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Introduction

Mitochondria, which are present in virtually all eukaryotic cells, are membrane-bound organelles that convert energy to forms that can be used to drive cellular reactions. The common pathway whereby mitochondria derive energy for biological purposes operates on a process known as chemiosmotic coupling [1]. The energy from the oxidation of food materials is used to drive a membrane-bound proton (H^+) pump that transfers H^+ from one side of the mitochondrial membrane to the other. The H^+ pump therefore generates an electrochemical proton gradient across the membrane [2]. The H^+ gradient drives many energy storing reactions when the H^+ flow back through membrane-embedded protein energy machines. The mitochondrion is therefore extremely important for the production of daily aerobic energy [3].

Mitochondria contain their own DNA (mtDNA) in a relatively small 16.5 kb circular molecule which is exclusively concerned with coding for 13 polymerases, 22 tRNA's, 2 rRNA's, and most of the components of the respiratory-chain/oxidative phosphorylation system [4]. The mitochondiral genome is about 10^{-5} times smaller than the size of an average nuclear genome [3]. The mtDNA genome is nearly the same size in animals as diverse as the common fruit fly (*Drosophila melanogaster*) and humans. All mitochondria contain multiple copies of the organelle DNA molecule which are usually distributed in several clusters in the matrix of the mitochondria where they are thought to be attached to the inner membrane [2]. Although it is not known how the mtDNA is packaged from replication to replication, the genome structure is thought to resemble that of bacteria rather than eukaryotic chromatin [3]. For example, a distinct

difference, is that mtDNA does not contain any histones for tight and efficient packaging [3]. Despite the fact that mtDNA encodes for only a small number of proteins in comparision to that of nuclear DNA, the mitochondria carries out its own DNA replication, DNA transcription, and protein synthesis [3]. These processes take place in the mitochondrial matrix where countless other important energy transforming reactions take place [2].

In comparison to the nuclear genome, the mitochondiral genome has several interesting features. (1) Unlike other genomes, nearly every nucleotide appears to be part of a coding sequence, for either a tRNA, rRNA, or protein. Because of these direct coding sequences, there is very little room for regulatory DNA sequences. (2) Whereas 30 or more tRNA's specify amino acids (aa) in the cytosol, only 22 tRNA's are required for mitochondrial DNA synthesis. (3) And most surprising is the comparison of mt gene sequences and aa sequences of the corresponding proteins which indicates that the genetic code is different. There are 4 of the 64 codons that have a different meaning from those of the same codons in other genomes [3].

Purpose

The project undertaken involved research into the mitochondrial (mt) DNA genome of *Drosophila melanogaster*. This mtDNA genome of *D. melanogaster* has been found to undergo spontaneous deletions over the lifetime of the organism. Previous research has indicated that there are several "hot-spots" where deletions have occurred within organisms as disparate as fungi and humans [4]. The purpose of this project was to optimize the "Polymerase Chain Reaction" (PCR) parameters and conditions for the stable amplification of a small region (approximately 250 nucleotide pairs) from the larval *Drosophila melanogaster* mtDNA genome.

Methods & Materials

Design of mtDNA Primers

The genome sequence of *Drosophila melanogaster* was obtained [5] and initially three primers (18, 18, and 20 nucleotides respectively) were designed (Table 1). The initial three primers targeted two mtDNA sequences; the first one 512 nucleotides in length and the second 3196 nucleotides in length. After several PCR analyses, a fourth primer was designed to complement one of the previous primers and target a 252 nucleotide sequence. Upon arrival, the lyophilized primers were resuspended in 100 µl to make a 20.0 µM stock primer suspension. The stock primer suspension was then diluted as needed to a 1.0 µM working concentration.

Table 1. Designed Primers for PCR Analysis

Primer[1] #	**Primer Sequence[2]**	**MW[3]**	**Span of Primer Sequence[4]**
Primer 1 (Fred-A)	GAACATAAACCATGAGCA	5495	8613 to 8630
Primer 2 (Edward-B)	GTTGAGGTTATCAGCCAG	5543	9159 to 9142
Primer 3 (Maggie-C)	GGAACTTTACCTCGATTTC G	6071	11,845 to 11,826
Primer 4 (Sue-D)	CTGGAGCTTCAACATGAGC	5799	8907 to 8926

[1] Primer name is given below each primer.
[2] Primer sequence is given in the 5' → 3' direction.
[3] Molecular weight is given in units of Daltons (Da).
[4] The span of the primer sequence is given using the GenBank numbering system [4].

mtDNA Isolation

Approximately twenty wild-type larval *Drosophila melanogaster* were isolated for DNA extraction. The *Drosophila melanogaster* larva were washed in 0.9% saline and frozen at -80°C. The larva were then homogenized in 1.0 ml of a buffer solution: 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 60 mM $CaCl_2$, and 0.5% SDS. The buffered homogenate was then centrifuged at a low speed for approximately two minutes. After the centrifugation, the supernatant was poured off and frozen at -80°C. The pelleted larval organelle debris was discarded. The frozen supernatant was later thawed and incubated in 10 µl of 5.3 mg/ml RNase at 37°C for 15 minutes. Proteins were extracted using a 1:1 phenol to chloroform solution mixed 1:1 with the supernatant and centrifuged at high speed for 10 minutes. The DNA was extracted with the aqueous supernatant solution and frozen at -80°C.

PCR

To date, sixteen PCR reactions were run which attempted to isolate and identify the variable conditions for the proper PCR amplification. Each reaction set had its own unique variable that could help solidify the purpose of the research project which was to design a set of primers and determine the optimum conditions to amplify a targeted sequence of *D. melanogaster* mtDNA. All of the PCR reactions were performed in a Thermojet™ temperature cycler. The following cycle times and temperatures were used in all trials unless otherwise indicated: 1 min. denaturation at 94°C, 1 min. annealing at 62-65°C, and 2 min. elongation at 72°C. Distilled de-ionized water (d^2H_2O) was added to all tubes in all reaction trials to increase the final tube volume

to 25 µl. Each PCR reaction included 30 cycles and ended with a 10 min. post-cycle at 72°C. The latitude of the Thermojet™ temperature cycler allowed that a temperature of 4°C to be reached as a final resting temperature following the 10 min. post-cycle.

Rxn Set-1 was performed to determine the viability of larval vs. adult mtDNA and also effectiveness of Epicentre™Tfl thermostable polymerase on mtDNA amplification (Table 2).

Table 2. Tfl Trial 1.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
★ 2 µl larval DNA	★ 2 µl adult DNA	★ 2 µl larval DNA	★ 2 µl adult DNA
2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)
2 µl primer (Edward)	2 µl primer (Maggie)	2 µl primer (Edward)	2 µl primer (Maggie)
4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)
2.5 µl 10X Buffer	2.5 µl 10X Buffer	2.5 µl 10X Buffer	2.5 µl 10X Buffer
★ 1 µl Tfl enzyme	★ 1 µl Tfl enzyme	★ 1 µl Tfl enzyme	★ 1 µl Tfl enzyme
2 µl $MgSO_4$ (25µM)	2 µl $MgSO_4$ (25µM)	2 µl $MgSO_4$ (25µM)	2 µl $MgSO_4$ (25µM)

★ Project defined variable(s) for each Rxn Set.

Rxn Set-2 was performed to determine the viability of larval vs. adult mtDNA and also effectiveness of Biometra™ PrimeZyme thermostable polymerase on mtDNA amplification (Table 3).

Table 3. PrimeZyme Trial 1.

Tube 5	Tube 6	Tube 7	Tube 8
★ 2 µl larval DNA	★ 2 µl adult DNA	★ 2 µl larval DNA	★ 2 µl adult DNA
2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)
2 µl primer (Edward)	2 µl primer (Maggie)	2 µl primer (Edward)	2 µl primer (Maggie)
4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)
2.5 µl 10X Buffer	2.5 µl 10X Buffer	2.5 µl 10X Buffer	2.5 µl 10X Buffer
★ 1 µl PrimeZyme	★ 1 µl PrimeZyme	★ 1 µl PrimeZyme	★ 1 µl PrimeZyme

Rxn Set-3 was performed to quantify two variables: 1) to determine whether larval mtDNA was properly extracted from *D. melanogaster* and 2) to determine which thermostable polymerase was more efficient: Epicentre™ Tfl or Biometra™ PrimeZyme (Table 4).

Table 4. Tfl Trial 2 and PrimeZyme Trial 2.

Tube 1	Tube 2	Tube 3	Tube 4
★ 2 µl larval DNA	★ 2 µl larval DNA	★ 2 µl larval DNA	★ 2 µl larval DNA
2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)
2 µl primer (Edward)	2 µl primer (Maggie)	2 µl primer (Edward)	2 µl primer (Maggie)
4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)
2.5 µl 10X Buffer	2.5 µl 10X Buffer	2.5 µl 10X Buffer	2.5 µl 10X Buffer
★ 1 µl Tfl enzyme	★ 1 µl Tfl enzyme	★ 1 µl PrimeZyme	★ 1 µl PrimeZyme
2 µl $MgSO_4$ (25µM)	2 µl $MgSO_4$ (25µM)		

Rxn Set-4 was performed to determine the effect of Promega™ Taq-A (1% Triton™ X-100 detergent) polymerase along with Promega™ PCR Enhancement Pre-Mix (Table 5). A 12X Master-Mix was prepared from the following 1X amounts: 2 µl larval DNA, 2 µl primer (Fred), 2 µl primer (Edward), 0.33 µl Promega™ Taq enzyme. Also 6.17 µl d^2H_2O were added to bring the final 1X volume to 12.5 µl. 12.5 µl Promega™ PCR Enhancement Pre-Mix (2X concentration) was added to each 1X Rxn tube for each pre-mix variable (A-L).

Table 5. Promega™ Taq-A with PCR Enhancement Pre-Mix Trial 1.

Pre-Mix	$MgCl_2$	Master Amp Enhancer
★ A	3 mM	0X
★ B	5 mM	0X
★ C	7 mM	0X
★ D	3 mM	4X
★ E	5 mM	4X
★ F	7 mM	4X
★ G	3 mM	6X
★ H	5 mM	6X
★ I	7 mM	6X
★ J	3 mM	8X
★ K	5 mM	8X
★ L	7 mM	8X

Rxn Set-5 was performed to quantify which mt DNA extraction was viable (Table 6). Promega™ Taq-A was the polymerase used. Larval *D. melanogaster* mtDNA was extracted on the following dates: 1-20-97, 2-15-97, 1-29-98, and 2-5-98.

Table 6. Promega™ Taq-A Trial 2.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
★ 2 µl larval DNA (1-20-97)	★ 2 µl larval DNA (2-15-97)	★ 2 µl larval DNA (1-29-98)	★ 2 µl larval DNA (2-5-98)
2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)
2 µl primer (Edward)	2 µl primer (Edward)	2 µl primer (Edward)	2 µl primer (Edward)
4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)
2 µl $MgCl_2$ (25µM)	2 µl $MgCl_2$ (25µM)	2 µl $MgCl_2$ (25µM)	2 µl $MgCl_2$ (25µM)
2.5 µl 10X Buffer-A	2.5 µl 10X Buffer-A	2.5 µl 10X Buffer-A	2.5 µl 10X Buffer-A
★ 0.375 µl Taq-A	★ 0.375 µl Taq-A	★ 0.375 µl Taq-A	★ 0.375 µl Taq-A

Rxn Set-6 was performed was performed to quantify which mt DNA extraction was viable (Table 7). Promega™ Taq-B (0.5% Tween™ 20 and 0.5% Nonident™ P-40 detergents) was the polymerase used. Larval *D. melanogaster* mtDNA was extracted on the following dates: 1-20-97, 2-15-97, 1-29-98, and 2-5-98.

Table 7. Promega™ Taq-B Trial 1.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
★ 2 µl larval DNA (1-20-97)	★ 2 µl larval DNA (2-15-97)	★ 2 µl larval DNA (1-29-98)	★ 2 µl larval DNA (2-5-98)
2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)
2 µl primer (Edward)	2 µl primer (Edward)	2 µl primer (Edward)	2 µl primer (Edward)
4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)
2 µl $MgCl_2$ (25µM)	2 µl $MgCl_2$ (25µM)	2 µl $MgCl_2$ (25µM)	2 µl $MgCl_2$ (25µM)
2.5 µl 10X Buffer-B	2.5 µl 10X Buffer-B	2.5 µl 10X Buffer-B	2.5 µl 10X Buffer-B
★ 0.375 µl Taq-B	★ 0.375 µl Taq-B	★ 0.375 µl Taq-B	★ 0.375 µl Taq-B

Rxn Set-7 was performed with two variables involved (Table 8). The first variable was the mtDNA template used combined a 1:1 ratio of extracted DNA on 1-25-98 and 2-5-98. The second variable was the annealing temperature was lowered 3 degrees to 62°C to decrease the stringency of annealing. A 12X Master-Mix was prepared from the following 1X amounts: 2 µl larval DNA, 2 µl primer (Fred), 2 µl primer (Edward), 0.33 µl Promega™ Taq-B Polymerase. Also 6.17 µl d^2H_2O were added to bring the final 1X volume to 12.5 µl. 12.5 µl Promega™ PCR Enhancement Pre-Mix (2X concentration) was added to each 1X Rxn tube for each pre-mix variable (A-L).

Table 8. Promega™ Taq-B with PCR Enhancement Pre-Mix Trial 2.

Pre-Mix	$MgCl_2$	Master Amp Enhancer
★ A	3 mM	0X
★ B	5 mM	0X
★ C	7 mM	0X
★ D	3 mM	4X
★ E	5 mM	4X
★ F	7 mM	4X
★ G	3 mM	6X
★ H	5 mM	6X
★ I	7 mM	6X
★ J	3 mM	8X
★ K	5 mM	8X
★ L	7 mM	8X

Rxn Set-8 was performed to determine the effect of two different concentrations of $MgCl_2$ and also the effect of mtDNA extracted on 1-29-98 and 2-5-98 (Table 9). Promega™ Taq-A was the polymerase used. The annealing temperature was also lowered 3 degrees to 62°C to decrease the stringency of annealing.

Table 9. Promega™ Taq-A Trial 3.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
★ 2 µl larval DNA (1-29-98)	★ 2 µl larval DNA (1-29-98)	★ 2 µl larval DNA (2-5-98)	★ 2 µl larval DNA (2-5-98)
2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)
2 µl primer (Edward)	2 µl primer (Edward)	2 µl primer (Edward)	2 µl primer (Edward)
4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)
★ 0.5 µl $MgCl_2$ (25µM)	★ 1.0 µl $MgCl_2$ (25µM)	★ 0.5 µl $MgCl_2$ (25µM)	★ 1.0 µl $MgCl_2$ (25µM)
2.5 µl 10X Buffer-A	2.5 µl 10X Buffer-A	2.5 µl 10X Buffer-A	2.5 µl 10X Buffer-A
★ 0.375 µl Taq-A	★ 0.375 µl Taq-A	★ 0.375 µl Taq-A	★ 0.375 µl Taq-A

Rxn Set-9 was performed to determine the effect of two different concentrations of $MgCl_2$ and also the effect of mtDNA extracted on 1-29-98 and 2-5-98 (Table 10). Promega™ Taq-B was the polymerase used. The annealing temperature was also lowered 3 degrees to 62°C to decrease the stringency of annealing.

Table 10. Promega™ Taq-B Trial 3.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
★ 2 µl larval DNA (1-29-98)	★ 2 µl larval DNA (1-29-98)	★ 2 µl larval DNA (2-5-98)	★ 2 µl larval DNA (2-5-98)
2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)	2 µl primer (Fred)
2 µl primer (Edward)	2 µl primer (Edward)	2 µl primer (Edward)	2 µl primer (Edward)
4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)
★ 0.5 µl $MgCl_2$ (25µM)	★ 1.0 µl $MgCl_2$ (25µM)	★ 0.5 µl $MgCl_2$ (25µM)	★ 1.0 µl $MgCl_2$ (25µM)
2.5 µl 10X Buffer-B	2.5 µl 10X Buffer-B	2.5 µl 10X Buffer-B	2.5 µl 10X Buffer-B
★ 0.375 µl Taq-B	★ 0.375 µl Taq-B	★ 0.375 µl Taq-B	★ 0.375 µl Taq-B

Rxn Set-10 was performed to determine the effect of a new primer, Sue-D on the targeted mtDNA sequence (Table 11). This primer was designed to complement with primers Edward-B and Maggie-C which targeted different sequences to be amplified. Both Promega™ Taq-A and Taq-B were used to optimize amplification conditions. The annealing temperature was also lowered 3 degrees to 62°C to decrease the stringency of annealing.

Table 11. Promega™ Taq-A and Taq-B Trials 4.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
2 µl larval DNA (1-29-98)	2 µl larval DNA (1-29-98)	2 µl larval DNA (1-29-98)	2 µl larval DNA (1-29-98)
★ 2 µl primer (Sue)	★ 2 µl primer (Sue)	★ 2 µl primer (Sue)	★ 2 µl primer (Sue)
★ 2 µl primer (Edward)	★ 2 µl primer (Maggie)	★ 2 µl primer (Edward)	★ 2 µl primer (Maggie)
4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)	4 µl dNTP's (1.25 µM)
2 µl $MgCl_2$ (25µM)	2 µl $MgCl_2$ (25µM)	2 µl $MgCl_2$ (25µM)	2 µl $MgCl_2$ (25µM)
2.5 µl 10X Buffer-A	2.5 µl 10X Buffer-A	2.5 µl 10X Buffer-B	2.5 µl 10X Buffer-B
★ 0.375 µl Taq-A	★ 0.375 µl Taq-A	★ 0.375 µl Taq-B	★ 0.375 µl Taq-B

Rxn Set-11 was performed to determine the effect of Sue-D on increasing concentrations of $MgCl^2$ (Table 12). Promega™ Taq-B was used to optimize the amplification conditions. A 7X Master-Mix was prepared from the following 1X amounts: 2 µl larval DNA (1-29-98), 2 µl primer (Fred), 2 µl primer (Edward), 4 µl dNTP's, 2.5 µl 10X Buffer-B, and 0.33 µl Promega™ Taq-B Polymerase. Also d^2H_2O was added to bring the final 1X volume per tube to 25.0 µl. Also, the annealing temperature was lowered 3 degrees to 62°C to decrease the stringency of annealing.

Table 12. Promega™ Taq-B Trial 5.

Tube #	$MgCl_2$ Concentration
★ 1	3.0 µl
★ 2	3.5 µl
★ 3	4.0 µl
★ 4	4.5 µl
★ 5	5.0 µl
★ 6	5.5 µl
★ 7	6.0 µl

Rxn Set-12 was performed to determine the effect of primer Sue-D on Promega^{Tm} PCR Enhancement Pre-Mix (Table 13). A 6X Master-Mix was prepared from the following 1X amounts: 2 µl larval DNA (1-29-98), 2 µl primer (Sue), 2 µl primer (Maggie), 0.33 µl Promega^{Tm} Taq-B Polymerase. Also 6.17 µl d^2H_2O were added to bring the final 1X volume to 12.5 µl. 12.5 µl Promega^{Tm} PCR Enhancement Pre-Mix (2X concentration) was added to each 1X Rxn tube for each pre-mix variable (A-F). The annealing temperature was also lowered 3 degrees to 62°C to decrease the stringency of annealing.

Table 13. Promega^{Tm} Taq-B Trial 6.

Pre-Mix	$MgCl_2$	Master Amp Enhancer
★ A	3 mM	0X
★ B	5 mM	0X
★ C	7 mM	0X
★ D	3 mM	4X
★ E	5 mM	4X
★ F	7 mM	4X

Rxn Set-13 was performed to determine the effect of Sue-D on increasing concentrations of $MgCl^2$ (Table 14). Promega^{Tm} Taq-A was used to optimize the amplification conditions. A 6X Master-Mix was prepared from the following 1X amounts: 2 µl larval DNA (1-29-98), 2 µl primer (Sue), 2 µl primer (Edward), 4 µl dNTP's, 2.5 µl 10X Buffer-A, and 0.33 µl Promega^{Tm} Taq-A Polymerase. Also d^2H_2O was added to bring the final 1X volume per tube to 25.0 µl. Also, the annealing temperature was lowered 3 degrees to 62°C to decrease the stringency of annealing.

Table 14. Promega^{Tm} Taq-A Trial 5.

Tube #	$MgCl_2$ Concentration
★ 1	2.0 µl
★ 2	2.5 µl
★ 3	3.0 µl
★ 4	3.5 µl
★ 5	4.0 µl
★ 6	4.5 µl

Rxn Set-14 was performed to determine the effect of Sue-D on increasing concentrations of $MgCl^2$ (Table 15). Primer Edward-B was used as the complement for annealing. Promega^{Tm} Taq-A was used to optimize the amplification conditions. A 3X Master-Mix was prepared from the following 1X amounts: 2 µl larval DNA (1-29-98), 2 µl primer (Sue), 2 µl primer (Edward), 4 µl dNTP's, 2.5 µl 10X Buffer-A, and 0.33 µl Promega^{Tm} Taq-A Polymerase. Also d^2H_2O was added to bring the final 1X volume per tube to 25.0 µl. Also, the annealing temperature was lowered 3 degrees to 62°C to decrease the stringency of annealing.

Table 15. Promega^{Tm} Taq-A Trial 6.

Tube #	$MgCl_2$ Concentration
★ 1	2.0 µl
★ 2	2.5 µl
★ 3	3.0 µl

Rxn Set-15 was performed to determine the effect of Sue-D on increasing concentrations of $MgCl^2$ (Table 16). Primer Maggie-C was used as the complement for annealing. Promega^{Tm} Taq-A was used to optimize the amplification conditions. A 3X Master-Mix was prepared from the following 1X amounts: 2 µl larval DNA (1-29-98), 2 µl primer (Sue), 2 µl primer (Maggie), 4 µl dNTP's, 2.5 µl 10X Buffer-A, and 0.33 µl Promega^{Tm} Taq-A Polymerase. Also d^2H_2O was added to bring the final 1X volume per tube to 25.0 µl. Also, the annealing temperature was lowered 3 degrees to 62°C to decrease the stringency of annealing.

Table 16. Promega^{Tm} Taq-A Trial 7.

Tube #	$MgCl_2$ Concentration
★ 1	2.0 µl
★ 2	2.5 µl
★ 3	3.0 µl

Rxn Set-16 was performed as a confirmation of the results of Rxn Set-13. This set was reperformed to determine the effect of Sue-D on increasing concentrations of $MgCl^2$ (Table 17). Promega^{Tm} Taq-A was used to optimize the amplification conditions. A 6X Master-Mix was prepared from the following 1X amounts: 2 µl larval DNA (1-29-98), 2 µl primer (Sue), 2 µl primer (Edward), 4 µl dNTP's, 2.5 µl 10X Buffer-A, and 0.33 µl Promega^{Tm} Taq-A Polymerase. Also d^2H_2O was added to bring the final 1X volume per tube to 25.0 µl. Also, the annealing temperature was lowered 3 degrees to 62°C to decrease the stringency of annealing.

Table 17. Promega^{Tm} Taq-A Trial 8.

Tube #	$MgCl_2$ Concentration
★ 1	2.0 µl
★ 2	2.5 µl
★ 3	3.0 µl
★ 4	3.5 µl
★ 5	4.0 µl
★ 6	4.5 µl

Gel Electrophoresis

All PCR products were electrophoresed in 1% agarose and 1X TAE gels. The DNA standards were as follows: 100 bp ladder, 200 bp ladder, 250 bp ladder, and λ cut with Hind III. Ethidium bromide was used to stain the gels.

Results

The results of each PCR reaction set were analyzed by gel electrophoresis. Each gel contained one or more lanes of size standards along with six or more lanes of PCR product.

Gel electrophoresis of Epicentre™ Tfl Trials 1 and 2 and also Epicentre™ PrimeZyme Trials 1 and 2 showed no amplification of DNA. However, there were primer-dimers at the end of the gel which gives evidence that there was primer annealing in the PCR tubes. The three standards, 100 and 250 bp ladders and λ/Hind III, were clearly visible and therefore electrophoresed and stained well.

Gel electrophoresis of Promega™ Taq-A Trials 1, 2, 3, 4, 6, and 7 showed no amplification of DNA. However, there were primer-dimers at the end of the gel which gives evidence that there was primer annealing in the PCR tubes. The three standards, 100 and 250 bp ladders and λ/Hind III, were clearly visible and therefore electrophoresed and stained well.

Gel electrophoresis of Promega™ Taq-B Trials 1, 3, 4, and 6 showed no amplification of DNA. However, there were primer-dimers at the end of the gel which gives evidence that there was primer annealing in the PCR tubes. The three standards, 100 and 250 bp ladders and λ/Hind III, were clearly visible and therefore electrophoresed and stained well.

Gel electrophoresis of Promega™ Taq-B Trial 2 showed a minute smear of amplified DNA. The lanes with 0X Master Amp Enhancer and with 3, 5, 7 mM of $MgCl_2$ gave a smear over approximately a 1500 bp range (between 200-1900 bp). These ranges were not the targeted areas on the mtDNA genome that were supposed to be amplified. There were again good primer-dimers at the end of the gel. The three standards, 100 and 250 bp ladders and λ/Hind III, were

clearly visible and therefore electrophoresed and stained well.

Gel electrophoresis of Promega™ Taq-B Trial 5 showed significant smears of amplified DNA (Table 12 and Fig.1). Lanes 2-4 showed smears that were approximately in the 2000 ntp range and increased in intensity from 3.5, 4.0, 4.5, 5.0, and 5.5 mM of $MgCl_2$. There were again primer-dimers at the end of the gel. The three standards, 100 and 250 bp ladders and λ/Hind III, were clearly visible and therefore electrophoresed and stained well.

Gel electrophoresis of Promega™ Taq-A Trial 5 showed significant amplification of DNA. This was the first observed reaction where amplified mtDNA was present in distinct and tightly packed bands (see duplicate results in Figs. 2 & 3). Furthermore, the bands were also in the correct range of the DNA being amplified. Each of the six bands was in the relative position of the 300 ntp range. In addition, the intensity of the bands decreased with increasing concentrations of $MgCl_2$ (Table 14). The primer-dimers were also further to the end of the gel, smaller than the amplified bands. The two standards, 100 bp and 200 bp ladders, were clearly visible and therefore electrophoresed and stained well (Fig. 2).

Gel electrophoresis of Promega™ Taq-A Trial 8 again showed significant amplification of DNA (Table 17 and Fig.3). The purpose for this reaction was to replicate the reaction of Promega™ Taq-A Trial 5 where distinct banding first appeared. This was the second observed reaction where amplified mtDNA was present in distinct and tightly packed bands. Once again, the bands were also in the correct range of the DNA being amplified (~250 ntp). There was greater resolution of each of the six bands due to a longer electrophoresis time and therefore a more accurate relative size of 250 ntp could be estimated. Again, the intensity of the bands decreased with increasing concentrations of $MgCl_2$, and the primer-dimers were present. The two

standards, 100 bp and 200 bp, were clearly visible and therefore electrophoresed and stained well (Fig. 3).

Discussion

The purpose of this research project was to make a qualitative analysis of larval *D. melanogaster* mtDNA via the PCR reaction. Minimal success was achieved in the beginning partly because of improper procedures such as non-sterile pipetting and procedure mix-ups. Many mistakes were made during the first few weeks of the project and were the result of a limited knowledge of the workings of PCR and gel electrophoresis. In the latter stages of the project, proper laboratory protocol and the design of a new primer, Sue-D (Table 11), helped to give a more definite amount of success. According to the results, Sue-D primed best in conjunction with Edward-B to produce an expected amplification product. The primers, Sue-D and Edward-B along with Taq-A polymerase, dNTP's, viable DNA, and correct concentrations of $MgCl_2$ allowed for the amplification of the targeted mtDNA sequence (Tables 14 & 17 and Figs.2 & 3).

Based upon research results from Rxn Sets 13 and 16 (Tables 14 & 17), mtDNA amplification was improved with the use of Primers Sue-D and Edward-B along with Promega™ Taq-A Polymerase. These reactions also identified which concentrations of $MgCl_2$ were most efficient for this particular targeted mtDNA sequence. The results of Rxn Sets 13 and 16 (Tables 14 & 17) showed that the optimal $MgCl_2$ concentration was approximately 5.0 mM. Distinct banding slowly faded as increased concentrations of $MgCl_2$ were added to the reaction tubes (Fig. 2 & 3). According to the results, a region of mtDNA was amplified under the appropriate

conditions which resulted in a segment of approximately 250 ntp (Fig. 2 & 3). According to calculations from the *D. melanogaster* genome [4], the use of primers Sue-D and Edward-B would amplify a targeted region of 252 ntp.

For reasons unknown, primer Sue-D did not complement well with Maggie-C. During the reaction sets involving Sue-D and Maggie-C, there were no amplification bands at all. The optimistic targeted region for this set of primers was 1800 ntp. According to results of this project, the proper reaction conditions for the amplification of the 252 ntp region differs from that of the 1800 ntp region. If bands were produced in the targeted 1800 ntp region, then there would be more conclusive evidence for the hypothesis that PCR reactions can be effectively used on the mtDNA genome of *D. melanogaster* as a tool to detect the presence of deletions.

As a reference to the continuation of this project, there are many other aspects yet to be elucidated. Future research needs to be attempted in this area concerning the longer targeted sequence between Sue-D and Maggie-C. To this point, there appears to be no reason why these particular primers did not complement to produce a proper amplification product. Once amplification can be performed of both targeted regions, another logical research project is the qualitative analysis of mtDNA deletions occuring during successive stages on the *D. melanogaster* life-cycle [6]. There is good evidence that mtDNA is more vulnerable to damage than nuclear DNA. It has a 10 fold higher mutation rate, lacks protective histones, has few and inefficient repair mechanisms, and a high rate of replication turnover [4]. Such research into the role that mtDNA plays in the senescence of an organism is of current concern and optimism.

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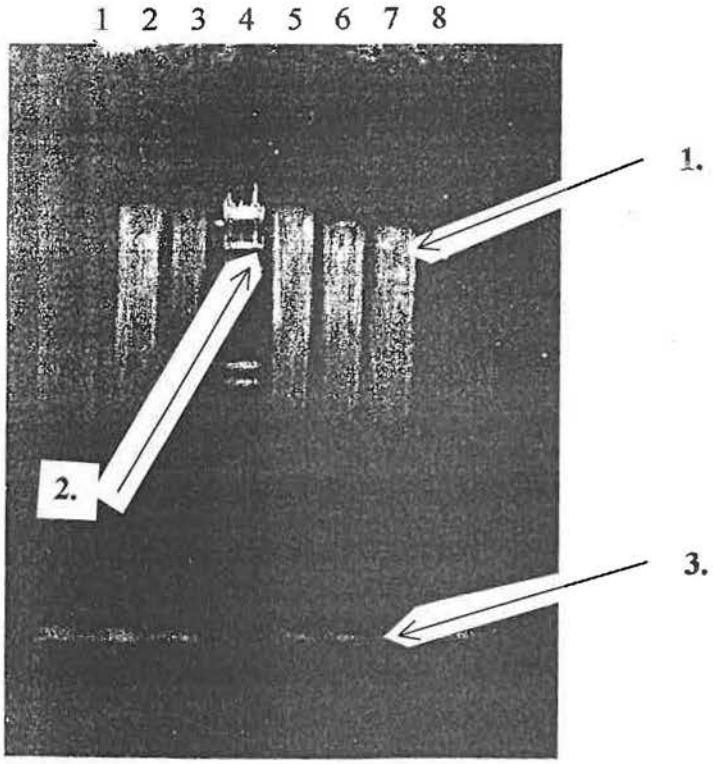

Figure 1. –Gel Electrophoresis of Rxn Set-11. Lane 4 contains λ/Hind III standard. Lanes 1-3 and 5-7 contain increasing concentrations of $MgCl_2$ (Table 12). Features of interest: 1- PCR amplification smears of mtDNA. 2- λ/Hind III standard. 3- Primer-dimers at bottom of gel.

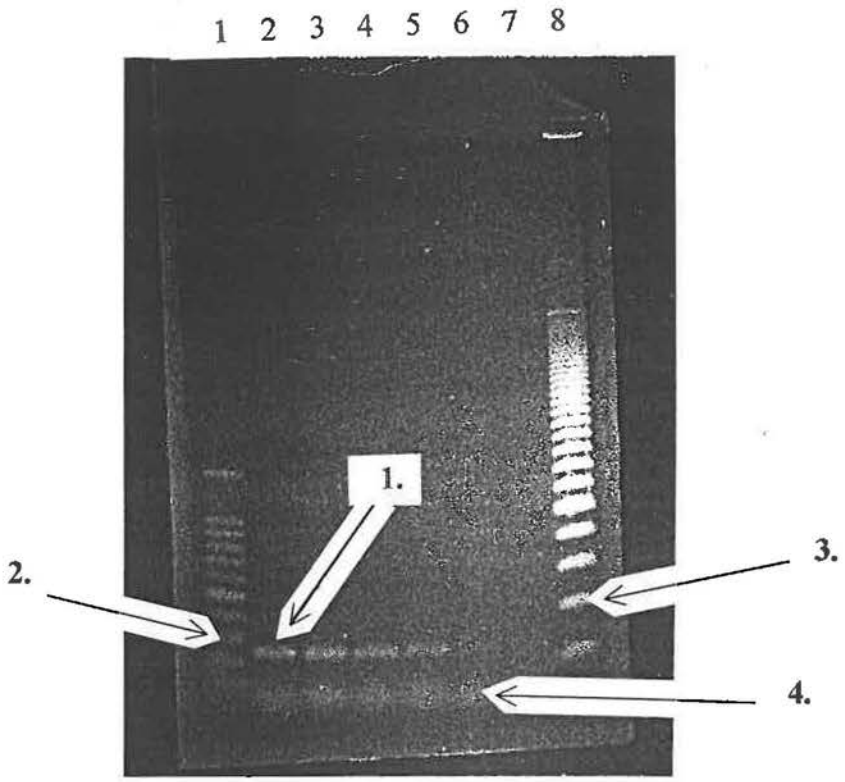

Figure 2. –Gel Electrophoresis of Rxn Set-13. Lanes 1 and 8 contain 100 and 200 bp ladders respectively. Lanes 2-7 contain increasing concentrations of $MgCl_2$ (Table 14). Features of interest: 1- Highly packed PCR amplification bands of mtDNA approximately 250 ntp in size. 2- 100 bp ladder. 3- 200 bp ladder. 4- Primer-dimers at bottom of gel.

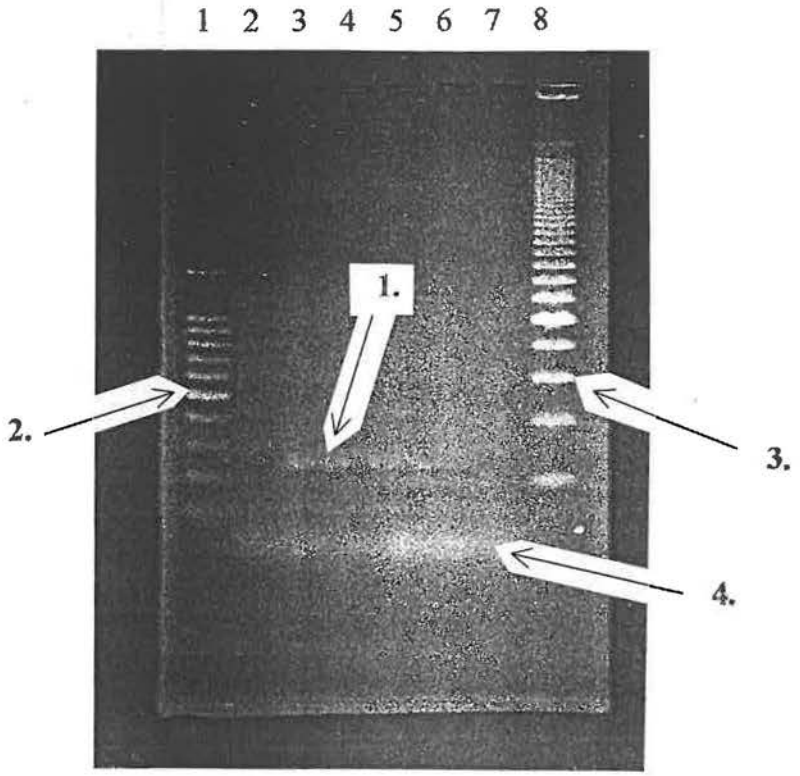

Figure 3. –Gel Electrophoresis of Rxn Set-16. The purpose of this reaction was to replicate the results of Rxn Set-13. Lanes 1 and 8 contain 100 and 200 bp ladders respectively. Lanes 2-7 contain increasing concentrations of $MgCl_2$ (Table 17). Features of interest: 1- Highly packed PCR amplification bands of mtDNA approximately 250 ntp in size. 2- 100 bp ladder. 3- 200 bp ladder. 4- Primer-dimers at bottom of gel.