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SCIENTIFIC NOTE

POLYMERASE CHAIN REACTION ASSAY TO IDENTIFY ALL IMMATURE STAGES OF TWO SPECIES OF THE *ANOPHELES QUADRIMACULATUS* SIBLING SPECIES COMPLEX (DIPTERA: CULICIDAE)

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ABSTRACT. A polymerase chain reaction assay that detects differences in the 2nd internal transcribed spacer of ribosomal DNA was tested as a means to identify all immature stages of 2 species in the *Anopheles quadrimaculatus* sibling species complex. The technique was successful in amplifying DNA from eggs, 1st–4th instars, and pupae of *An. quadrimaculatus* Say and *An. inundatus* Reinert.

KEY WORDS *Anopheles quadrimaculatus, An. inundatus,* immature stages, polymerase chain reaction

Anopheles quadrimaculatus Say is a sibling species complex that includes 5 sibling species. Reinert *et al.* (1997) published a comprehensive taxonomic treatment describing *An. quadrimaculatus* sensu stricto and 4 new species. What were formerly known as species A, B, C1, C2, and D are now known, respectively, as *Anopheles quadrimaculatus* Say, *An. smaragdinus* Reinert, *An. diluvialis* Reinert, *An. inundatus* Reinert, and *An. maverlius* Reinert.

Additional studies that have added to the general knowledge of these 5 species include work on species genetics, adult identification, species distribution, filarial susceptibility, and host preferences. These works, along with others on *An. quadrimaculatus sensu lato* are listed in Reinert's (1997) bibliography. Reinert et al. (1997) summarized all current information on habitats.

In an effort to further characterize the larval habitats of this complex of mosquitoes, we encountered a need for a quick, reliable method to identify the immature stages soon after collections were made. Starch gel electrophoresis (SGE) of isozymes has been used in the past to identify individual adult mosquitoes; however, this method is limited to the adult stage of the mosquito and is not reliable for individual specimens of the immature stages of *quadrimaculatus* complex species (Janet McAllister, personal communication).

We tested the polymerase chain reaction (PCR) system of Cornel et al. (1996) for its ability to amplify ribosomal DNA from each immature stage of *An. quadrimaculatus* sibling species. In this assay, 5 oligonucleotide primers were combined in a single PCR reaction. Comparisons were made between SGE and PCR.

Adult mosquitoes were collected from natural resting sites identified during a 2-year distribution survey of *An. quadrimaculatus* sibling species in Louisiana (Rutledge and Meek 1998). Bloodfed females from these collections were placed in individual mosquito breeders (BioQuip, Gardena, CA) after a wing was removed from each female to induce oviposition. After the eggs were laid, each adult female was frozen and stored at −70°C for subsequent identification by SGE. The eggs were placed into white enamel larval rearing pans with tap water for further immature development. A portion of the eggs, 1st-, 2nd-, 3rd-, and 4th-stage larvae and pupae were removed as they developed. The immature stages were stored individually in 1.5-ml microcentrifuge tubes at −70°C awaiting PCR. Larvae were fed daily with ground Gaines Cycle Light® dog food. The mosquitoes were maintained in a rearing room at ~25°C, 75% relative humidity, and a photoperiod of 12:12 (light : dark).

The bloodfed females that laid eggs in the mosquito breeding containers were identified by SGE by examination of hydroxyacid dehydrogenase, isocitrate dehydrogenase, malic dehydrogenase, malic enzyme, mannose phosphate isomerase, and phosphoglucose isomerase following Narang et al. (1989) and Rutledge (1995).

The immature stages of *An. quadrimaculatus* and *An. inundatus* were tested with oligonucleotide primers that amplify the 2nd internal transcribed spacer region of ribosomal DNA (Cornel et al. 1996). These primers included a universal primer (UNAQ) and 4 species-specific primers (AQA,

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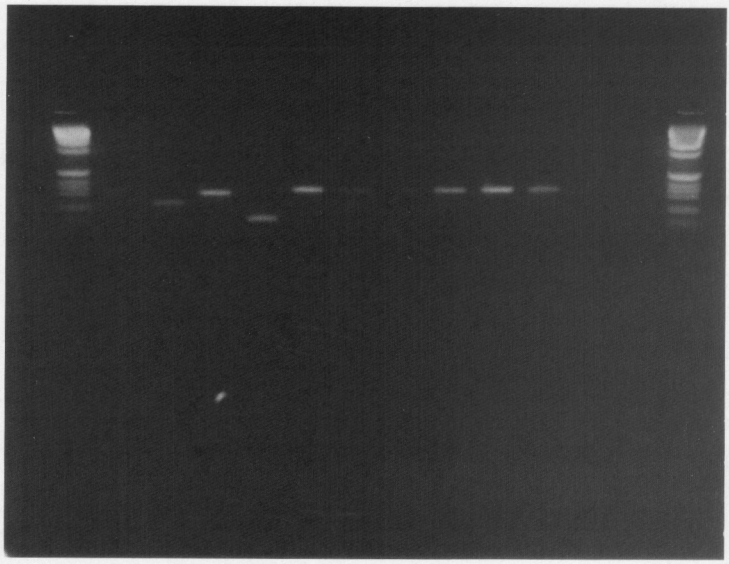

Fig. 1. Photograph of a 2% agarose gel showing amplified DNA of *Anopheles quadrimaculatus* sibling species. Left to right: lanes 1 and 14, 1-kb DNA ladder; lanes 2–5, positive controls: *An. quadrimaculatus, Anopheles smaragdinus, Anopheles inundatus,* and *Anopheles maverlius*; lanes 6–11, *An. quadrimaculatus* eggs, individual specimens of 1st-, 2nd-, 3rd-, and 4th-stage larvae and a single pupa; lanes 12–13, negative controls: *Anopheles franciscanus* and sterile water.

AQB, AQC, and AQD) in a single reaction mixture. The fragment size generated for each species is as follows: *An. quadrimaculatus,* 319 bp; *An. smaragdinus,* 227 bp; *An. diluvialis* and *An. inundatus,* 293 bp; and *An. maverlius,* 141 bp.

Eggs, 1st–4th-stage larvae, pupae, and adults of *An. quadrimaculatus* and *An. inundatus* were examined by the PCR assay. DNA was extracted according to Collins et al. (1990). Reagents used were from the Perkin Elmer GeneAmp PCR core reagents kit (Hoffman-La Roche Inc., Branchburg, NJ). For each 25-μl reaction, the following volumes were used: 2.5 μl 10× buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 2.5 μl $MgCl_2$ (25 mM), 0.5 μl each deoxynucleoside triphosphate (10 mM) and 0.865 units Ampli-Taq polymerase, 10 pmol (pm) UNAQ, 8 pm AQA, 28 pm AQB, 14 pm AQC, 38 pm AQD, and 1 μl target DNA ($1/_{50}$ of DNA isolated from a single mosquito). For each run, positive controls of known *An. quadrimaculatus, An. smaragdinus, An. inundatus,* and *An. maverlius* were used. The 2 negative controls utilized were *Anopheles franciscanus* McCracken and sterile water.

Twenty-five-microliter reactions were placed in a GeneAmp PCR System 2400 thermocycler (Hoffman-La Roche Inc.) with a denaturing temperature of 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 2 min. After 25 cycles, the product along with a loading dye (bromophenol blue) was electrophoresed through a 2% agarose gel containing ethidium bromide. Electrophoresis was stopped when the loading dye had migrated approximately 2.5 cm. Electrophoresis was conducted at 55 ma for about 0.5 h. The fragments were visualized with ultraviolet light, and black and white Polaroid photographs were taken of each gel.

Eggs, 1st–4th-stage larvae, pupae and adults of *An. quadrimaculatus* and *An. inundatus* were tested. Diagnostic bands were amplified in all mosquito life stages for the 2 sibling species evaluated in this study. Fig. 1 shows a gel representing each immature stage of *An. quadimaculatus*. In conclusion, PCR is a highly sensitive technique that can be used to identify immature stages as well as adults of the *An. quadrimaculatus* sibling species complex. This technique also serves as a considerable time saving alternative to SEE for biochemical identification of mosquitoes.

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