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Effects of Preservation Methods, Parasites, and Gut Contents of Black Flies (Diptera: Simuliidae) on Polymerase Chain Reaction Products

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Abstract

Molecular analysis of biological specimens usually requires extraction of high-molecular-weight DNA free of foreign DNA contaminants. DNA was extracted from black flies at different life stages that had been preserved by 4 methods: larvae and adults in ethanol, larvae in Carnoy's solution, adults on card-points, and adults hand-swatted and sun-dried. Using specific primers for the mitochondrial ND4 gene, a 257-bp amplicon was obtained from specimens preserved by ethanol, card-point mounting, and sun-drying. Successful amplification often required DNA dilutions ≥ 1:20 (<1–10 ng). DNA from specimens preserved in Carnoy's solution (ethanol: acetic acid, 3:1) yielded degraded DNA, resulting in fewer successful amplifications. Parasitic nematodes and, to a lesser extent, gut contents resulted in extra products when amplified with randomly amplified polymorphic DNA (RAPD) primers. Sufficient DNA was extracted from the head of a larva for a successful polymerase chain reaction (PCR), eliminating the need to remove the contaminating gut and parasites.

Keywords: black flies, *Simulium vittatum*, insect preservation methods, DNA extraction, polymerase chain reaction, randomly amplified polymorphic DNA

Molecular genetics has undergone a revolution over the past decade, in large part due to in vitro amplification of DNA by means of the polymerase chain reaction (PCR). The power of the PCR in insect population genetic studies is well established because it allows analysis of multiple loci from an individual insect, or even just a portion of an individual. Analysis of amplified DNA has provided a diagnostic technique to distinguish sibling species of black flies (Ballard 1994, Tang et al. 1995). Most black fly studies have analyzed mitochondrial DNA, but the increasing use of randomly amplified polymorphic DNA (RAPD) has provided characters for identification of mosquitoes (Kambhampati et al. 1992) and many other species (Wilkerson et al. 1993).

Museum collections represent potentially important sources of specimens for genetic analysis, and future specimens should be preserved in a manner that allows DNA analysis. We investigated the effects of various preservation methods on the quality of DNA suitable for analysis by PCR. We assessed both specific primer and RAPD-PCR. Because RAPD analysis does not use specific primers, we suspected that the presence of foreign DNA, such as endoparasites or ingested biological material, might lead to the appearance of spurious banding patterns. Black fly larvae ingest a variety of bacteria, plant, and animal material and are prone to infection by nematodes (Mermithidae) and other pathogens. The body mass of parasitic nematodes can approach a significant fraction of that of their hosts and could contribute much foreign DNA. We performed experiments to determine whether the presence of gut contents or nematode parasites can affect RAPD banding patterns.

The ability to extract high-molecular-weight DNA and then perform PCR may be influenced by the method of preservation. Numerous insect preservation methods have been used. Post et al. (1993) found that black flies frozen in liquid nitrogen had the least degraded DNA and that silica gel also conserved the linear structure of DNA. Of the alcohols tested, only ethanol maintained intact DNA, and the quantity of high-molecular-weight DNA increased with the increasing concentration of ethanol. DNA extracted from black flies preserved in Carnoy's solution (ethanol: acetic acid, 3:1), a standard fixative for cytogenetic studies, was highly degraded. Reiss et al. obtained results concordant with those of Post et al. (1993) for specimens preserved in liquid nitrogen, ethanol, and Carnoy's fixative. Reiss et al. (1994) were unable to isolate intact DNA from specimens preserved in ethyl acetate.

Post et al. (1993) did not examine the influence of biological contaminants or confirm that extracted DNA could be amplified by PCR. The latter is of particular concern for specimens stored in highly acidic Carnoy's solution because low pH is likely to chemically damage DNA (Lindahl 1993).

The research reported herein had 3 primary objectives: (1) to determine the quality of DNA obtained from black flies preserved in ethanol or Carnoy's solution, sun-dried adults, or adults pinned on card points and air-dried; (2) to determine which DNA, regardless of appearance on agarose gels, could be amplified by PCR with specific mitochondrial primers; and (3) to determine the effects of naturally occurring foreign DNA on RAPD-PCR banding patterns. Several alternative methods of DNA extraction were also investigated.

Materials and Methods

Black Flies

Simulium vittatum Zetterstedt from Nebraska comprised most of the specimens used in this study (Table 1). Larvae were killed and preserved in ethanol or Carnoy's solution. Adults were reared from pupae and killed and preserved 1 d after emergence. Sun-dried adults were from the state of Washington and were not identified by species.

DNA Extraction

The gut and nematodes (when present) were removed when larval bodies were the source of DNA, unless otherwise indicated (i.e., RAPD analysis). Specimens showing signs of being infected with pathogens were not used. Proteinase K digestion, RNase treatment, phenol/chloroform extraction, and ethanol precipitation followed Wilkerson et al. (1993) in all experiments. DNA was resuspended in 100 μl TE (10 mM Tris, pH 7.5, and 1 mM EDTA) and stored at –20°C until used. Specimens 4–8 were also extracted by Dtab:Ctab (Phillips and Simon 1995), microwave using mercaptoethanol (Goodwin and Lee 1993), and Chelex (Cano et al. 1993) in exploratory trials.

Quantification and Inhibition

Total DNA was quantified by the agarose plate method (appendix E.6, Sambrook et al. 1989). Serial dilutions of sample DNA (5 μl) were spotted on the surface of a 1% agarose gel containing ethidium bromide, photographed under UV light, and compared with known DNA concentrations (1–40 ng/μl) of BioMarker Low (BioVentures, Murfreesboro, Tennessee). Samples containing high-molecular-weight DNA were cut with HinfI (Promega, Madison, Wisconsin) according to manufacturer's instructions. Samples with degraded DNA were directly evaluated.

To test for the presence of inhibitors, known amounts of alfalfa weevil, *Hypera postica* Gyllenhal, DNA which had been extracted and stored in the same way as black fly DNA, was tested over a range of concentrations with the ND4 mitochondrial PCR primer set described below. After it was determined that positive amplification products were obtained, PCR assays with mixtures of black fly and alfalfa weevil DNA were tested.

PCR

DNA extractions were diluted 1:5–1:100 (best results determined empirically) and amplified by PCR. Specific amplifications of a portion of the mitochondrial ND4 gene were carried out in a solution containing 0.125 mM each dNTP, 0.4 μM each primer, 1.5 mM $MgCl_2$, 1X Taq buffer (Promega), 1 unit Taq polymerase, 2 μl of diluted DNA sample, and sterile deionized distilled H_2O for a total volume of 25 μl. The reaction mixture was overlaid with 30 μl mineral oil. Amplification was performed in a Perkin Elmer Cetus (Norwalk, Connecticut) Model 480 thermocycler with 1 min of denaturation at 94°C followed by 35–40 cycles of 1 min denaturation at 94°C, 1 min annealing at 40°C, and 1 min elongation at 72°C, all with minimum ramp times. Using the nomenclature of Simon et al. (1994) the primers were

N4-J-8718 5′-GCTTATTCATCGGTTGCTCA-3′
N4-N-8502 5′-CTAGGAGGAGCTGCTATATTAG-3′.

This ND4 fragment was chosen as a representative mitochondrial protein coding gene because this primer set amplifies well from a wide range of arthropods.

RAPD-PCR amplifications were done in MicroAmp tubes (Perkin Elmer Cetus) in 10-μl volumes with final concentrations of 0.05 mM each dNTP, 0.4 μM F8 primer (5′-GGGA TATCGG-3′, Operon Technologies, Alameda, California), 2.25 mM $MgCl_2$, IX Taq buffer, and 0.5 unit Taq polymerase. All amplifications were done in a Perkin Elmer Cetus GeneAmp PCR System 9600 thermocycler programmed for 1 min of denaturation at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 35°C, and elongation for 1.5 min at 72°C, all with minimum ramp times.

Electrophoresis

All DNA extractions and amplifications were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and photographed with exposure to UV light. Visual estimates of the quantity and quality of genomic DNA were made by running 5 μl (5%) of the extraction in 0.7% gels. Specific mitochondrial and RAPD amplifications were analyzed in 1.5 and 1.0% gels, respectively. All gels were run for 1–2 h at ≈ 5 V/cm in 0.5X TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0).

Results and Discussion

Extraction Methods

All extraction methods yielded DNA suitable for amplification. Dtab:Ctab (Phillips and Simon 1995) usually yielded high quantity and quality of DNA but was more expensive and time-consuming and, at best, was comparable to proteinase K digestion. The microwave method (Goodwin and Lee 1993) using mercaptoethanol, followed by phenol/chloroform extraction, was rapid, but yields were often low. Chelex, used by Cano et al. (1993) to extract DNA from fossil insects, was also rapid, but the DNA appeared to be degraded relative to the proteinase K method which we used. Cano and Poinar (1993) found that DNA extracted in this manner could no longer be amplified after storage at –20°C for 20 d. All 4 methods may be suitable if only low yields of DNA are required from well-preserved specimens.

Preservation

Some preservation methods yielded high-molecular-weight DNA (> 4 kb), visualized on gels, whereas other methods provided only degraded or no visible DNA (Table 1). We tested specimens that had been stored in ethanol for various periods of time. As is common in older collections, ethanol concentrations were unknown (70–95%). Of the 104 insects preserved in ethanol, visible DNA was present in all, although substantial amounts of high-molecular-weight DNA were observed in only 84.

Table 1. Visual quality and amplification success for DNA extracted from black flies preserved in different manners

Set	Method of preservation	Life stage	Collection date	No. analyzed	No. with high-molecular-weight DNA	No. amplifying[a]
1	100% EtOH	Larvae	19-IX-93	32	22	22[b,c]
2	100% EtOH	Larvae	19-IX-93	56	52	56[b]
3	100% EtOH	Larvae	10-VI-94	6	6	6
4	Ethanol[d]	Adults	20-XI-86	2	0	2
5	Ethanol[d]	Adults	28-X-86	2	0	2
6	Ethanol[d]	Adults	7-VII-86	2	2	1
7	Ethanol[d]	Adults	30-X-87	2	2	2
8	Ethanol[d]	Adults	27-III-90	2	0	2
9	Sun-dried	Adults	l-VIII-92	8	7	5
10	Dry, card point	Adults	13-V-88	8	7	8
11	Carnoy's	Larvae	19-IX-93	8	4	0
12	Carnoy's	Larvae	11-VI-86	2	0	2
13	Carnoy's	Larvae	6-III-88	2	0	0
14	Carnoy's	Larvae	13-IV-88	2	0	0
15	Carnoy's	Larvae	7-V-90	2	2	2

a. Sets 1 and 2 amplified with RAPD primers, sets 3–15 with mitochondrial ND4 primers.
b. Some bodies contained nematodes and/or guts.
c. Only 22 larvae were tested for amplification.
d. Ethanol concentration unknown (70–95%).

Specimens preserved in Carnoy's solution yielded mostly degraded DNA. It was only from the 2 most recent samples that some high-molecular-weight DNA was obtained, indicated by a smear extending to > 6 kb on agarose gels.

In both "hand-swatted/sun-dried" (sample 9) and card-point mounted (sample 10) specimens, 7 of 8 individuals contained some high-molecular-weight DNA. The sun-dried specimens were more degraded than the card-point mounted specimens which, in addition to a smear, usually contained a relatively crisp band of high-molecular-weight DNA.

Our results showed that there is a high probability of recovering high-molecular-weight DNA from specimens preserved in ethanol. Based on experience, initial preservation may be as important as subsequent storage. We suggest killing specimens in 100% ethanol and changing the ethanol at least once within 1 h. Although in this study we worked with material which had been preserved at room temperature, we have found that material stored at 4°C for up to 9 yr almost always yields high-quality DNA. Although less practical, we have also had excellent results with specimens transported from the field alive on ice and stored at –70°C in TE (10 mM Tris, pH 7.5, and 1 mM EDTA). Again, specimens stored for up to 9 yr have yielded excellent DNA (data not shown).

Quantification and Inhibition

Yields were 2,000–16,000 ng per black fly for samples containing high-molecular-weight DNA, 100–1,000 ng for heads alone. Digestion of samples containing high-molecular-weight

DNA confirmed that we were dealing with DNA and not with other substances which might fluoresce under UV light. Post et al. (1993) recovered < 2,000 ng from specimens in the *S. damnosum* Theobald complex. *S. vittatum* is a much larger black fly which likely accounts for the greater yields. Degraded samples yielded 500–8,000 ng total DNA.

Inhibition appeared to be a common problem with black fly DNA. Alfalfa weevil DNA when used alone readily amplified at DNA concentrations from 2–160 ng/25-μl amplification. But when 40 ng of black fly DNA was added to 2–80 ng of alfalfa weevil DNA, neither template produced amplification products. Only when black fly DNA was diluted to ≤ 20 ng were PCR amplifications successful. Quantification, done after these experiments were completed, indicated that positive amplifications actually contained < 1–10 ng DNA/25-μl amplification, consistent with results of the inhibition study.

Mitochondrial PCR Analysis

Amplification of a portion of the ND4 gene (257-bp fragment) was carried out on samples with various amounts of high-molecular-weight DNA to test for intact-template DNA that could be amplified. Amplification failures were common when undiluted DNA was used. Samples 4–8, which had been stored in ethanol of unknown concentration for various periods of time, could be amplified only when DNA was diluted 20-fold (Fig. 1). Positive amplification of the ND4 fragment appeared to be only slightly related to the presence of high-molecular-weight DNA. After dilution, most samples (99%) were amplified successfully. It would seem reasonable that, as target DNA sequences increase in length, the quality of DNA becomes increasingly important.

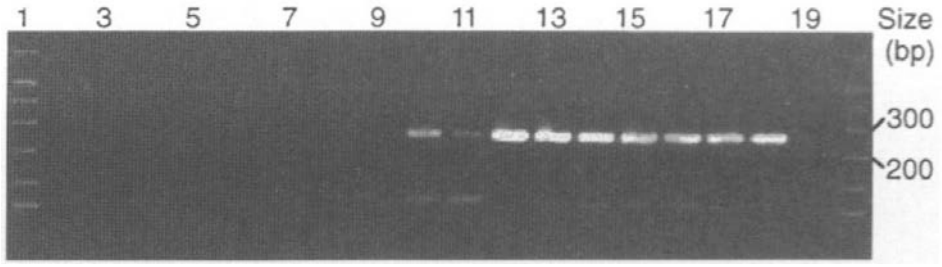

Figure 1. Agarose gel of PCR products of a portion of ND4 gene. Lanes 1 and 20 are size standards. Lanes 2–9 show undiluted DNA from card-point mounted insects (set 11); lanes 10–17, same specimens diluted 1:20; lanes 18–19, undiluted DNA of sun-dried insects (set 10).

An inverse relation between length of storage and presence of high-molecular-weight DNA was observed for samples stored in Carnoy's solution, but ability to amplify was independent of age of samples (Table 1). Amplification was successful for only 4 of 16 larvae tested, indicating possible chemical damage to the DNA by acidic depurination in Carnoy's solution. Of the sample sets preserved in Carnoy's, only sets 12 and 15 could be amplified, again from diluted (1:20) DNA. These 2 samples were the most recent and oldest

specimens, respectively. We were unable to amplify from sample set 11 in which 4 of 8 individuals appeared to have high-molecular-weight DNA, further indicating DNA damage.

DNA from all 8 specimens of a 1:20 dilution of card-pointed adults (sample 10) could be amplified, but the undiluted samples could not be amplified. Three of 8 of the undiluted sun-dried specimens (sample 9) amplified, whereas 5 of 8 of the 1:20 diluted samples amplified.

RAPD-PCR Analysis

Foreign DNA can be a problem even when using specific primers if they bind to regions conserved widely among various taxa. Moreover, because the short, nonspecific primers used for RAPDs can be expected to produce bands from any type of DNA, there is the potential for additional bands in RAPD analysis if foreign DNA is present. To test this potential, 5 sets of RAPD-PCR amplifications were run: DNA extracted from (1) a larval black fly head, (2) the remaining carcass with gut and nematodes removed, (3) the head and nematodes removed but gut retained, (4) the head, gut, and one-half of the nematodes removed, (5) the other one-half of the nematodes which had been cut with a razor blade. The heads from 22 of 32 individuals from sample 1, which had been stored in 100% ethanol, yielded small amounts of high-molecular-weight DNA as exemplified by lanes 2–9 in Figure 2. Very little degraded DNA was observed in these samples. The low concentration of DNA may be attributed to the small mass of the head. The bodies of sample 1, which also included different combinations of gut and nematodes, gave a higher yield of total DNA with high-molecular-weight bands and some degraded DNA (Fig. 2, lanes 10–17) than heads alone. Faint, but distinct, high-molecular weight bands were produced by the half-nematode samples (Fig. 2, lanes 18–25).

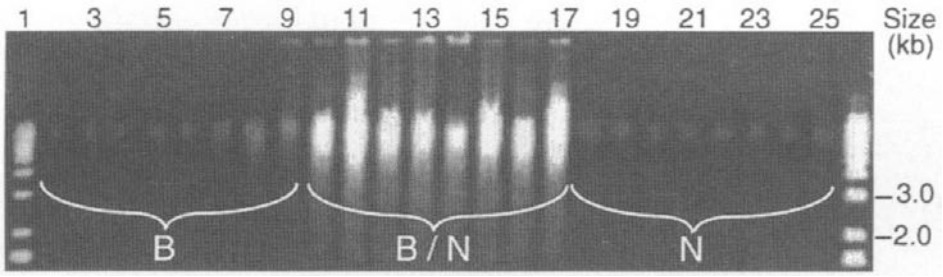

Figure 2. Agarose gel of genomic DNA. Each lane contains 5% of DNA extracted from various parts of 1 black fly larva (set 1), except lanes 1 and 26 (size standards). Lanes 2–9 are DNA from heads; lanes 10–17 are from bodies of the same specimens without guts but with one-half of the nematodes; lanes 18–25 are from the remaining one-half of the nematodes from the same specimens.

RAPD banding patterns of DNA from the heads of all 8 individuals (4 each from sets 1 and 2) matched those of the respective 1:20 and 1:50 diluted body carcass banding patterns. When parasitic nematodes were present in the hemocoel, they caused additional bands specific to the nematode. This is demonstrated by lanes 1–4 (Fig. 3) with RAPD patterns

from different portions of 4 nematode-infected black flies. Lane 1 for each black fly, with DNA only from the larval head, gave bands specific to the black fly. Lane 4, with DNA from one-half of the nematode, gave bands specific to the nematode. Lanes 2 and 3, with DNA from the black fly body carcass containing one-half of the nematode, gave both black fly and nematode bands.

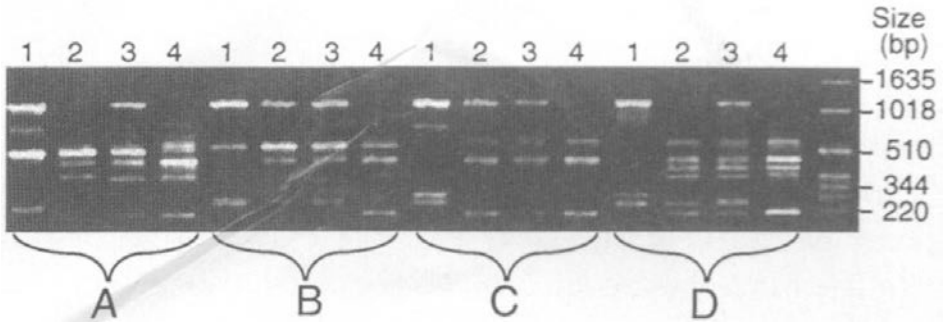

Figure 3. Each group of 4 lanes (denoted by braces) consists of RAPD-PCR products from a specific part of 1 black fly larva. Lane 1 of each group is a 1:5 dilution of DNA from the head; lanes 2 and 3 are 1:20 and 1:50 dilutions from the body plus one-half of the nematodes; lane 4 is a 1:20 dilution of the remaining one-half of the nematodes.

RAPD banding patterns of the heads of 8 larvae were compared to their respective carcass plus gut (without nematode) banding patterns. Gut contents caused faint extra amplicons in only 2 of 8 specimens (not shown).

Our results demonstrated that foreign DNA co-extracted with DNA from a target specimen can seriously affect RAPD banding patterns. Insects as well as other target organisms commonly can be expected to be infected with a variety of parasites and pathogens. Further, gut contents also have the potential to contribute anomalous banding patterns. In our study, the source of exogenous DNA in the gut contents was unknown and might have been derived from ingested food or microbes normally occurring in the black fly gut. The effect of gut contents in black fly larvae was relatively minor but could be greater in other insects, depending on diet (obvious examples being blood-fed adult mosquitoes or black flies). In any case, it is clear that care must be exercised in the design and interpretation of RAPD studies. Otherwise, RAPD bands that actually are due to foreign DNA might be confused as markers of differentiation or speciation in the populations under study. This problem can largely be avoided by using specific portions of the specimen, such as the heads of black fly larvae, which are generally free of foreign DNA. Specific primers may be used to detect microorganisms pathogenic to either black flies or vertebrates, and quality of DNA would be the more important factor.

We found, as have others, that ethanol was the best preservative for subsequent DNA experiments. Storage is preferably at 4°C, or lower. We have had some success by cutting living larvae with a razor blade and preserving the abdomen (containing the salivary glands) in Carnoy's solution for cytological analysis and the head and thorax in ethanol for DNA analysis. Alternatively, separate samples may be preserved in each fluid. With

properly collected and stored specimens, sufficient high-molecular-weight DNA can be obtained from even a small portion of a single individual to permit analysis of multiple markers.

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