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USE OF DNA PROBES TO DISTINGUISH SIBLING SPECIES OF THE *ANOPHELES QUADRIMACULATUS* COMPLEX

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ABSTRACT

Isozyme electrophoresis has traditionally been the method of choice for analyzing the genetic structure of populations. It can also be useful for distinguishing morphologically identical sibling species. More recently mitochondrial DNA restriction analysis has become popular as an alternative for studying population structure; and the isolation of species-specific DNA probes has become the method of choice for distinguishing closely related species. We have evaluated the usefulness of these DNA based techniques in mosquitoes and discuss their advantages and disadvantages.

RESUMEN

La electroforesis de isoenzyma tradicionalmente ha sido el método preferido para analizar la estructura genética de poblaciones. También puede ser utilizada para distinguir entre especies hermanas morfológicamente idénticas. Más recientemente, análisis de la restricción de la mitocondria del DNA se ha hecho más popular como una alternativa para estudiar la estructura de la población; y el aislamiento de sondas de DNA específicas a especie ha sido el método preferido para distinguir especies cercamente relacionadas. Nosostros hemos evaluado la utilida de estas técnicas basadas en el DNA de mosquitos y se discuten sus ventajas y desventajas.

The *A. quadrimaculatus* species complex consists of at least four sibling species: species A, species B (Lanzaro 1986), species C and species D (unpublished data). We have used species A, B, and C of this complex to investigate the usefulness of DNA techniques in mosquito evolution research. Our first aim was an estimate of the degree of diversity between different populations (in this case the three sibling species) and the second was a rapid identification tool for wild-caught mosquitoes. These are quite different questions and have involved different approaches.

DNA restriction analysis has been widely used in vertebrate population and evolution studies (Brown 1985), and is becoming more popular in entomology (DeSalle et al. 1986, DeSalle & Giddings 1986, Hale & Beckenbach 1985, Hale & Singh 1986, Harrison et al. 1987, Hall 1986, LaTorre et al. 1986, Powell 1983, Shah & Langley 1979, Solignac

et al. 1986a, 1986b, Solignac & Monnerot 1986). The basic approach is to isolate DNA from individual insects, cut it at specific sites using a restriction endonuclease, separate the fragments by size by agarose gel electrophoresis, and then probe to identify specific fragments of interest. Except for the restriction endonuclease step, this is analogous to the technique of isozyme electrophoresis. The resulting data is even very similar, consisting of a series of bands in lanes on a gel.

DNA probes have been used to identify species or strains of microorganisms when no reliable phenotypic key was available. The approach is to identify a DNA sequence that is present in only one species or strain, clone that sequence in bacteria, and then use the cloned DNA as a probe against DNA of an unknown specimen. This is similar to the use of antibodies for serotyping. Techniques such as this are not in general use for identifying higher eucaryotes, though several groups have used them to identify individual insects infected with parasites (e.g. Kirkpatrick et al. 1987).

RESULTS

Restriction analysis

The most widely used DNA for restriction analysis is mitochondrial DNA (mDNA). There are several reasons why this molecule is preferred: it is relatively small (about 16,000 base pairs in mosquitoes), it is present in thousands of copies per cell, and it can be isolated away from the bulk of the DNA in the nucleus (Brown 1985). Mammals are large enough that it is possible to isolate enough mDNA from a single individual to analyze it by staining. That is not possible in mosquitoes because of their small size, so we were forced to take a different approach to identifying the mDNA.

DNA fractionated on a gel can be transferred to a nitrocellulose membrane and hybridized to a radioactive probe sequence to detect specific fragments. We investigated various DNAs to use to detect the mDNA fragments. *Heliothus zea* mDNA (courtesy of S. Miller) hybridized to most of the *A. quadrimaculatus* mDNA fragments, but about 20% of the molecule did not hybridize. In addition, the sensitivity of this probe was rather low and it would have been difficult to detect the mDNA of a single individual.

We devised a procedure to isolate *A. quadrimaculatus* mDNA and used this as a probe. All of the fragments were easily detectable. Two problems were that contaminating nuclear DNA gave minor background bands and in some cases the patterns were too complex to easily analyze. As an alternative we used three cloned fragments of *Aedes albipictus* mDNA (Dubin et al. 1986, HsuChen et al. 1984) as probes. These hybridized as intensely as the *A. quadrimaculatus* probe, gave no background, and lit up only a subset of the bands (which made interpretation easier).

The sensitivity of the technique was studied by serial dilutions of the DNA from a single mosquito. The mDNA fragments could be scored in the 1/64 dilution. Therefore theoretically 64 different enzymes could be scored for each mosquito.

Different methods of preservation of material for DNA preparation were tried. Live mosquitoes gave the highest yields, but mosquitoes stored for several days in ethanol also produced DNA, and the banding patterns were unchanged (though less intense). This method of preservation could be of great utility when collecting field material in areas where dry ice or liquid nitrogen is unavailable.

Analysis of the three *A. quadrimaculatus* sibling species with thirteen different restriction endonucleases showed ten differences among the three mitochondrial genomes. Species C was most divergent but species A and B also differed. This work was done on pooled samples, so only the major forms would have been detected. Further work on individual samples will be necessary to determine if these variants are fixed in the different species.

The ribosomal RNA genes (rDNA) (Gerbi 1985) could also useful for restriction analysis. They are also repeated hundreds of times per cell and cloned probes are available. We probed the same blots used to analyze the mDNA with an *A. gambiae* rDNA probe (courtesy of V. Finnerty) and found that the sensitivity was about the same. The results when the rDNA patterns of the *A. quadrimaculatus* species complex were compared were similar to the mDNA results. With the thirteen restriction endonucleases tested, six differences were seen among the three species. This is slightly less variability than in the mDNA, but the *A. gambiae* probe only hybridized to the coding sequences, not the non-transcribed spacer region. If this region had been included the differences probably would have been about the same. This is similar to the situation in *Drosophila* (Powell et al. 1986) but different from mammals (Brown 1985) where the mitochondrial DNA evolves at a much faster rate than the nuclear genes. Reprobing the filters was little additional effort and generated a great deal of additional information, so it is highly recommended that both probes be used rather than just one.

Restriction enzyme analysis has to compared to isozyme analysis to determine its usefulness.

Disadvantages could be:

1. More time consuming.
2. Slightly more expensive.
3. Requires new expertise.

Advantages could be:

1. DNA is invariant in all cells (no artifacts based on comparing different life stages).
2. DNA is much more stable than enzymes.
3. Only one type of gel and DNA isolation are used for all insects, therefore results can be directly compared between different workers.
4. Restriction endonucleases are commercially available.
5. Polymorphisms usually result in changed numbers of bands, not slight changes in mobility, making analysis much simpler.

Species specific probes

In applied entomology it is often important to be able to identify field specimens rapidly and cheaply. For example, one member of a species complex might be a serious disease vector while another is not. Chromosome analysis, isozyme analysis, and other techniques that have been useful in research situations are slow and require expert interpretation.

We have developed a simple means of isolating cloned DNA sequences that are species-specific and used it to obtain aclone (Arpl) that is specific to *A. quadrimaculatus* species A. Arpl can be used as a probe to identify DNA from individual mosquitoes of the species in question. Since the assay is for presence or absence of the Arpl sequence, it is not necessary to digest the DNA or run gels. Hundreds of "bug blots" can be analyzed by a single person in a day.

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