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Southern Scholars Senior Research

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INTRODUCTION

The circular mitochondrial genome of most animals differs greatly from their nuclear genome. It is usually much smaller than the nuclear genome, and unlike nuclear DNA, mitochondrial DNA (mtDNA) contains almost no introns or non-coding regions. MtDNA codes for proteins, rRNAs, and tRNAs which are made in the matrix of the mitochondrion[1], and are used to create portions of the respiratory enzyme complexes. For a cell's respiratory and oxidative phosphorylation systems to function properly, the entire mitochondrial genome must be expressed[6].

However, the mitochondrial genome is very susceptible to mutations. In humans, for example, the mutation rate for mtDNA is ten times that for nuclear DNA[5]. Many factors cause the difference in susceptibility to mutation. For example, unlike nuclear DNA, mtDNA has no repair enzymes. It also lacks the histone proteins that protect nuclear DNA. Because mtDNA is located in the matrix of the mitochondrion, it is constantly exposed to oxygen free radicals created by respiration. In addition, because there are almost no non-

coding regions, mutations that occur are likely to be deleterious[5].

MtDNA mutations are believed one factor that affects aging and disease in eukaryotic cells. As an organism ages, mtDNA is damaged, impairing the ability of the mitochondria to create energy, and eventually leading to degeneration of the tissues[5]. A correlation between mtDNA deletions and aging in adult humans has been documented[5,4].

Drosophila melanogaster, the common fruit fly, is used in genetics research more than any other animal. Its usefulness is well established. However, few studies involving mtDNA mutations and aging have been done on *Drosophila melanogaster*. The polymerase chain reaction (PCR) and primer shift PCR techniques which have been used to detect mtDNA deletions in other animals[5] have not been used on *Drosophila melanogaster*[2].

PURPOSE

To optimize PCR parameters for amplifying a three kilobase segment of the mtDNA of larval *Drosophila melanogaster* from total cellular DNA extracts.

MATERIALS AND METHODS

Primer Design

The mtDNA sequences of *Drosophila yakuba* and *Drosophila melanogaster* are highly conservative. The mtDNA sequence of *Drosophila yakuba* was obtained[3] and four primers of sixteen nucleotides each were designed. The four primers bracketed two sequences, each about three kilobases long (Table 1). The bracketed regions lie between the light and heavy chain origins of replication, a region particularly susceptible to deletion mutations in human mtDNAs[4].

Table 1. PCR Primers[1].

Primer #	Primer Sequence	MW[2]	corresponding nucleotides in mtDNA	gene location
Primer 1	AAGCCAAAATAAAACT	4862	14,923 to 14,938	small rRNA
Primer 2	TTAGGTTGTGATGTAT	4950	11,942 to 11,927	ND1[3]
Primer 3	CGAAACCGAGGTAATG	4926	11,848 to 11,863	ND1
Primer 4	TGCTCATGGTTTATGT	4886	8,645 to 8,630	ND4

1 The primers were diluted to a 1 uM concentration.

2 Daltons

3 NADH dehydrogenase subunit

DNA Isolation

DNA was isolated from twenty wild-type larval *Drosophila melanogaster*. Because the primers were specific, mtDNA was not separated from nuclear DNA. The *Drosophila* in each

group were washed in .9% saline and frozen at -80°C. They were then homogenized in 1 ml of buffer solution which contained 10 mM Tris-HCl at pH 7.5, 10 mM EDTA, 60 mM CaCl, and .5% SDS. The homogenate was centrifuged at low speed for two minutes to pellet the debris. The supernatant was poured off and frozen at -80°C. The supernatant was then incubated in 10 ul of 5.3 mg/ml pure RNAse at 37°C for 15 minutes. Proteins were extracted using a 1:1 phenol:chloroform solution mixed 1:1 with the supernatant and centrifuged at high speed. The aqueous solution containing DNA was extracted and frozen at -80°C.

PCR

The effectiveness of Taq polymerase in the amplification of 3 kb segments of the mtDNA of larval *Drosophila melanogaster* was tested in a series of three PCR trials. In all trials, the following cycle times and temperatures were used unless indicated otherwise: 1 min. denaturation at 94°C, 1 min. annealing at 59°C, 5 min. elongation at 72°C. Each PCR included 30 cycles, ending with a 10 min. post-cycle at 72°C.

Trial 1 was performed to determine the effect of $MgCl_2$ on amplification with Taq (Table 2).

Table 2[1]. Taq Trial 1.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
2.5 ul Taq buffer	2.5 ul Taq buffer	2.5 ul Taq buffer	2.5 ul Taq buffer
2.5 ul primer one	2.5 ul primer one	2.5 ul primer three	2.5 ul primer three
2.5 ul primer two	2.5 ul primer two	2.5 ul primer four	2.5 ul primer four
1.5 ul 25 mM $MgCl_2$.	**No $MgCl_2$.**	**1.5 ul 25 mM $MgCl_2$.**	**No $MgCl_2$.**
4.0 ul dNTP's, 1.25 mM each	4.0 ul dNTP's, 1.25 mM each	4.0 ul dNTP's, 1.25 mM each	4.0 ul dNTP's, 1.25 mM each
5 units Taq pol	5 units Taq pol	5 units Taq pol	5 units Taq pol
2 ul larval mtDNA	2 ul larval mtDNA	2 ul larval mtDNA	2 ul larval mtDNA

1 In all tubes in trials, d2H20 was added to increase the total volume of the tube to 25 ul.

Trial 2 was performed to determine the effect of different $MgCl_2$ concentrations on Taq amplification (Table 3). New Taq polymerase was used. A 5 minute, 90°C hot start was performed on trial 2 and on all following trials.

Table 3. Taq Trial 2.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
2.5 ul 10X Taq buffer	2.5 ul 10X Taq buffer	2.5 ul 10X Taq buffer	2.5 ul 10X Taq buffer
2.5 ul primer one	2.5 ul primer one	2.5 ul primer three	2.5 ul primer three
2.5 ul primer two	2.5 ul primer two	2.5 ul primer four	2.5 ul primer four
1.5 ul 25 mM $MgCl_2$	**3.0 ul 25 mM $MgCl_2$**	**1.5 ul 25 mM $MgCl_2$**	**3.0 ul 25 mM $MgCl_2$**
5 units Taq pol	5 units Taq pol	5 units Taq pol	5 units Taq pol
4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's
2 ul larval mtDNA	2 ul larval mtDNA	2 ul larval mtDNA	2 ul larval mtDNA

Trial 3 was performed to determine the effect of careful thawing and mixing of the contents of the PCR solutions on Taq amplification (Table 4). All solutions were completely thawed and thoroughly vortexed before addition to the PCR tube.

Table 4. Taq Trial 3.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
2.5 ul 10X Taq buffer	2.5 ul 10X Taq buffer	2.5 ul 10X Taq buffer	2.5 ul 10X Taq buffer
2.5 ul primer one	2.5 ul primer one	2.5 ul primer three	2.5 ul primer three
2.5 ul primer two	2.5 ul primer two	2.5 ul primer four	2.5 ul primer four
1.5 ul 25 mM $MgCl_2$	**3.0 ul 25 mM $MgCl_2$**	**1.5 ul 25 mM $MgCl_2$**	**3.0 ul 25 mM $MgCl_2$**
4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's
5 units Taq polymerase	5 units Taq polymerase	5 units Taq polymerase	5 units Taq polymerase
2.0 ul larval mtDNA	2.0 ul larval mtDNA	2.0 ul larval mtDNA	2.0 ul larval mtDNA

Ten more PCR trials were performed to determine the effectiveness of Tfl polymerase in the amplification of 3 kb segments of the mtDNA of larval *Drosophila melanogaster*.

Trial 1 was performed to determine the effect of differing Tfl polymerase concentrations on amplification (Table 5).

Table 5. Tfl Trial 1.

Tube 1	**Tube 2**
2.5 ul Tfl buffer	2.5 ul Tfl buffer
2.5 ul primer one	2.5 ul primer one
2.5 ul primer two	2.5 ul primer two
1.5 ml 25 mM $MgCl_2$	1.5 ml 25 mM $MgCl_2$
5 units Tfl polymerase	**1 unit Tfl polymerase**
4.0 ul dNTP's	4.0 ul dNTP's
2 ul larval mtDNA	2 ul larval mtDNA

Trial 2 was performed to determine the effect of different $MgCl_2$ concentrations on amplification (Table 6). A 5 minute, 90°C hot start was performed on this trial and on all following trials.

Table 6. Tfl Trial 2.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**
1.25 ul 20X Tfl buffer	1.25 ul 20X Tfl buffer	1.25 ul 20X Tfl buffer	1.25 ul 20X Tfl buffer
2.5 ul primer one	2.5 ul primer one	2.5 ul primer three	2.5 ul primer three
2.5 ul primer two	2.5 ul primer two	2.5 ul primer four	2.5 ul primer four
1.5 ul 25 mM $MgCl_2$	**3.0 ul 25 mM $MgCl_2$**	**1.5 ul 25 mM $MgCl_2$**	**3.0 ul 25 mM $MgCl_2$**
5 units Tfl pol	**5 units Tfl pol**	**5 units Tfl pol**	**5 units Tfl pol**
4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's
2 ul larval mtDNA	2 ul larval mtDNA	2 ul larval mtDNA	2 ul larval mtDNA

Trial 3 was performed to determine the effect of an increased annealing temperature (Table 7). The annealing temperature was raised 6°C from 59°C to 65°C.

Table 7. Tfl Trial 3.

Tube 1	Tube 2
1.25 ul Tfl buffer	1.25 ul Tfl buffer
2.5 ul primer 1	2.5 ul primer 3
2.5 ul primer 2	2.5 ul primer 4
3.0 ul 25 mM $MgCl_2$ solution	3.0 ul 25 mM $MgCl_2$ solution
4.0 ul dNTP's	4.0 ul dNTP's
5 units Tfl polymerase	5 units Tfl polymerase
2.0 ul larval mtDNA	2.0 ul larval mtDNA

Trial 4 was performed to determine the effect of an annealing temperature of 61.5°C (Table 8). (The 5 minute, 90°C hot start reached a temperature of 108°C for 1 minute during this trial).

Table 8. Tfl Trial 4.

Tube 1	Tube 2
1.25 ul Tfl buffer	1.25 ul Tfl buffer
2.5 ul primer 1	2.5 ul primer 3
2.5 ul primer 2	2.5 ul primer 4
3.0 ul 25 mM $MgCl_2$ solution	3.0 ul 25 mM $MgCl_2$ solution
4.0 ul dNTP's	4.0 ul dNTP's
5 units Tfl polymerase	5 units Tfl polymerase
2.0 ul larval mtDNA	2.0 ul larval mtDNA

Trial 5 was performed to determine the effect of a post-cycle length of 20 minutes, and an annealing temperature of 59°C (Table 9). The 20 minute post-cycle was continued in all following trials.

Table 9. Tfl Trial 5.

Tube 1	Tube 2
1.25 ul 20X Tfl buffer	1.25 ul 20X Tfl buffer
2.5 ul primer 1	2.5 ul primer 3

2.5 ul primer 2	2.5 ul primer 4
3.0 ul 25 mM $MgCl_2$	3.0 ul 25 mM $MgCl_2$
4.0 ul dNTP's	4.0 ul dNTP's
5 units Tfl polymerase	5 units Tfl polymerase
2.0 ul larval mtDNA	2.0 ul larval mtDNA

Trial 6 was performed to determine the effect of higher $MgCl_2$ concentrations, and the effect of increased primer concentrations (Table 10).

Table 10. Tfl Trial 6.

Tube 1	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**
1.25 ul 20X Tfl buffer	1.25 ul 20X Tfl buffer	1.25 ul 20X Tfl buffer	1.25 ul 20X Tfl buffer	1.25 ul 20X Tfl buffer	1.25 ul 20X Tfl buffer
2.5 ul primer 1	**2.5 ul primer 3**	**2.5 ul primer 1**	**2.5 ul primer 3**	**4.0 ul primer 1**	**4.0 ul primer 3**
2.5 ul primer 2	**2.5 ul primer 4**	**2.5 ul primer 2**	**2.5 ul primer 4**	**4.0 ul primer 2**	**4.0 ul primer 4**
3.0 ul 25 mM $MgCl_2$	**3.0 ul 25 mM $MgCl_2$**	**5.0 ul 25 mM $MgCl_2$**	**5.0 ul 25 mM $MgCl_2$**	**3.0 ul 25 mM $MgCl_2$**	**3.0 ul 25 mM $MgCl_2$**
5 units Tfl pol	5 units Tfl pol	5 units Tfl pol	5 units Tfl pol	5 units Tfl pol	5 units Tfl pol
4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's
2.0 ul larval mtDNA	2.0 ul larval mtDNA	2.0 ul larval mtDNA	2.0 ul larval mtDNA	2.0 ul larval mtDNA	2.0 ul larval mtDNA

Trial 7 was performed to determine the effect of a 65°C annealing temperature, and the addition of 5 extra units of polymerase during the amplification process (Table 11). Five units of Tfl polymerase were added after the first 10 cycles.

Table 11. Tfl Trial 7.

Tube 1
1.25 ul 20X Tfl buffer
2.5 ul primer 1
2.5 ul primer 2
3.0 ul 25 mM MgCl2
4.0 ul dNTP's
5 units Tfl polymerase, 5 extra units added after 10 cycles
2.0 ul larval mtDNA

Trial 8 was performed to determine the effect of increased $MgCl_2$ concentration, and the effect of decreased polymerase concentration, at an annealing temperature of 65°C (Table 12).

Table 12. Tfl Trial 8.

Tube 1	**Tube 2**	**Tube 3**
3.0 ul 10X Tfl buffer	3.0 ul 10X Tfl buffer	3.0 ul 10X Tfl buffer
2.5 ul primer 1	2.5 ul primer 1	2.5 ul primer 1
2.5 ul primer 2	2.5 ul primer 2	2.5 ul primer 2
3.0 ul $MgCl_2$	**5.0 ul $MgCl_2$**	**3.0 ul $MgCl_2$**
5 units Tfl pol	**5 units Tfl pol**	**1 unit Tfl pol**
4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's
2.0 ul larval mtDNA	2.0 ul larval mtDNA	2.0 ul larval mtDNA

Trial 9 was performed to determine the effect of an elongation time of 7 minutes and an increased $MgCl_2$ concentration (Table 13). The annealing temperature was 65°C.

Table 13. Tfl Trial 9.

Tube 1	**Tube 2**
3.0 ul 10X Tfl buffer	3.0 ul 10X Tfl buffer
2.5 ul primer 1	2.5 ul primer 1
2.5 ul primer 2	2.5 ul primer 2
3.0 ul 25 mM $MgCl_2$	**5.0 ul 25 mM $MgCl_2$**
4.0 ul dNTP's	4.0 ul dNTP's
5 units Tfl pol	5 units Tfl pol
2.0 ul larval mtDNA	2.0 ul larval mtDNA

Trial 10 was performed to determine the effect of different polymerase concentrations (Table 14). The annealing temperature was 65°C, and the elongation time was 5 minutes.

Table 14. Tfl Trial 10.

Tube 1	**Tube 2**	**Tube 3**
3.0 ul 10X buffer	3.0 ul 10X buffer	3.0 ul 10X buffer
2.5 ul primer 1	2.5 ul primer 1	2.5 ul primer 1
2.5 ul primer 2	2.5 ul primer 2	2.5 ul primer 2
3.0 ul 25 mM $MgCl_2$	3.0 ul 25 mM $MgCl_2$	3.0 ul 25 mM $MgCl_2$
4.0 ul dNTP's	4.0 ul dNTP's	4.0 ul dNTP's
2.0 ul larval mtDNA	2.0 ul larval mtDNA	2.0 ul larval mtDNA
5 units polymerase	**5 units pol, 5 extra units added after 20 cycles**	**1 unit polymerase**

Gel Electrophoresis

All PCR products were electrophoresed in 1% agarose, 1X TAE gels. Gels were run at 105 V for approximately fifty minutes. Two ul of tracking dye were added to 10 ul of PCR product or 5 ul of standard DNA. Forty ul of 2.5 mg/ml ethidium bromide were used to stain the gels. All gels were

stained for 15 minutes and destained for 5 minutes. Photographs were taken of all gels.

RESULTS

Gel electrophoresis of Taq trials 1, 2, and 3 showed no amplified DNA, and no primers. The standard DNAs electrophoresed well, and were clearly visible.

Gel electrophoresis of Tfl trial 1 showed amplification in tube 1, which contained 5 units of Tfl polymerase. The amplification product was smeared, extending from the well (over 4 kb) to about 100 bp. Tube 2, which contained 1 unit of Tfl polymerase did not show amplification. In this gel and all following gels, the most intense staining occurred in and around the 3 kb length region of the gel. Primers were not visible in either lane. The standard DNAs were clearly visible.

Gel electrophoresis of Tfl trial 2 showed amplification in tubes 2 and 4, which both contained 3 ul of $MgCl_2$. The products in both lanes were smeared. The product of tube 2 ranged from about 3 kb to 100 bp. The product of tube 4 ranged from the well (over 20 kb) to about 1.5 kb. Tubes 1 and 3, which both contained 1.5 ul $MgCl_2$, showed no

amplification. No primers were visible in any lanes. The standard DNAs were visible.

Gel electrophoresis of Tfl trial 3 showed no amplification in any tube. No primers were visible. The standard DNAs were clearly visible.

Gel electrophoresis of Tfl trial 4 showed no amplification in tube 1. Amplification product was visible from tube 2. The product was smeared, and ranged from over 10 kb to about 100 bp. No primers were visible in any lane. The standard DNAs were clearly visible.

Gel electrophoresis of Tfl trial 5 showed amplification in both tubes. Primers were visible in both lanes. The amplification products ranged from over 10 kb to under 100 bp. The standard DNA was clearly visible.

Gel electrophoresis of Tfl trial 6 showed amplification in all six tubes. Primers were visible in all lanes. All PCR products were smeared in the gel. Tubes 1 and 2 showed the largest smear. Tubes 5 and 6 showed a slight decrease in product greater than 3 kb in length. Tubes 3 and 4 showed a significant decrease in product greater than 3 kb in length. The standard DNA was clearly visible.

Gel electrophoresis of Tfl trial 7 showed amplification, and primers were visible. There was almost

no DNA greater than about 6 kb in length. There was no visible decrease in the amount of amplification product less than 3 kb in length.

Gel electrophoresis of Tfl trial 8 showed amplification in tube 1, but not tubes 2 or 3. Primers were only visible in tube 1. Because the standard DNA was not clearly visible in this gel, the length of the DNA smear from tube 1 was not determined.

Gel electrophoresis of Tfl trial 9 showed amplification in both tubes, and primers were visible in both lanes. Tube 2 had almost no DNA over 3 kb in length. The lower ends of the smears were not visibly different from those in previous trials. The standard DNA was clearly visible.

Gel electrophoresis of Tfl trial 10 showed amplification only in tube 2. Primers were visible in the corresponding lane, but not in the lanes that showed no amplified DNA. The standard DNAs were clearly visible.

DISCUSSION

There are various possible causes for the lack of amplification in the Taq trials. One possible reason is careless preparation of the PCR tubes. However, it is not probable that this would cause problems in all tubes of all

three trials. Another possible cause is the presence of exonucleases in the PCR tubes. The lack of visible primers in the gels of all three trials supports this hypothesis. However, the lack of visible primers is insufficient evidence to prove the presence of exonucleases, as Tfl trial 1 indicates. A third possible cause of lacking amplification is the possibility that Taq polymerase is simply not suitable for amplification of 3 kb segments of the mtDNA of larval *Drosophila melanogaster*. Although this hypothesis cannot be proven, it must be considered because Tfl polymerase successfully amplified DNA under the same reaction conditions, and because the presence of exonucleases in the Taq trials cannot be demonstrated conclusively.

The Tfl trials were more successful in amplifying 3 kb segments of the mtDNA of larval *Drosophila melanogaster*.

Tfl trial 1 suggests that a higher concentration of Tfl polymerase (5 units/25 ul) is preferable to a lower concentration (1 unit/25 ul). The most intense staining occurred in and around the 3 kb region of the gel, suggesting that most of the amplification occurred due to the correct annealing of the primers. However, amplification product much larger and smaller than the

target 3 kb was made, suggesting that the reaction conditions were not ideal. Also, in trial 1 as in all trials, the presence of exonucleases may have been the cause of the lack of amplification product.

Tfl trial 2 suggests that a higher concentration (3 ul) of $MgCl_2$ is preferable to a lower concentration (1.5 ul). Although amplification occurred in trial 1 with 1.5 ul of $MgCl_2$, and no amplification occurred in trial 2 with the same amount of $MgCl_2$, the results of trial 2 suggest that when all other conditions are equal, a higher concentration of $MgCl_2$ is preferable.

At first, Tfl trial 3 seems to suggest that an annealing temperature of 65°C is too stringent for amplification. However, later trials (such as trial 10) show that it is not. The lack of amplification may have been due to careless mixing and/or thawing of PCR solutions, or to the presence of exonucleases.

Tfl trial 4 demonstrates the resilience of Tfl polymerase. Although the PCR solutions reached a temperature of 108°C for about a minute, the polymerase was still functional. Improper thawing and mixing, or the presence of exonucleases probably caused the lack of amplification in tube 1. Trial 4 also demonstrated that an

annealing temperature of 61.5°C is not too stringent for amplification.

Tfl trial 5, with a post-cycle time of 20 minutes, was performed to lower the amount of amplification product smaller than 3 kb in length by increasing extension. However, there was no noticeable decrease in the intensity of stain in this region of the gel, suggesting that a post-cycle increased to 20 minutes has little effect.

Tfl trial 6 clearly demonstrates the effects of increased $MgCl_2$ concentration and of increased primer concentration. The tubes with increased primer concentration showed a very slight decrease in product greater than 3 kb in length. However, the tubes with increased $MgCl_2$ concentration (5 ul 10X $MgCl_2$) show a significant decrease in product greater than 3 kb in length.

Tfl trial 7 shows that at an annealing temperature of 65°C, and with the addition of 5 extra units of polymerase during the PCR process, almost no DNA greater than 3 kb in length is amplified. This is most likely due to the stringent annealing temperature, and not the addition of extra polymerase. The results suggest that the amplification product greater than 3 kb in length was due to misannealing of primers. Also, because there was no visible

decrease in product less than 3 kb in length, this product was probably not the result of primer misannealing.

Because there was no amplification except in the control tube (tube 1), the effects of the conditions of Tfl trial 8 are unknown. The lack of amplification in tubes 2 and 3 may have been caused by exonucleases.

Tfl trial 9 shows that at a stringent annealing temperature (65°C), an increased $MgCl_2$ concentration further decreases the occurrence of product over 3 kb in length. Trial 9 was run with an elongation time of 7 minutes in an attempt to decrease amplification product less than 3 kb in length. However, the results suggest that increasing the elongation time from 5 to 7 minutes does not significantly decrease the smaller amplification product.

Trial 10 suggests that the addition of extra polymerase during the PCR reaction may be useful in amplification. However, the lack of amplification in tubes 1 and 3 could simply be the result of exonucleases or of poor preparation of the PCR solution.

CONCLUSION

Taq polymerase may not be ideal for amplification of 3 kb segments of the mtDNA of larval *Drosophila melanogaster*.

However, the possibility that improper mixing and thawing of PCR solutions, or that the presence of exonucleases caused the problems cannot be dismissed.

Tfl polymerase is suitable for amplification of 3 kb segments of the mtDNA of larval *Drosophila melanogaster*. To eliminate amplification products above 3 kb in size, an annealing temperature of 65°C is needed. The stringent annealing temperature presumably eliminates misannealing of primers. An increased $MgCl_2$ concentration (5 ul 25 mM $MgCl_2$ in 25 ul solution) also lowers the amplification of products above 3 kb in size. The amplification product less than 3 kb in size is probably not due to misannealing of primers. Adding polymerase during the PCR reaction does not noticeably lessen this product, nor does an elongation time of 7 minutes or a post-cycle of 20 minutes.

It would be useful to test the effect of increased nucleotide concentrations on the amplification product less than 3 kb in length, or to test the effectiveness of other thermostable polymerases. Other related experiments include determining the optimum PCR conditions for amplification of 3 kb segments of the adult mtDNA of *Drosophila melanogaster*, and optimum PCR conditions for the amplification of sequences of mtDNA that have deletion mutations.

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