ALTERING THE GENOME OF THE MICROCRUSTACEAN, DAPHNIA, WITH EXPOSURE TO THE MUTAGEN, PROFLAVINE

by

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Presented to the Faculty of the Honors College of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

HONORS BACHELOR OF SCIENCE IN BIOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON

December 2021

ACKNOWLEDGMENTS

I gratefully acknowledge Dr. Sen Xu for being my mentor on this project and allowing me the opportunity to conduct research in his Genetics lab. I would also like to express appreciation to Marelize Snyman for taking her time to work with me and guide me through this project. Further, I would like to express gratitude to my partner in research, Aleema Haq, for her cooperation in this project, as well as for staying by my side throughout my college journey. I would like to thank the Honors College staff, especially Bobbie Brown and Rebekah Chojnacki for their continued assistance and guidance on the completion of my thesis.

I would like to thank my family for their endless support and encouragement, which includes my parents, whose dedication and sacrifices are the reason I was able to complete my degree. I would like to thank my brothers for inspiring me and providing me with unconditional support. I am grateful for everyone who has made a contribution to my life, motivating me to succeed in my academic career.

November 16, 2021

ABSTRACT

ALTERING THE GENOME OF THE MICROCRUSTACEAN,

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The University of Texas at Arlington, 2021

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DNA can be altered by introducing mutagens that can induce mutations and alter the genomes of organisms. The microcrustacean, *Daphnia*, was exposed to Proflavine, a mutagenic disinfectant, and the effects were seen by breeding the organisms and sequencing their genomes. Isolates of *Daphnia* were collected, and then solutions with the mutagen were prepared to determine the adequate concentration to utilize for exposure. After successive reproductions, the babies were monitored, then sent to sequencing to examine mutations present in the DNA. After many trials, the optimal concentration for Proflavine was determined to be 35 ug/mL, showing the ability of *Daphnia* to reproduce in subsequent generations in the presence of a mutagen. Sequenced genomes illustrated an increased rate of insertions and deletions (indels). On average, the indel mutation rate was 4.22×10^{-5} higher than the control for the Aromoose isolates and 1.01×10^{-4} for the Warner

5, signifying the presence of frameshift mutations. Research will continue being conducted to see the phenotypical effects in growing generations, thus creating a successful forward genetics screening method.

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CHAPTER 1

INTRODUCTION

1.1 Forward Genetic Screening

Changing genomes and inducing mutations in organisms has been a key point in directing the advancement of genetics. Scientists have been looking into different technologies such as CRISPR, which function to do the same, alter genomes, but in a wider context. It has the capability to further its potential by causing DNA mutations that lead to diseases and other genetic conflicts. However, on a narrower basis, multiple studies have been focused on introducing mutations into a genome and then sequencing it to obtain more information about identifying the types of mutation and its effects in successive generations. Forward genetic screening is commonly used as an approach to identify the set of genes that correspond to a specific phenotype. With this knowledge, the phenotype of interest can be identified and used for further studies which annotate the genes involved.

1.1.1 Impracticality of Utilizing Spontaneous Mutations

It is difficult to accomplish genetic screening utilizing the spontaneous mutations that occur within organisms. This is due to the mutation rates being extremely low, as spontaneous mutations usually arise from an error in normal cellular processes such as DNA replication or transcription or from mutagenic nucleotide substrates (Maki 2002). It is easier to study mutagenesis for bacterial systems due to their fast growth rate and thus higher mutation rate (Schroeder et al. 2017). However, it is futile to depend on this for experiments involving multicellular eukaryotic organisms as it would take an excessive

number of animals to be screened to find any mutations that occur. Therefore, it is more practical to induce mutations by using an external factor, such as a mutagen, to alter the genome. The mutations can occur through changes in a single base or fully rearranging segments of DNA referred to as a frameshift mutation. These mutations consist of indels, which are the insertions and deletions in the sequence. The addition of an extra nucleotide in the sequence is known as an insertion while skipping the replication of a nucleotide is known as a deletion.

1.1.2 Past Mutagens and their Effects

Many mutagens such as N-ethyl-N-nitrosourea (ENU), trimethylpsoralen with ultraviolet light (UV/TMP), and Ethyl Methanesulfonate (EMS) have been used in past research to implement genetic screening (Snyman et al. 2020). The genome mutation rate and the spectrum were determined by introducing the mutagen, Ethyl Methanesulfonate (EMS), into three different species of *Daphnia*. The research illustrated that the treatment-induced base substitutions at a rate of 1.17×10^{-6} across the three varying freshwater species at 10 mM and even higher, 1.75×10^{-6} at 25mM (Snyman et al. 2020). The study also illustrated that the EMS-exposed female *Daphnia* of the F^0 generation was able to asexually reproduce to create the mutant offspring of the F^1 generation. This illustrated the capacity of the mutagen to sustain the mutation, altering the genome of the microcrustaceans and displaying similarities present in the location of the genome mutations.

1.2 Proflavine

1.2.1 Proflavine Characteristics and Uses

There are numerous mutagens with varying properties that could be used for genetic screening. Among them, Proflavine, a surface disinfectant, has been utilized in

many instances to contribute to genetic experiments. Proflavine can be used as an antimicrobial agent as it is bacteriostatic. It is usually used as a topical agent for superficial wounds; thus it is found in many wound dressings. This mutagen denatures the DNA of bacteria which leads to bacterial lysis. Because it is able to affect the host DNA, it has carcinogenic properties (Calberg-Bacq et al. 1977).

1.2.2 Past Research with Proflavine

Past research has been done to see the effects of Proflavine (PF) on other types of DNA. On contact with skin, Proflavine was able to alter the epidermal DNA, thus leading to mutations (Gatasheh et al. 2017). It was shown that Proflavine is able to penetrate further into the epidermal and dermal structures. Frameshift mutations were observed during an in vitro DNA replication of *Escherichia Coli*, concluding that the mutation was produced due to a consequence of exonuclease or DNA polymerase activity (Gatasheh et al. 2017). In bacteriophages, the insertion of Proflavine also caused frameshift mutations to occur, with most of the mutations being deletions or duplications of bases. Deletions were less common than duplications (Ripley and Shoemaker 1983). The effects of single-strand breaks in DNA were also investigated, and it showed that it enhanced Proflavine mutagenesis (Sarabhai and Lamfrom 1969). Other research also included other factors as it illustrated that exposing bacteria to Proflavine in the presence of visible light resulted in both frameshift and base substitutions (Speck and Rosenkranz 1980). This shows that other factors such as light can alter the experiment; therefore, the research must be closely monitored to avoid any outlying variables that could invalidate the outcome.

1.3 Daphnia

1.3.1 Daphnia, The Model Organism

Proflavine proves itself to be an adequate candidate to research mutagenesis with the numerous successful results it had in past studies. It will be utilized to induce mutations in the freshwater microcrustacean *Daphnia*. These organisms, also known as water fleas, reside in eutrophic water bodies and are filter feeders, utilizing algae to survive. They are easily cultured in the laboratory as long as adequate food and aeration are guaranteed (Peake 2016). These creatures are used for genetic screening purposes due to their reproduction methods, short generation time, and easy maintenance. *Daphnia,* which consist of a cyclic parthenogenetic life cycle, can reproduce both sexually and asexually. (Alekseev and Lampert. 2001). They are able to switch their reproduction methods in response to environmental stimuli.

1.3.2 Reproduction Methods

Typically, in asexual reproduction, diploid eggs are produced, which develop into genetically identical clones of the parent. Sexual reproduction is induced under adverse conditions, such as extreme temperatures or low food availability. Females produce haploid eggs, which are fertilized by males and encased in ephippia, which are dormant eggs. The eggs enter diapause, stay in ephippia, and are able to hatch when conditions are favorable (Alekseev and Lampert. 2001). The fast growth rate of the water fleas also contributes to successful genetic screening. In their breeding season, females can produce eggs as often as every four days. Usually, the generation time is around 7 to 10 days, also producing multiple broods at a time. Due to the favorable characteristics of *Daphnia,* it has been a key organism to utilize to study mutagenesis and implement forward genetic screening.

1.4 Hypothesis

According to the research noted above, due to Proflavine's effects on DNA, as well as the favorable characteristics of *Daphnia*, insertion and deletion (indels) mutations are hypothesized to occur and alter the genome. This is a type of frameshift mutation, which is known to completely alter the amino acid sequence of a protein, thus affecting gene expression. In order to introduce Proflavine to the water fleas, an adequate concentration must be synthesized, which is hypothesized to be stronger than the ones used for past mutagens due to its strength, high tolerance, and carcinogenic properties. The concentration cannot be too lethal or too survivable as it should be around a 50/50 percent survival and death rate in order to obtain an adequate concentration in which the *Daphnia* are indeed affected but not completely eradicated. After the concentration is found, the Proflavine-treated organisms will then reproduce and be sent to sequencing and analyzed in order to find any mutations present and the specific types. This will be a contribution to the ongoing research illustrating the effects of multiple mutagens and their ability to alter genomes. Although this research will be done with minuscule microcrustaceans, this research will aid in devising screening variations that can identify genes leading to a mutant phenotype of interest. It is expected that the insertion of the mutagen will lead to the mutations in random areas of the genome; however, this research can help determine if there are any similarities or consistency within the changes in the genome. Forward genetic screening or mutagenesis screening is the target of this research to further identify and change genes that alter the phenotype of organisms.

CHAPTER 2

METHODOLOGY

2.1 Experimental Preparations

2.1.1 Breeding the Experimental Animals

This study utilized six cyclically parthenogenetic (CP) *Daphnia pulex* isolates were utilized in this study; however, the only two isolates sent to sequencing were Aromoose and Warner-5 clones. The isolates of *Daphnia* were collected from different bodies of water in the country. The animals were prepared and grown in the lab in *Daphnia* combo, which is artificial lake water. They were allowed to continuously reproduce in a 16:8 hour light and dark cycle at around 18 degrees (Snyman et al. 2020). The organisms were fed with green algae *Scenedesmus obliquus* at least twice a week. The beakers in which the Daphnia resided were consistently changed in order to avoid algae from accumulating and clouding the beaker. The transfer of the animals was done in careful conditions to avoid contamination.

2.1.2 Obtaining the Adequate PF Concentration

Solutions with the mutagen were prepared and examined to find the adequate concentration in which the Daphnia is not thriving but still shows the capability to survive in. The optimum concentration was one with a 50% death and survival rate in order to allow the *Daphnia* to be affected by the mutagen, but not too lethal, which would eradicate

all of them. A total of six varying concentrations, 25 ug/mL, 35 ug/mL, 45 ug/mL, 55 ug/mL, 65 ug/mL, and 75 ug/mL were utilized in order to determine the adequate one. Proflavine was inserted into the six tubes along with the Daphnia combo, and around 10-12 organisms were placed in each. The animals were exposed for four hours and then placed again in artificial lake water. The survival rate of the organisms was recorded after 24 hours and analyzed by line graphs. The favorable concentration was then utilized for the remainder of the experiment. The F0 generation was then allowed to reproduce under adequate conditions to create the F1 generation.

2.2 Genome Sequencing

2.2.1 DNA Extraction by CTAB

The F1 generation, which was affected by the optimal Proflavine concentration, was then subjected to DNA extraction. The extraction was completed by the Cetyl Trimethyl Ammonium Bromide Method, otherwise known as CTAB. In this method, a homogenization buffer was first prewarmed at 65 degrees. 200 uL of the buffer was then inserted into a microcentrifuge tube that contained around 50-150 *Daphnia*. The sample was then ground by a blue pestle which also was washed with another 300 uL of the homogenization buffer. The sample was incubated for 60 minutes under a 65 degrees water bath. It was then centrifuged for five minutes. 500 uL of chloroform and 5-10 uL RNase. was added after successive incubating and centrifuging periods. The supernatant was transferred to a new tube and mixed with two volumes of cold ethanol. It was incubated and centrifuged again. 50 uL of H20 was added, and then the sample was subjected to re-dissolve overnight. The concentrations of the samples of DNA were measured by utilizing a Thermo Fisher Qubit 4.0 Fluorometer. The DNA's quality was examined on a 2% agarose

gel by electrophoresis. DNA was then sequenced on an illumina platform with 150-bp paired-end reads (Snyman et al. 2020).

2.2.2 Computational Mutation Analysis

The Burrows-Wheeler Alignment Tool BWA-MEM was used to align the raw reads of each mutant line to the reference genome. SAMtools (Li *et al.*, 2009) was used to remove reads that mapped to multiple locations in the genome. This aided in reducing potential false-positive calls of mutations. The MarkDuplicates function of Picard tools was utilized for locating and tagging PCR duplicates, further reducing conflicting reads (Snyman et al. 2020). BCF tools were used to call indels; furthermore, the BCF stat function compared the number of indels in each sample and ensured that the indels mapped were unique and not shared between the other samples. The total insertions and deletions in the isolates were then analyzed by graphs and compared to the control to see if there was indeed an increase in the number of indels and if a quantified rate of increase could be determined. To find the rate, the number of indels in the control was subtracted from the total amount of indels in the isolates, then divided by the total number of base pairs in the *Daphnia* which is approximately 200,000,000 bp. If it is a viable rate showing at least a tenfold increase, it would indicate that frameshift mutations did indeed occur. The indel mutation rates of the three clones in each isolate were then averaged together to roughly provide a quantified value to see what extent the genome changed by.

2.2.3 Analyzing the F2 Generation

After sequencing the animals of the F1 generation, the mutant lines were maintained and allowed to reproduce to create the next generation, which is to be known as the F2 generation. The F1 mutants are creating ephippia, which are resting eggs. They

are being dissected then closely monitored to find any signs of development. Currently, the ephippia, dormant eggs of the F1 *Daphnia* generation are being dissected, removing the 1-2 eggs which are in the ephippium pouch. First, these eggs were incubated for about 2-3 weeks in artificial lake water, then placed under ultraviolet light. If no signs of development are present after checking each day for around 5-6 days, then the eggs are removed from the ultraviolet light and placed in the incubator again for a week. Signs of development are checked once again, but if there is none, the eggs are moved under natural light. Hatched eggs are grown under natural light and fed with a minimal amount of algae corresponding to their size.

CHAPTER 3

RESULTS

3.1 Determined PF Concentration

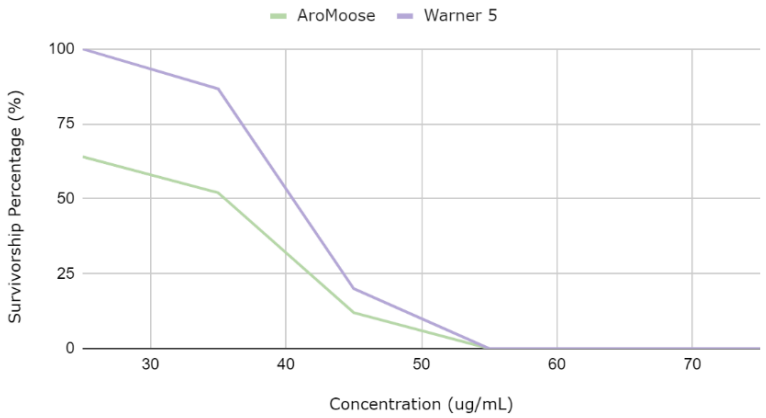
3.1.1 Survival Rates of Proflavine-Exposed Daphnia

Many trials occurred to obtain the optimum Proflavine concentration needed to proceed with the experiment. When the *Daphnia* was exposed to the low concentrations of the mutagen, it had a high survival rate, which was not in accordance with the optimum concentration needed for the experiment to continue. The Proflavine concentrations tested were 25 ug/mL, 35 ug/mL, 45 ug/mL, 55 ug/mL, 65 ug/mL, and 75 ug/mL on the Daphnia isolates Aromoose, Pine 1, 3L2-1, and Warner 5. For the 25 ug/mL, the survival rate was high in almost all of the isolates except Aromoose, which had a rate of 64 % (Table 1). For the 35 ug/mL trial, there were relatively lower survival rates nearing the fifty/fifty mark. The 45 ug/mL trial illustrated exceedingly lower rates ranging from 12-20% in all of the isolates. The 45-65 ug/mL solutions had no survivors in any of the isolates (Table 1). The survival rates of Aromoose and Warner 5 were closest to the 50/50 survival/death rate. The concentrations of the two isolates were compared to see the effects of the mutagen concentration on survivorship. The survival rate ranged from 64-100% on the first exposure at 25 ug/mL. However, there was a drastic decline in the survival rate starting from the 35 ug/mL concentration and ending at 45 ug/mL for both isolates (Graph 1). The rate then terminated to zero at 55 ug/mL.

Table 3.1: Survival Rates of Multiple Daphnia Isolates

Multiple *Daphnia* isolates were subjected to a four-hour exposure of Proflavine, then placed in artificial lake water. The survival rate of *Daphnia* was calculated 24 hours after the exposure with the mutagen.

Proflavine Concentration	AroMoose	Pine 1	3L2-1	Warner 5
25	64	93.33	93.33	100
35	52	80	80	86.6
45	12	33.33	13.33	20
55	0	0	0	0
65	0	0	0	0
75	0	0	0	0



Graph 3.1: Survival Rates of Warner 5 and Aromoose Isolates

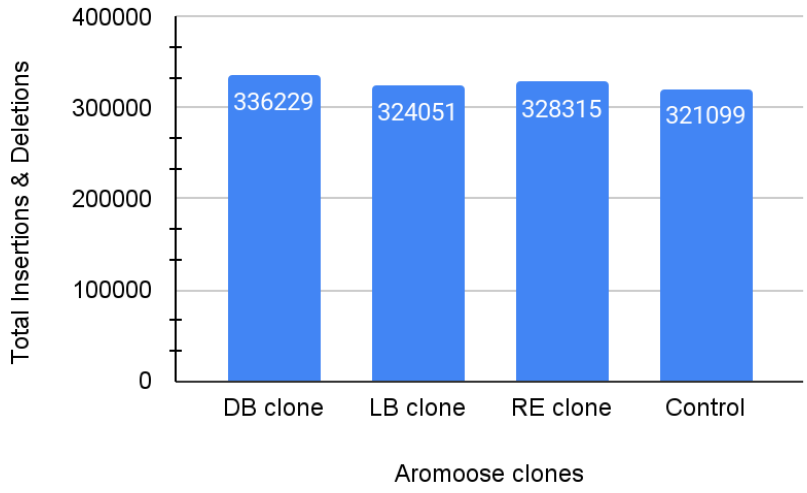
Daphnia isolates were graphed and compared to the varying Proflavine concentrations.

3.2 Presence of Indels in *Daphnia* Isolates

3.2.1 Aromoose Clone Indels

The isolates with the best results were Aromoose and Warner 5, which were then sent to sequencing. The 3L2-1 and Pine-1 clones were disregarded for the remainder of the

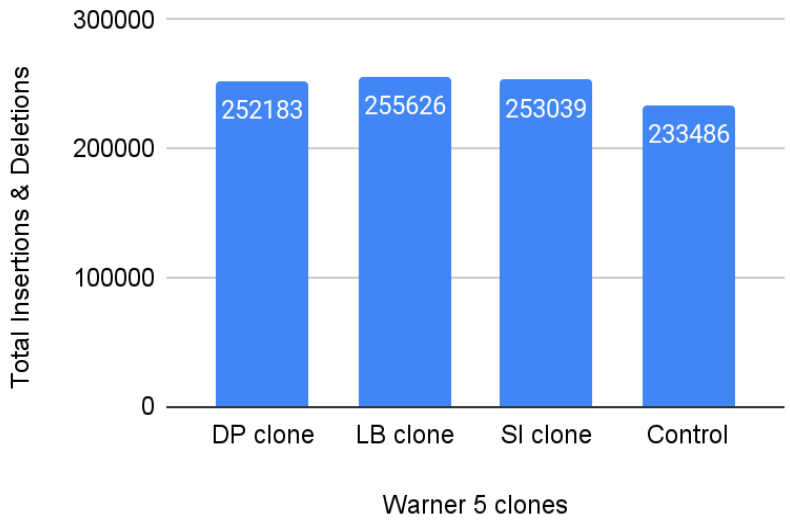
experiment. BCF tools were then used to call the indels from the Aromoose and Warner 5 isolates after sequencing. There were varying Aromoose clones which were distinguished by their respective labels. In the clone labeled dark blue (DB), there were 336229 indels, in the light blue (LB) clones, there were 324051, the red (RB) clones had 328315, while the control of the isolates had 321099 total indels (Graph 2).



Graph 3.2: Total Indels in Varying Aromoose Clones
Multiple Aromoose clones were sequenced, dark blue (DB). light blue (LB), red (RE) and the control. The total number of indels were called in each and portrayed in the figure above.

3.2.2 Warner 5 Clone Indels

The total indels in varying Warner 5 clones were less than the ones called in the Aromoose clones. The Aromoose clones each had approximately 100,000 more indels than the ones seen in Warner 5. There were around 252183 indels in the dark purple (DP) clone and 253039 in the silver (SI) clone (Graph 3). The light blue (LB) clone had the greatest amount of indels at 255626, while the control had the least, containing 233486 (Graph 3). The control in both samples had the least amount of indels in their respective isolates. The other clones all varied near the same amount.



Graph 3.3: Total Indels in Varying Warner 5 Clones
Multiple Warner 5 clones were sequenced, dark purple (DP). light blue (LB), silver (SI), and the control. The total number of indels were called in each and portrayed in the figure above.

3.2.3 Indel Mutation Rate

The Indel mutation rate, or ratio of insertions/deletions to the control, was calculated by comparing the clone indels to control indels then dividing it by the total number of base pairs in *Daphnia* (200,000,000 bp). This rate gave us a quantified rate of increase of indels in the Proflavine-treated isolates. For the Aromoose clones, the dark blue had the highest mutation rate at 7.57×10^{-5}, while the light blue clone had the lowest rate at 1.48×10^{-5} (Table 2). Pertaining to the Warner 5 clones, the light blue clone had a rate of 1.11×10^{-4} which was significantly higher than all of the other clones in both isolates. The other two Warner 5 clones, dark purple and silver, were around the same rate 9.35×10^{-5} and 9.78×10^{-5} respectively (Table 3).

Table 3.2: Indel Mutation Rates of Varying Aromoose Clones

Aromoose clones	Indel mutation rate
DB clone	7.57E-05
LB clone	1.48E-05
RE clone	3.61E-05

Table 3.3: Indel Mutation Rates of Varying Warner 5 Clones

Warner 5 clones	Indel mutation rate
DP clone	9.35E-05
LB clone	1.11E-04
SI clone	9.78E-05

CHAPTER 4

DISCUSSION

In this study, Proflavine-induced mutations were observed, adding to the advancement of a forward genetics screening method. It was illustrated that the mutagen, Proflavine, was able to alter the genome of the microcrustacean, Daphnia. Exposing the organisms to the mutagen at a concentration of 35 ug/mL for four hours affected the oocytes of female Daphnia, allowing mutations to occur and carry on to successive generations.

4.1 Hypothesis Analysis

4.1.1 Determined Concentration

The concentration of Proflavine was the determinant of the entire experiment, as that amount was utilized to expose the Daphnia and observe the long-term effects. Among the six concentrations that were tested, 35 ug/mL was chosen to proceed forward due to its optimal survival rate in the isolates. The lower concentrations had too many *Daphnia* surviving, which illustrates that the mutagen did not affect the animals as needed; therefore, utilizing those concentrations would have a low mutation rate. The higher concentrations did not have organisms surviving; therefore, they had to be disregarded. The survivorship graphs indicated that the optimal concentration was nearing the fifty/fifty survival rate needed for the study. Aromoose and Warner 5 illustrated the closest rates, and although Warner 5 had a slightly higher rate than wanted, the organisms were still affected. This high survival rate illustrates why there were ultimately fewer indels present in this isolate.

As hypothesized, a relatively greater concentration was needed as compared to other mutagens such as Acridine Orange (AO) and ethyl methanesulfonate (EMS). The minimal concentration for AO was 1.5 ug/mL, while for EMS, exposure with 10mM or 25 mM caused mutations to be induced in *Daphnia* (Snyman et al. 2020). Proflavine needed to be highly concentrated to induce mutations, which illustrates the high tolerance of the *Daphnia* to this mutagen. Potentially, the reason for some clones being unaffected by the treatment could be due to some animals having resistant properties; thus, they would not be susceptible to the mutagen. With experiments done on *Daphnia magna*, it is understood that clonal differences make it difficult to draw general conclusions about resistance; however, most observations indicate that the resistance of *Daphnia* initiates in their early development and increases over time (Garbutt et al. 2014). Nevertheless, the optimal concentration of the PF was sufficient to produce an effect on the animals, indicating the presence of mutations.

4.1.2 Presence of Mutations

The presence of indels confirmed that frameshift mutations did indeed occur with the exposure as anticipated due to the results of past studies in other organisms. The sequenced F1 generation exhibited that there was a greater amount of indels in the clones than in the control. This shows that the organisms were indeed affected as the increase of insertions and deletions in the clones signify that Proflavine was the factor that induced the mutations. The clones were all kept in the same environment and subjected to the same treatment in order to reduce any bias or errors. However, the differences in indels within the clones could also be attributed to any contamination or uneven distribution of treatment that could have occurred in the duration of the experiment. The amount of mutagen that

each animal was exposed to is subjected to variation as it is not guaranteed the extent to each organism is affected. The average indel mutation rate for the three Aromoose clones was 4.22E-05, while for the Warner 5 clones, it was 1.01E-04. The mutation rates for both the Aromoose and Warner 5 isolates illustrated at least a tenfold increase from the control, further quantifying the effect Proflavine had on the number of indels. The effects of the mutagen were hypothesized due to the studies that illustrate Proflavine has previously induced frameshift mutations in the past. In the bacterium, *Salmonella typhimurium*, Proflavine caused frameshift mutagenic activity for multiple strains, illustrating its tendency to affect bacteria (Speck and Rosenkranz 1980). However, this experiment displays that the mutagen can also induce frameshift mutations in heterotrophic, multicellular organisms, opening new possibilities for mutagenesis research

4.2 Future Research

As mentioned previously, scientists are continuously looking at options to advance on CRISPR (clustered regularly interspaced short palindromic repeats) research. Research such as these aid in developing genome editing techniques that can further introduce genomic mutations. In an experiment with *Daphnia magna*, genome alteration was mediated by the CRISPR system to affect the gene associated with eye development (Takashi et al. 2014). It was illustrated that when Cas9 mRNAs were inserted into the eggs, abnormal eye morphology was depicted in around 18-47% of the survived animals. After the *Daphnia* was monitored and able to mature, approximately 8.2% of the adults produced offspring with deformed eyes, carrying out the mutation to the successive generation. CRISPR was successfully utilized to target and affect a specific gene in the eye loci, and further creating heritable mutations and phenotypic effects. For my study, The F2

generation is also being currently analyzed as well to see any phenotypic effects the mutagen caused in the creatures. There is a large scope for advancement towards experiments, seeing how mutations can be induced, affecting the genome; moreover, specificity towards the alteration is in the realm of future research. Due to the associated genetic risks, exposing humans and animals is out of reach. With more research and knowledge, especially on other multicellular organisms, scientists can look into effective methods pertaining to inducing mutations in the mammalian germline.

4.3 Conclusion

In conclusion, it was demonstrated that the adequate concentration of 35 ug/mL Proflavine can successfully induce frameshift mutations that are able to carry on to successive generations, possibly causing phenotypic changes. On average, there was a significantly higher amount of indels in the mutant lines than in the control. The mutant lines are still being maintained and focused on for further research. The concentrations and mutation rates aided in creating effective genetic screening methods, which will be utilized for future studies in mutagenesis and *Daphnia* genomics. The goal was to produce F2 mutants with an altered phenotype; the phenotype of interest can be further studied in depth to annotate the genes that are involved. In Dr. In Xu's genetics lab, the focus is maintained on generating the F2 generations and analyzing them for any abnormalities specifically pertaining to reproductive or physical changes. This generation will be screened to find any phenotypic changes, which include having an amount of limbs, the ability to form ephippia, differences in swimming behaviors, or even varying colors and size. The results of the study aided in directing the future of mutagenesis research, illustrating how a

mutagen is able to alter the genome of an organism and potentially create an altered phenotype in future generations.

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BIOGRAPHICAL INFORMATION

Eesha graduated in Fall 2021 from the University of Texas at Arlington with an Honors Bachelor of Science in Biology and Maverick Distinction. She worked in Dr. Xu's Genetics lab conducting research on mutagenesis and developing new forward genetic screening methods. Her motivation for success stems from her curiosity and thirst for knowledge. The hard work and dedication of her family further inspire her to achieve her goals. Her parents immigrated from Pakistan to seek new opportunities and create a better life for their kids. She credits them for all her accomplishments: without their support she would not have the opportunities that currently are presented to her. It is because of them that she now stands with doors open wide. She is passionate about pursuing a career in the medical field as she wants to make a positive impact on the lives of others. The experience she gained from working at a clinic as a scribe and volunteering with underprivileged communities reaffirmed her passion for following this route. Her other interests include traveling, learning new languages, and photography. She is excited for the next chapter in her journey and is eager to see what awaits her.