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MULTIPLE-TECHNIQUE IDENTIFICATION OF SIBLING SPECIES OF THE *ANOPHELES QUADRIMACULATUS* COMPLEX

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ABSTRACT. In the past, most researchers used a single technique for identification of cryptic taxa, population structures, biosystematics, and phylogenetic studies. Our experience with the *Anopheles quadrimaculatus* complex shows the importance of using several methods on individual mosquitoes. This approach consists of analysis of the polytene chromosomes in ovarian nurse cells, gas chromatographic profiles of cuticular hydrocarbons, isozyme electrophoresis, and restriction site analysis of mitochondrial or genomic DNA. We recommend use of this multiple-technique approach when analyzing feral populations for the first time, or for correlating information obtained by investigators using different techniques.

Population genetic studies on a number of insect species have shown that there is no single population that represents the entire gene pool of a species. A species is an assemblage of variable populations dynamically evolving in response to geographical, temporal, clinal, and other ecologically related patterns, that is genetically distinct from other species. The genetic structure of each population is a unique response to a local environment. Because of the complexity of population analysis, the resolution possible with a single technique can be unsatisfactory, and analysis of individuals of populations using a combination of different techniques becomes desirable. The literature on anopheline mosquitoes contains numerous examples of situations where sympatric sibling species were independently investigated with different techniques. In many cases, the correlations between the data sets remain undetermined (Steiner et al. 1982, Narang and Seawright 1990a).

Generally, complex variation patterns and analytical limitations require the analysis of populations through a combination of different techniques, but use of more than one technique usually requires labor-intensive procedures. For example, gravid females are collected and morphologically examined, and their progeny are split into groups for analysis of chromosomes, isozymes, mitochondrial DNA restriction fragments, and cuticular hydrocarbons.

Anopheles quadrimaculatus Say was once regarded as one species, but recent research divulged a complex of at least 5 sibling species (Narang et al. 1990). During our studies on the *An. quadrimaculatus* complex, we developed methods for analysis of an individual mosquito by 4 techniques, *viz.,* 1) cytology (Narang et al. 1989), 2) isozyme electrophoresis (Narang and Seawright 1990a), 3) gas chromatography of cuticular hydrocarbons (Carlson and Service 1980), and 4) mitochondrial (mt) DNA restriction site analysis (Cockburn et al. 1990). This approach is summarized in the present paper.

Seventy-five adult females (collected near Panama City, FL) from a mixed field population of members of the *An. quadrimaculatus* complex were analyzed to determine the validity of the multiple-technique methods described below.

Ovarian chromosomes and hydrocarbon extraction: First, the ovarian polytene chromosomes were prepared and analyzed by standard methods (Kaiser and Seawright 1987). The female was then submerged in 0.5 ml cold hexane for 10–30 min for extraction of the cuticular hydrocarbons. The column chromatography and gas chromatographic analyses of the hexane extract were performed by the method of Carlson and Service (1980).

Isozyme electrophoresis and isolation of DNA: Individual mosquitoes were homogenized in 1.5 ml Eppendorf tubes in 0.06 ml ice-cold buffer (0.03 M tris HCl, pH 8.0). Two 1 × 6 mm filter paper wicks (Whatman 3 mm) were dipped in each tube and analyzed by starch-gel electrophoresis (Narang et al. 1989). Electromorphs of up to 17 enzyme systems (29 loci) were studied.

A modified lysis buffer (0.14 M NaCl, 0.27 M sucrose, 0.1 M EDTA, 0.8% SDS, and 0.2 M tris HCl, pH 9.0) was added to individual homogenates and total genomic DNA was isolated according to established procedures (Cockburn and Seawright 1988). The DNA pellets were dissolved in 0.04 ml TE (10 mM Tris, 1 mM EDTA, pH 8.0) containing preboiled RNAse (0.01 mg/ml) and stored at −20 or −70°C. One-fifth to one-eighth fraction of the above DNA solution was used for digestion with individual restriction endonucleases (according to the protocols of the supplier, Bethesda Research Laboratories). The methods of agarose gel electrophoresis and Southern blotting were as described in Maniatis et al. (1982). Clones of 3 EcoR I fragments of mitochondrial DNA of *An. quadrimaculatus* species A were used to probe the Southern blots of

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mtDNA profiles (Cockburn et al. 1990) and hybridization conditions were as in Cockburn and Seawright (1988). The fragment sizes of the mtDNA digested with restriction endonucleases were determined using molecular weight standard markers run on the same gel (lamda Hind III digest or 1 kb ladder).

Use of hexane for extraction of the cuticular hydrocarbons did not interfere with analysis of enzymes (Narang and Seawright 1990b) or DNA. However, it was necessary to prepare the chromosomes before hexane extraction. There was a perfect correlation for using chromosomes, isozymes, and mtDNA for identification of the 4 sibling species. As described by Narang et al. (1989) 4 cytotypes were observed. Similarly, the 4 expected diagnostic restriction fragment patterns were observed for digests of mtDNA samples. The interpretation of electromorph clusters of isozyme loci also agreed in identifying 4 sibling species; however, the analysis divulged substructuring within species C. Subsequent examination of the isozymes of additional mosquitoes led to a better description of types C_1 and C_2 (Narang et al. 1990).

An analysis of the hydrocarbons of individual mosquitoes revealed a complex series of hydrocarbons from 26 to 50 carbons, with a series of *n*-alkanes, methyl branched alkanes, and dimethyl branched alkanes. We were unable to discern diagnostic markers for the 4 sibling species. However, the methodology for extraction of the hydrocarbons was shown to be compatible with the other techniques.

Our results indicated the feasibility of using either of the 3 effective diagnostic techniques for specimen identification. This analysis of individual insects by several techniques is most appropriate for the examination of new populations. Once this is done, a single technique can be chosen to monitor temporal, cyclic, and other kinds of changes in the genetic structure of populations or relative composition of sibling species.

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