



Factors affecting mitochondrial DNA quality from museum preserved *Drosophila simulans*

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Abstract

In this study we investigate how traditional killing methods and storage regimes affected mitochondrial DNA quality in *Drosophila simulans*. Here we define quality with three criteria: (1) size of extracted DNA, (2) extraction yield, and (3) ability to amplify from four target regions. Killing methods had a significant effect on extraction yield, but not on PCR success. Highest DNA yields were extracted from specimens exposed to cyanide, while the lowest were from specimens killed in 70% ethanol. Specimens stored for two years contained badly sheared DNA, which translated into a significant decrease in PCR success compared to freshly assayed specimens. The most dramatic decrease in PCR success occurred in the 1822 bp and 1332 bp amplicons, compared to the 959 bp and 291 bp fragments. Naphthalene did not affect any aspect of DNA quality; time of storage affected PCR success regardless of naphthalene environment. This study serves to further refine our understanding of DNA degradation.

Introduction

As more efficient methods are developed for gathering DNA sequence, many researchers wish to integrate molecular analyses into their evolutionary studies. Unfortunately, not all specimens contain DNA of suitable quality for molecular analysis. Without a clear understanding of the parameters that influence the degradation of DNA in stored specimens, destructive sampling of specimens could be unnecessarily wasteful (Quicke et al., 1999).

The purpose of this study was to investigate how four killing methods and three storage conditions affect DNA quality. These methods were chosen because they are traditionally employed by museum scientists. We define quality with three criteria: (1) size of extracted DNA, (2) extraction yield, and (3) ability to amplify from four target regions. Past studies have helped identify potential factors that directly affect DNA. Post et al. (1993) reported that black flies preserved in liquid nitrogen or cold ethanol yielded more DNA than those preserved in formal saline, Carnoy's

solution, methanol, or propanol. Reiss et al. (1994) and Koch et al. (1998) also found that cold ethanol yielded higher quality DNA than Carnoy's solution. Quicke et al. (1999) found a relationship between age of the specimen and PCR success, but these data were analyzed a posteriori, without prior design of specific experiments targeting effects on DNA quality. These studies have advanced our understanding of the parameters that influence DNA preservation. The present study is the first to assess effects of killing method and preservation on the ability of the Polymerase Chain Reaction (PCR) to amplify differently sized products in a balanced experimental design.

Shearing and interstrand cross-linking are two poorly understood mechanisms that can compromise DNA integrity. Shearing, or the breaking of DNA into smaller fragments, depends on a number of factors, including radiation (mainly UV), temperature, pH, and salt concentration of the environment (Dessauer et al., 1990; Seutin et al., 1991; Lindahl, 1993; Walker & Sikorska, 1994). Interstrand cross-linking may be caused by a variety of chemical reactions, including

alkylation, which bind or modify DNA and inhibit denaturation (Kornberg, 1980).

Extractions containing sheared DNA should contain shorter fragments. It may be expected that very short fragments should precipitate less efficiently, thus yields based on precipitation techniques should decrease with increased shearing. In the 140-year-old tissue of an extinct quagga, Higuchi et al. (1984) observed extraction yields of approximately 1% that of fresh samples. With shearing, PCR success should be correlated with the amount of continuous template; all else being equal, shorter fragments should be easier to amplify because they are more likely to remain continuous. With cross-linking, we would not necessarily expect shorter DNA fragments or lower yields. Instead, PCR success should correlate with the ability to denature the cross-linking bonds.

Materials and methods

Insects. The *Drosophila simulans* DSR isofemale line was used for all experiments. Ary Hoffmann (LaTrobe University, Bundoora) collected this line at Riverside, California. The line was maintained at 25 °C.

Treatments. Four traditional killing methods and three storage conditions were chosen. The four killing methods included 7–9 min exposure to cyanide, ethyl acetate, freezing (–20 °C), and 70% ethanol (room temperature). One full set of treatments was stored in the presence of naphthalene and one in the absence of naphthalene for two years. All two-year-old specimens were pinned immediately following death, using Karlovarské size 00 insect pins. After the two year storage period, we exposed a third full set of treatments to the same killing methods and assayed them immediately.

Extraction. Five individuals were sampled from each of 12 treatments. DNA was extracted according to the Puregene[®] DNA isolation kit D-7000A (Gentra Systems), employing one-third of the standard volumes. Individuals were homogenized and the cells lysed before Proteinase K and RNase A digestion. The protein was precipitated prior to precipitation of DNA with cold (4 °C) isopropanol. DNA precipitates were washed, dried, and rehydrated with 25 μ l autoclaved ddH₂O. Extraction yields were quantified using

a GeneQuant spectrophotometer, model 80-2103-98 (Pharmacia).

We tested for differences in extraction yield with a two-way analysis of variance, using time of storage and killing method as the two factors. DNA concentrations were log-transformed in order to satisfy the assumption of normality. Since we also used a Mann–Whitney Rank Sum Test to test for effects of naphthalene, we used the Dunn–Sidak method to correct for multiple tests (Sokal & Rohlf, 1995). To visualize size of extracted DNA, samples were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and the images captured with an EagleEye[™] II (Stratagene[®]).

PCR amplification. For each of four primer pairs, three individuals from each of 12 treatments were sampled. Prior to PCR amplification, all extracts were diluted to 10 ng μ l^{–1}. Four regions were amplified using a common minor-coding-strand primer (6210- 5'-GTTAGCAGCTTTTACTTGATC-3') and four major-coding-strand primers (4448+ 5'-CATACACAACATATATTTGCTCA-3', 4937+ 5'-ACGAGATGTATCACGAGAAGG-3', 5309+ 5'-GAGCTCCATTTACTATTGC-3', and 5945+ 5'-TATATCATGAATGAAATCAAGG-3') that amplified regions of length 1822 bp, 1332 bp, 959 bp, and 291 bp, respectively. All primers exactly match the mtDNA sequence of *D. simulans* DSR GenBank #AF200841 (Ballard, 2000). All PCR reactions consisted of the following ingredients: 2 μ l DNA template (at 10 ng μ l^{–1}), 2 μ l of each primer (at 10 ng μ l^{–1}), 2 μ l 8 mM dNTP, 2 μ l 4.0 mM MgCl₂⁺ buffer, 0.1 μ l *Taq* polymerase (Roche), and 9.9 μ l ddH₂O. The cycling profile was 94 °C for 8 s, 52 °C for 10 s, and 72 °C for 80 s for 35 cycles, and all reactions were hot-started. There was a pre-cycling hold of 94 °C for 60 s and a post-cycling hold of 72 °C for 180 s. Each amplicon was electrophoresed on a 1.5% agarose gel as described above. Negative controls were routinely performed to test for contamination. In no case was a contaminant amplified.

Each PCR reaction was scored 0 or 1 for the absence or presence of a discernible band. A Mann–Whitney Rank Sum Test was used to test for differences in PCR success (1) due to effects of naphthalene and (2) among two-year-old specimens versus those freshly killed. Kruskal–Wallis one way analyses of variance were used to compare PCR success among (1) killing methods and (2) primer pairs. We used the

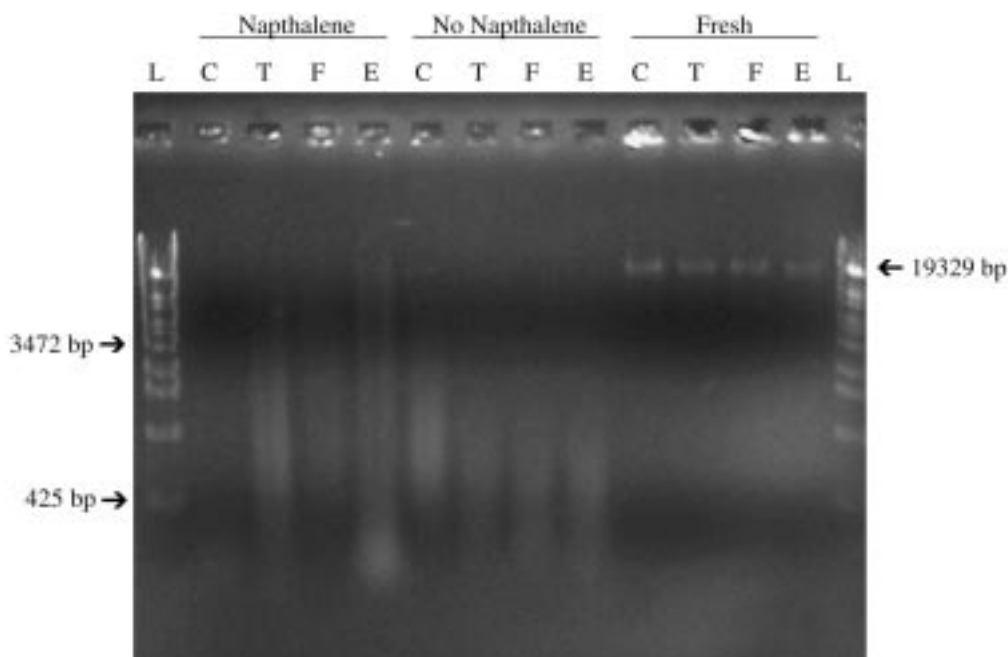


Figure 1. One of two replicates investigating sizes of DNA extractions. The other gel showed a similar pattern. Two-year-old specimens possessed smaller fragments (between about 3472 bp and <425 bp) than fresh specimens (about 19329 bp), regardless of killing or storage method. L = Eco130I (*StyI*) marker (MBI Fermentas), C = cyanide, T = Ethyl acetate, F = Freezing, E = 70% ethanol.

Dunn-Sidak method to correct for performing multiple tests on a single data set (Sokal & Rohlf, 1995).

Results

Treatment. A specific goal of this study was to test if naphthalene storage compromised DNA quality. To test its effect, we compared two-year-old specimens stored in the presence of naphthalene to those stored in its absence. Naphthalene did not reduce DNA quality, either in terms of extraction yield ($t = 0.15$, $P = 0.34$) nor PCR success (Mann-Whitney $T = 1958$, $P = 0.99$). Subsequent statistical tests pooled two-year-old specimens into a single category.

Extraction. Two outliers were removed prior to the analyses presented here. These outliers caused our data to violate the assumption of equal variances necessary to perform an ANOVA, and we feel it is more conservative to exclude them. As shown below, including these data only changed one result: the interaction term between killing method and storage time became significant when outliers were included.

A two-way ANOVA showed that killing method significantly affected extraction yield ($F_{3,67} = 3.62$,

$P = 0.02$), while time of storage did not ($F_{1,67} = 2.66$, $P > 0.1$). There was no significant interaction between the two factors when the outliers were excluded ($F_{3,67} = 2.41$, $P = 0.08$) but it was significant when they were included ($F_{3,69} = 3.32$, $P = 0.02$). A *post hoc* Bonferroni test showed that specimens killed with cyanide and ethanol yielded significantly different amounts of DNA ($t = 3.12$, $P < 0.05$). Cyanide treatments yielded 46.21 ± 6.35 (SE), while ethanol treatments yielded 24.85 ± 5.48 ng μl^{-1} specimen $^{-1}$. The other two treatments, freezing and ethyl acetate, yielded intermediate amounts of 26.74 ± 5.36 and 25.87 ± 3.46 ng μl^{-1} specimen $^{-1}$, respectively.

Genomic DNA extracted from fresh specimens showed a distinct band on the electrophoreses gel, while two-year-old specimens (regardless of killing method) contained a wide range of short DNA fragments (Figure 1).

Amplification. Time of storage had a significant effect, with fresh specimens showing greater success than specimens stored for two years (Mann-Whitney $T = 3718.00$, $P < 0.001$). The reduced success of two-year-old specimens, particularly dramatic in the two longer fragments, appears to be correlated with the fragment length (Figure 2). Killing method did

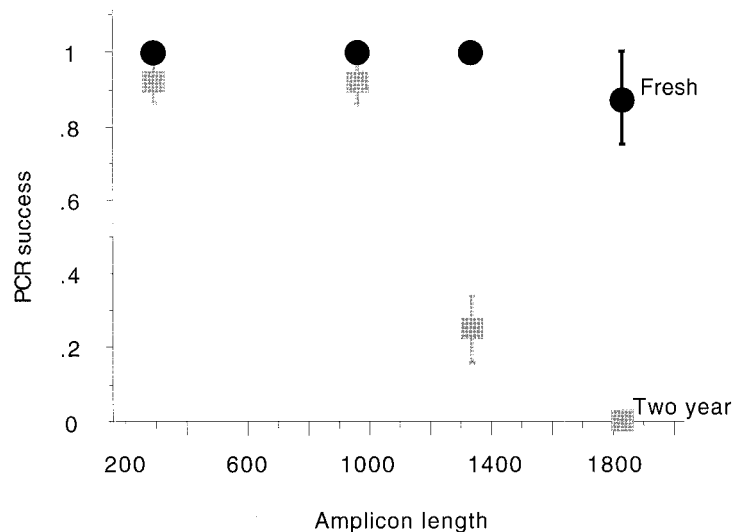


Figure 2. PCR success is influenced by length of amplicon and time of storage. As the size of the target region increases, PCR success drops most precipitously in two-year-old specimens. Standard error bars are shown where applicable.

not have a significant effect on PCR success (Kruskal-Wallis $H_3 = 2.13$, $P = 0.55$).

Discussion

Mining specimens from museum collections allows investigators to sample a greater range of inter- and intraspecific diversity. Unfortunately, many specimens may give negative results as a consequence of DNA degradation, making the destructive sampling of them a great loss. The goal of this study was to investigate how traditionally used killing and storage methods affect quality of insect DNA.

DNA extracted from two-year-old specimens was more sheared than that from freshly assayed ones. One possible source of shearing is from cellular endonucleases. DNA-damaging nucleases remain active for longer periods of time in a moist environment (Dessauer et al., 1990), and desiccating the specimen can inhibit degradation (Doyle & Dickson, 1987; Chase & Hills, 1991; Seutin et al., 1991; Lindahl, 1993; Rogstad, 1992).

To further assess DNA quality in this study we sequenced amplicons from individuals that differed in PCR success. One specimen that amplified successfully from all four amplicons, one from only the shortest three, one from the shortest two, and one from the shortest were sequenced. In all cases, sequence accuracy exceeded 96% (data not presented), indicating that if chimeric strands formed, they did not compromise sequence accuracy. Chimeric strand for-

mation may compromise sequence accuracy in ancient insect DNA through PCR jumping (Pääbo et al., 1990; DeSalle et al., 1993).

We found no evidence that naphthalene affected DNA quality, either in terms of extraction or amplification. Time of storage rather than storage environment appeared to be the primary factor responsible for DNA degradation. Two-year-old specimens contained shorter fragments than fresh specimens (Figure 1), which likely led to the significant reduction in PCR success of two-year-old specimens (Figure 2).

Killing methods and time of storage affect DNA yield, fragment size of extracted DNA, and/or PCR success. Fortunately, naphthalene does not compromise these measures of DNA quality. This study represents the first attempt to assay DNA quality in a balanced design, and had the potential to detect an interaction between killing method and preservation regime. This study should facilitate future experiments to determine the optimal method for storing insects intended for DNA analysis.

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