TECHNIQUES FOR MITOCHONDRIAL AND RIBOSOMAL DNA ANALYSIS OF ANOPHELINE MOSQUITOES

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ABSTRACT. Methods are described for the isolation of mitochondrial and total cellular DNA from mosquitoes. The mitochondrial and ribosomal DNA restriction patterns could be detected in the total DNA of an individual mosquito by the use of cloned probes. DNA restriction analysis may prove to be a useful alternative to isozyme electrophoresis for the study of insect population genetics.

INTRODUCTION

Restriction analysis of mitochondrial DNA (mt DNA) has several advantages over other DNA based techniques that have led to its use in studying mammalian population genetics (Brown 1985, Avise and Lansman 1983). The mitochondrial genome is present in hundreds to thousands of identical copies per cell, so there is much more of it than a nuclear sequence that is only present in two copies per cell. Second, it is possible to isolate mt DNA free of nuclear DNA by physical methods, so that it is not necessary to clone the DNA or to use radioactive detection methods. Finally, since the mammalian mitochondrial genome has evolved at a much faster rate than the nuclear genome mt DNA analysis is more sensitive than nuclear DNA analysis.

Mitochondrial DNA analysis has also been used to study insect evolution. Because of the small size of insects it is generally not possible to isolate pure mt DNA from individual insects, so two approaches have been taken. One is to rear large families of insects from the populations under investigation; since all descendants of a single female share her mitochondrial phenotype sufficient material can be obtained by pooling members of a family (Shah and Langley 1979, Solignac et al. 1986, Hale and Singh 1986, LaTorre et al. 1986). Another approach is to isolate total DNA from individual insects and to use a radioactively or chemically labelled probe to identify the mt DNA pattern (Harrison et al. 1985, 1987; De Salle et al. 1986a). This requires cloned or purified insect mt DNA sequences to use as probes.

The ribosomal RNA genes (rDNA) in most organisms are also present in hundreds of copies per cell, but are arranged in tandem repeats in the nuclear chromosomal DNA (Gerbi 1985). These genes have been cloned from many insect species and can also be analyzed in individual insects using cloned probes (Collins et al. 1987). Powell et al. (1987) have reported that the rates of evolution of mt DNA and of total nuclear DNA in *Drosophila* are about the same, so in insects rDNA analysis may be as valuable as mt DNA analysis. In this paper we describe techniques to analyze the mitochondrial and rDNA restriction profiles of individual mosquitoes.

MATERIALS AND METHODS

Anopheles quadrimaculatus sp. A was from the ORLANDO colony strain, while *An. quadrimaculatus* sp. B was from a colony established from mosquitoes collected at Lake Octahatchee, Florida. *Anopheles quadrimaculatus* sp. C was collected at Shell Mound, Levy County, Florida.

Mosquito DNA was isolated by an adaption of a technique commonly used to prepare *Drosophila* DNA (Bender et al. 1983). About 2 g of frozen (−80°C) adult mosquitoes were powdered in a mortar and pestle on dry ice. This was suspended in 100 ml of ice cold homogenization buffer (0.1 M NaCl, 0.2 M sucrose, 0.01 M ethylenediaminetetraacetic acid (EDTA), 0.03 M tris HCl, pH 8.0), homogenized for a few strokes in a Wheaton dounce type tissue grinder, and transferred to a centrifuge bottle. Twenty-five ml of lysis buffer (0.25 M EDTA, 2.5% SDS, 0.5 M tris HCl, pH 9.2) was vigorously blown in using a 60 ml syringe, 1 mg of proteinase K added, and the bottle gently inverted a few times to insure complete mixing. The mixture was incubated at 55°C for 1 hr, 17 ml of 8 M KCH_3COOH added, and the bottle gently inverted until mixed. After 1 hr at 4°C the protein/cuticle/SDS precipitate was pelleted by centrifugation at 17,000 g for 20 min. Three-hundred ml ethanol was added to the supernatant, mixed in, and incubated at 4°C for 1 hr. The nucleic acid pellet was pelleted at 17,000 g for 10 min, washed two times with 70% ethanol, and the pellet air dried. The pellet was redissolved in 5 ml of TE (10 mM tris HCl, 1 mM EDTA, pH 8.0), transferred to a smaller tube, 0.5 ml 4M NaCl added, and reprecipitated with 10 ml ethanol. The pellet was dissolved in a minimal volume of TE (generally 0.5–1.0 ml) containing 10 μg/ml preboiled RNase A. This crude DNA preparation was acceptable for restriction digestion. If necessary it was further purified by phenol extraction or CsCl gradient centrifugation (Maniatis et al. 1982).

Individual mosquito DNA preparations were made using a modification of the above technique. Individual mosquitoes were homogenized in 1.5 ml Eppendorf tubes in a mixture of 0.1 ml homogenization buffer and 0.025 ml lysis buffer. (We make pestles for grinding mosquitoes by casting about 0.2 ml epoxy resin in an Eppendorf tube with a dissecting needle for a handle.) The tubes were incubated at 65°C for 30 min, 0.05 ml 8M KCH_3COOH added to each tube, and then incubated at 4°C for 1 hr. The protein-detergent precipitate was pelleted by centrifugation in a microcentrifuge for 15 min and the supernatant transferred to new Eppendorf tubes. Two volumes of ethanol were added and the nucleic acid precipitate recovered by centrifugation for 5 min in a microfuge. The pellets were washed twice with 70% ethanol and air dried. The nucleic acids were redissolved in 0.05 ml TE containing 10 μg/ml preboiled RNase A.

Mitochondria were prepared by differential centrifugation. About 2 g of live mosquitoes were thoroughly homogenized in 100 ml homogenization buffer on ice (at least 20 strokes in a dounce homogenizer) and centrifuged at 1,600 g for 5 min, the supernatant decanted and the nuclei pelleted by centrifugation at 1,600 g for 10 min, the supernatant decanted and the mitochondria pelleted by centrifugation at 17,000 g for 30 min. The pellet was resuspended in 25 ml homogenization buffer, centrifuged at 1,600 g for 10 min, decanted, and centrifuged at 40,000 g for 10 min. The mitochondrial pellet was resuspended in 8 ml of homogenization buffer, lysed by the addition of 0.5 ml 20% sodium lauryl sarcosinate, 8.5 g CsCl, and 0.4 ml 10 mg/ml ethidium bromide, and centrifuged at 35,000 RPM for 40 hr. The lower DNA band was visualized by UV irradiation and removed. The purified mt DNA was further processed using standard techniques (Maniatis et al. 1982).

Heliothis zea mt DNA was provided by Stephen Miller. Cloned *Aedes albipoctus* mt DNA was provided by Donald Dubin, clone K8 is described in Dubin et al. (1986), clone K14 is described in HsuChen et al. (1984), and clone K50 has not been previously described. A rDNA clone from *An. gambiae* Giles was provided by Victoria Finnerty and is described in Collins et al. (1987).

Restriction digestion and nick translation were performed according to protocols provided by the manufacturer, generally Bethesda Research Laboratories. Agarose gel electrophoresis, southern blotting, and hybridization were as in Maniatis et al. (1982), except that 2% sodium dodecyl sulfate (SDS) was substituted for Denhardt's solution in the blocking and hybridization steps.

For rehybridizations, blots were stripped of bound probe by briefly immersing them in boiling water followed by several washes in warm water. The filters were then blocked with SDS and hybridized as before.

RESULTS

A southern blot of total DNA from *An. quadrimaculatus* sp. A (pooled Orlando colony samples) was probed with several insect mt DNA sequences to determine which would be effective at detecting the mt DNA fragments. *Heliothis zea* mt DNA hybridized to most of the *An. quadrimaculatus* mt DNA bands (Fig. 1A) but the efficiency of the detection was low and a small region (about 20% of the total) did not hybridize. It is unlikely that the DNA from a single mosquito could be reliably analyzed under these conditions.

Since lepidopteran mt DNA did not give satisfactory results we decided to use mt DNA from *An. quadrimaculatus* sp. A as a probe. A crude mitochondria preparation was made using differential centrifugation, DNA was extracted, and the closed circular form isolated by CsCl-

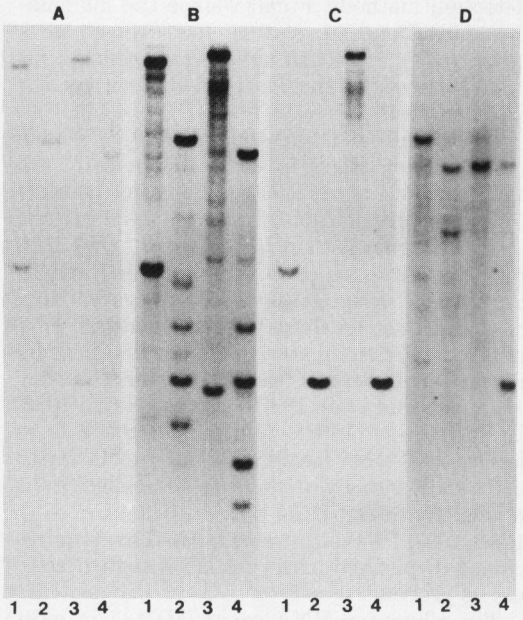

Fig. 1. Total DNA from ORLANDO strain was digested with 1) Pvu II, 2) Pvu II + Hind III, 3) Bgl II, 4) Bgl II + Hind II. Restricted DNA was run on a 0.8% agarose gel in tris-borate-EDTA buffer (Maniatis et al. 1982) and transferred to nitrocellulose. The filter was probed as described in Materials and Methods. A) *Heliothis zea* mtDNA, 48 hr exposure, B) *Anopheles quadrimaculatus* ORLANDO mtDNA, 5 hr exposure, C) K50 (*Aedes albopictus* mtDNA clone), 20 hr exposure, D) *An. gambiae* rDNA clone, 120 hr exposure.

ethidium bromide density gradient centrifugation. The yield was very low, only about 10 μg of mt DNA from 3 g of adult mosquitoes. This could be due to either loss of mitochondria in the differential centrifugation or to nicking of the closed circular DNA before the gradient centrifugation. It was critical to use live mosquitoes because frozen material did not yield any mt DNA.

The blot shown in Fig. 1A was stripped and re-hybridized to the density gradient purified mosquito mt DNA. All of the mitochondrial bands were easily detected (Fig. 1B). Because this preparation was still contaminated with nuclear DNA, the rDNA bands also hybridized to some extent and can be seen as faint bands (compare with Fig. 1D).

This procedure was still not satisfactory. The isolation of mosquito mt DNA was tedious and inefficient, and the resulting probe also hybridized faintly to other bands. With some restriction enzymes the pattern of fragments was complex and it was difficult to determine how many differences were occurring between different species (see below). Therefore we tried using the cloned mosquito mt DNA sequences from *Aedes albopictus* (Skuse) as probes.

Three clones of *Ae. albopictus* mt DNA were obtained and used to probe the *An. quadrimaculatus* sp. A blots. One of these (K50) is apparently contained within another (K8). Figure 1C shows hybridization of K50 to the same blot. The two mt larger clones (K8 and K14) each hybridized to about 20% of the mt DNA (data not shown). The intensity of hybridization with all of the clones was comparable to that with *An. quadrimaculatus* sp. A mt DNA, indicating that the sequence divergence between these two species did not interfere with hybridization. There was no detectable hybridization to the rDNA or to any other nuclear DNA sequences. Each of these clones hybridizes to a subset of the mt DNA bands, so the patterns are generally simpler and easier to interpret than the total mt DNA pattern. For this reason we believe that these and other clones will be the most valuable tools for mt DNA analysis of mosquitoes.

The rDNA is tandemly duplicated several hundred fold in insects and can be detected using appropriate probes. Since the blot of total DNA that we used for mt DNA analysis also contains the rDNA, we obtained a rDNA clone from *An. gambiae* and used that to probe the same blot (Fig. 1D). Normally, restriction analysis of a tandemly repeated sequence generates fragments that correspond to a circularly permuted unit repeat. These results are more difficult to interpret because the ribosomal repeat unit consists of a highly conserved coding sequence that cross-hybridizes and a spacer region that varies between species and does not cross-hybridize. If an enzyme cuts more than once in the diverged spacer, a fragment is generated that is not detectable, leading to gaps in the map when certain restriction enzymes are used. (In addition some residual hybridization of mitochondrial DNA can be seen due to incomplete removal of probe from the earlier experiments. This is one problem with reusing blots.) The total length of the unit is about 9.5 kb when this is taken into account.

The previous results were all obtained using DNA samples pooled from a large number of mosquitoes, and several times the amount of DNA in a single mosquito was run in each gel lane. To determine if this technique would work with the DNA from an individual mosquito we ran serial dilutions on southern blots, from 1/4 to 1/64 of an individual DNA sample. As can be seen in Fig. 2, we can detect mitochondrial bands in 3/64 of an individual DNA sample and can detect some of the mitochondrial bands in 1/64 of an individual DNA sample. Therefore the same individual DNA preparation can be split into many fractions and digested with different enzymes. This will greatly increase the information that can be obtained from a given mosquito.

Preservation of field collected material for isozyme electrophoresis is frequently a major problem. Compared to proteins DNA is relatively resistant to denaturation and will survive much harsher treatments. We have compared two methods of sample preservation to use of live material. Mosquitoes were stored for two days in 100% ethanol either at room tempera-

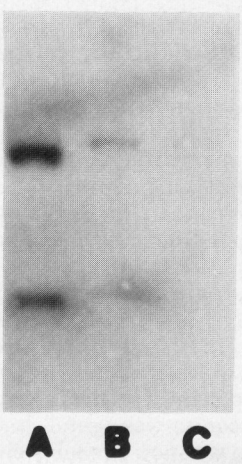

Fig. 2. DNA was extracted from a single mosquito and digested with Eco RI. Serial dilutions were made, run on a 0.8% agarose gel, transferred to nitrocellulose, and probed with K8 + K14. A) 3/16 of total DNA, B) 3/64 total DNA, C) 1/64 of total DNA.

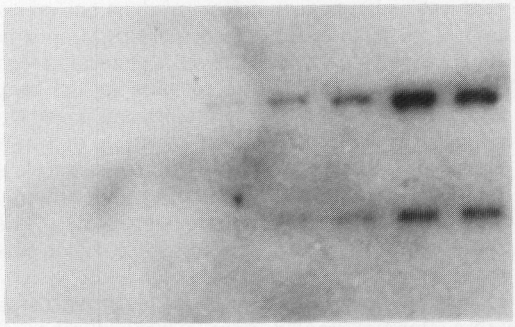

Fig. 3. DNA was extracted from individual mosquitoes and digested with Eco RI. 1/4 of each preparation was run per lane on a 0.8% agarose gel, transferred to nitrocellulose, and probed with K8 + K14. A–C) Mosquitoes preserved in ethanol at room temperature two days, D–E) mosquitoes preserved in ethanol at −15°C two days, F–H) live mosquitoes.

ture or at −15°C. The mt DNA bands were identical in all three types of samples (Fig. 3) although considerably less DNA was obtained from mosquitoes stored in ethanol at room temperature. This is probably due to irreversibly denatured proteins trapping some of the DNA, which would reduce the overall yield but would not change the banding patterns. Perhaps yields could be improved by the addition of proteinase K during the lysis step to release DNA from insoluble protein. It is also possible to preserve mosquitoes for DNA analysis by air drying (F. Collins and V. Finnerty, personal communication).

DISCUSSION

The techniques that we have described extend the use of DNA restriction analysis to individual mosquitoes. These techniques will be useful for the determination of the amount of variation within and between populations. Since it is much easier to preserve material for DNA analysis than for isozyme electrophoresis, this may become the method of choice for population analysis when collecting must be done under difficult conditions.

Mitochondrial DNA analysis can be done using total DNA if a suitable probe is available. Cloned mt DNA from other insect species is effective as a probe as long as the two species are not too distantly related, e.g., mt DNA from another mosquito was useful, but that from a lepidopteran was not. Probably cloned mt DNA from any other dipteran would work reasonably well. Cloned probes from other species can also be used for rDNA analysis, but the results are less satisfying. If it is desirable to identify all of the restriction fragments and construct a complete restriction map it is probably necessary to use a rDNA probe from the species being analyzed. However, for the purpose of distinguishing species this is not necessary, and a heterologous probe is sufficient.

Since both mitochondrial and rDNA sequences can be detected in total DNA digests, it is possible to probe a southern blot with both in succession. This can double the amount of information generated and entails only a small additional amount of work, since most of the effort in these studies is involved in the isolation of DNA from individual mosquitoes, digestion, and electrophoresis. Preliminary studies on the *An. quadrimaculatus* complex indicate that restriction polymorphisms of both the mt DNA and the rDNA exist among the four species, and that these polymorphisms can be used to differentiate the species (Cockburn, unpublished).

Previously, analysis of individual insect DNA has only been done on crickets (Harrison et al. 1985, 1987) and Hawaiian *Drosophila* (De Salle et al. 1986a, 1986b; De Salle and Giddings 1986), both relatively large insects. *Anopheles quadrimaculatus* weighs 2–3 mg, so we can efficiently detect mitochondrial and rDNA from as little as 0.1 mg of tissue. We do not know if the number of copies of these DNA sequences is exactly proportional to insect size, it might vary due to changes in cell number, polytene, and mitochondria number; however, our results suggest that insects as small as *Culicoides* could be studied using these techniques. This is about the same amount of tissue required for isozyme electrophoresis.

The method that we have used to detect DNA hydrids is autoradiography. Laboratories that are not currently working with radioactivity may be reluctant to start. We have also used nonradioactive methods for the detection of DNA, specifically biotin labelling followed by streptavidin/alkaline phosphatase detection (Mitchell and Cockburn 1988). This technique is not quite as sensitive as radiolabelling, but it should be sensitive enough to detect the mt DNA from a mosquito, especially if the whole sample is used for a single enzyme digest.

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