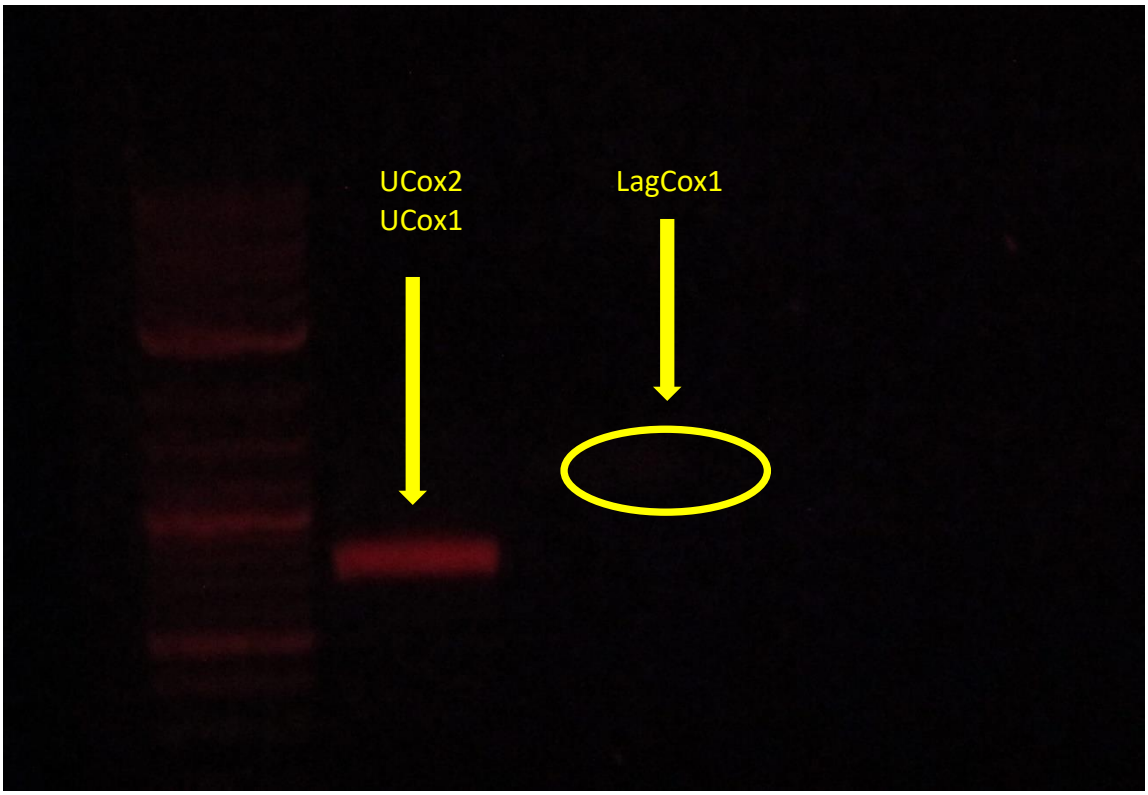
Figure 1: Gel Electrophoresis of Cox1 Primer Set and OomCoxLev Primer Set



In the figure above, the gel results for both the Cox1 primer set and OomCox Lev primer set are photographed. For Cox1, the Cox1 forward and Cox 1 reverse primers were used in a PCR reaction with the template DNA and the bright band above confirms the success of the PCR reaction at the expected sequence length. For the OomlevCox primer set, the OomlevCox lev Up and Lo primers were used in a PCR reaction. The result for the OomlevCox primer set also shows a bright band, thus confirming that the PCR reaction was successful.

b. Part II: Sequencing using UCox2F and UCox1R Primer Set and LagCox1 Primer Set

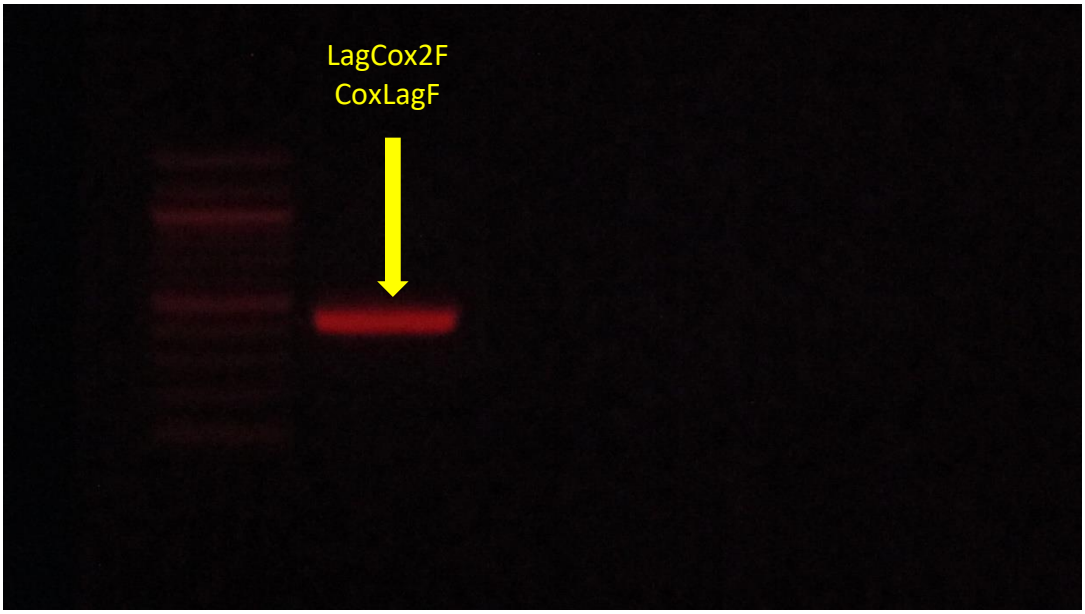
Figure 2: Gel Electrophoresis of UCox2F and UCox1R and LagCox1



In the figure above, the gel results for both the UCox2F & UCox1R primer set and LagCox1 primer set are photographed. For the UCox2F & UCox1R primer, both primers were used in a PCR reaction with the template DNA and the bright band above confirms the success of the PCR reaction at the expected sequence length. For the LagCox1 primer set, the LagCox1F and LagCox1R primers were used in a PCR reaction. The result for the LagCox1 primer set shows a very dull band, thus it showed that the PCR reaction did not amplify enough DNA but still can be sent for sequencing.

c. **Part III: Sequencing using LagCox2F & CoxLagF Primer Set**

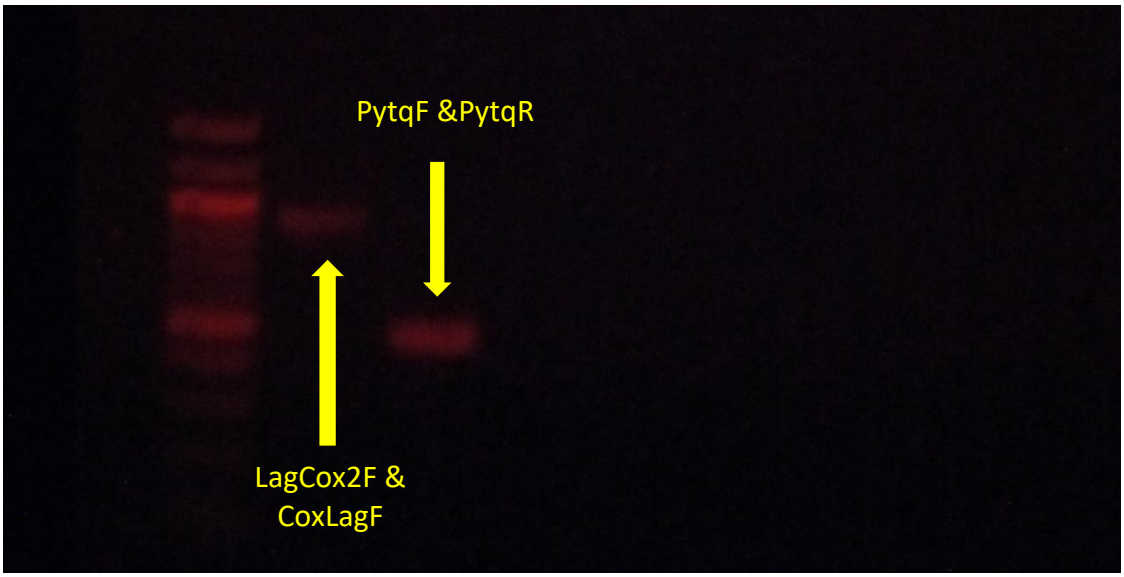
Figure 3: Gel Electrophoresis of LagCox2F & CoxLagF



In the figure above, the gel results for the LagCox2F & CoxLagF primer set is photographed. For the LagCox2F & CoxLagF primer set, both primers were used in a PCR reaction with the template DNA and the bright band above confirms the success of the PCR reaction at the expected sequence length.

d. Part IV: Gel Electrophoresis of LagCox2F & CoxLagF and PytqF &PytqR

Figure 4: Gel Electrophoresis of LagCox2F & CoxLagF Primer Set and PytqF & PytqR Primer Set



In the figure above, the gel results for both the LagCox2F & CoxLagF primer set and PytqF & PytqR primer set are photographed. For the LagCox2F & CoxLagF primer set, both primers were used in a PCR reaction with the template DNA and the bright band above confirms the success of the PCR reaction at the expected sequence length. For the PytqF & PytqR primer set, the LagCox1F and LagCox1R primers were used in a PCR reaction. The result for the PytqF & PytqR primer set also shows a bright band, thus confirming that the PCR reaction was successful.

e. Part V: Compiling a larger mtDNA genome sequence fragment

Figure 5: Combined Sequence of Lagenidium Juracyae

Lagenidium juracyae ARSEF 3327
mtDNA – 1,108 bp

TCGTTTAAATCAAACTTCAATGTTTATTAAAAGAGAAGGTGTTTTTTATGGTCAATGTAGTGAA
ATTTGTGGAGTGAATCATGGTTTTATGCCTATTGTTGTTGAAGCGGTTTCATTAGATGATTATT
TAACTTGGTTAAAAAACAAAGTTAATTTTGATTTTGATAAATAAGTCGAAAATATAATTTATGT
TTATAAAAACTTTTAGTGTAATAATTATTATATTATTATTATTTACAGATATAAAAAATATTAA
AAATAAAATTAAACATTTTTTAAAAAAATAAAAATTAAACATTGCGTAATTTTTAATTTAATAA
TTTATTAAAAAGTAATTTTCAATTAAAAAATTTTTAAAACATTTCATAAATGAATATTCAAAAT
ATTAAAAATTGGTCAACAAGATGGCTTTTTTCAACAAATCATAAAGATATTGGTACTTTATATT
TAATTTTTGGTGCTTTTTCTGGTGTAGTTGGTACAACTTTATCTATTTTAATTAGAATGGAATT
AGCACAACCAGGTAATCAAATTTTTATGGGAAATCATCATTTATATAACGTGGTTGTAACTGCA
CATGCGTTTATTATGATTTTCTTTATGGTTATGCCTGTATTAATTGGTGGTTTTGGTAATTGGT
TTATACCTTTAATGATTGGTGCTCCAGATATGGCTTTTCCAAGAATGAATAATATTAGTTTTTG
GTTATTACCACCATCTTTATTATTATTAGTATCATCAGCTATAGTAGAATCAGGAGCAGGTACA
GGTTGGACAGTATATCCACCTTTATCTAGTGTTCAAGCACACTCAGGACCTTCTGTAGATTTAG
CTATTTTTAGTTTACATTTATCAGGTATTTCATCATTATTAGGTGCAATTAATTTTTTATCAAC
TATTTATAATATGAGAGCACCTGGTTTAAGTTTTCATAGATTGCCTTTATTTGTTTGGGCTATA
TTTATTACAGCTTTTTTATTATTATTAACATTACCTGTTTTAGCTGGTGCGATTACAATGTTAT
TAACTGATAGAAATTTAAATACTTCGTTTTATGATCCATCAGGAGGAGGAGATCCAGTGTTATA
TCAACATTTATTTTGGTTTT

UCOX2F- AGATGCTTGTCCAGGTCGTT
UCOX1R- CCAACCAGTACCTGCTCCTG (reverse: CAGGAGCAGGTACTGGTTGG)
OOcoxLevup- TCAWCWMGATGGCTTTTTTCAAC
OOcoxLevlo- CYTCHGGRTGWCCRAAAAACCAAA
COX.LagF = 5'- CCACAAATTTCACTACATTGA-3' (rev: TCAATGTAGXXXXXX)

In the figure above, the resulting sequence generated by this project is shown, along with the location (and sequences) of the primers used. Sequence analysis showed that the obtained sequence is consistent with the Lagenidium genus and offer new information to characterize L. juracyae

IV. Discussion

After reviewing the results obtained from the PCR and sequencing reactions for *Lagenidium juracyae*, the final compiled sequence presented in Figure 5 was consistent with other *Lagenidium* species which had previously been sequenced. Using gel electrophoresis, the bright bands which were seen provide confirmation that Lagenidium juracyae DNA was successfully extracted as well as amplified. In the first gel, presented in Figure 1, bands for the Cox1 Primer Set and OomCoxLev Primer Set show successful PCR reactions for both segments of DNA. In the second gel, Figure 2 shows bands for the UCox2F and UCox1R Primer Set and LagCox1 Primer Set, although the band for LagCox1 was not as bright as expected. In the next part of the project, PCR reactions involving the LagCox2F & CoxLagF Primer Set resulted in bright bands(Figure 3. The same was observed for the LagCox2F & CoxLagF Primer Set and PytqF & PytqR Primer Set(Figure 4).

While the results were favorable, more can still be done to further understand the relationship between *Lagenidium juracyae* oomycetes and other similar species. One possible course of action is to finish sequencing more base pairs and genes from the mitochondrial DNA of *Lagenidium juracyae*. In order to do this, the use of more primers in different regions of the mtDNA is needed as well as the use of more PCR reactions. Through these reactions a much larger portion of mtDNA will be sequenced and can be compared to the mtDNA of the other *Lagenidium* oomycetes.

Another possible route for future research can involve using the Basic Local Alignment Search Tool or BLAST to further compare the sequences of *Lagenidium juracyae* and other species even outside of the Lagenidium oomycetes. This step would

be important for understanding more about the parasitic nature of *Lagenidium juracyae* and would help determine if it is unique

The most important next step would be to determine if the mtDNA found on the skin of mammals with skin disorders matches the mtDNA of *Lagenidium juracyae*. The purpose of this step would be to determine if this species is also a risk to mammals or if it is safe to use as a pesticide in the future. By analyzing discrepancies between the two DNA sequences, more ca be understood about which genes are at fault for developing the skin disorders. While this will require a much greater understanding of the Lagenidium species as a whole, the results will give a larger glimpse of how these oomycetes negatively impact other organisms.

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