DNA-BASED METHODS FOR IDENTIFYING MALARIA MOSQUITOES

:Species-diagnostic Polymerase Chain Reaction Assay for The Identification of *Anopheles* Vectors of Human *Plasmodium SPP.*

Corey L. Brelsfoard[1], Gary N. Fritz[1], Roberto Rodriguez[2].

[1]Department of Biological Sciences, Eastern Illinois University, Charleston IL 61920

[2]Laboratorio de Entomología Médica, Escuela Técnica de Salud Pública, Cochabamba, Bolivia

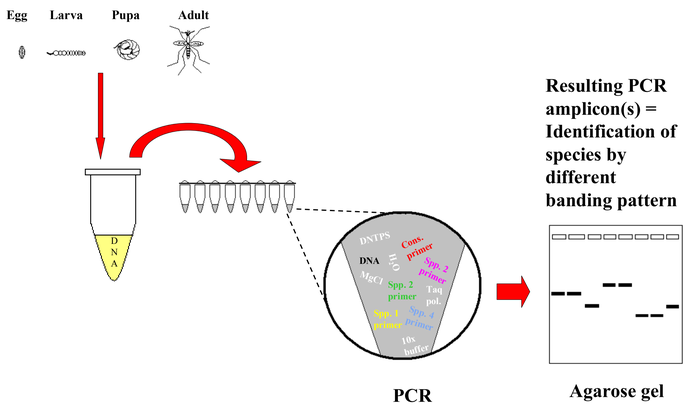
Introduction:

The mosquitoes of the genus *Anopheles* (subfamily Anophelinae) are vectors of human malaria, an important tropical disease that threatens >40% of the world's population in >90 countries and territories. According the World Health Organization, it is estimated to kill 1-3 million people worldwide each year. Five species groups are known to be responsible for most cases of malaria in the Neotropics. Of these groups, four are within the subgenus *Nyssorhynchus*.

Unfortunately, many species are almost identical in appearance and can only be identified with great difficulty using current morphology keys. Consequently, the lack of reliable and efficient means of identifying malaria mosquitoes in S. America has been a major obstacle for studies on their biology, ecology, and behavior, and responsible for our fragmentary understanding of the epidemiology of malaria in this region of the world. Therefore, the development of simple and inexpensive methods to differentiate *Anopheles* species is essential for epidemiological studies. Fortunately, recent advances in DNA analysis, such as the polymerase chain reaction (PCR) and improved cloning and sequencing techniques, have facilitated the application of molecular biology to the taxonomic problems of this subgenus.

Purpose:

The main objective of this study was to develop DNA-based methods for identifying malaria mosquitoes in a region of Bolivia where there is epidemic malaria, and a great diversity of mosquito species and breeding habitats. My goal was to develop a method using multiplex PCR (multiple species specific primers) that was easy, efficient, accurate, and could identify any life stage (egg, larva, pupa, or adult).



Results:

Two multiplex PCR were developed to identify two sets of morphologically similar species of malaria mosquitoes incorporating species-specific primers derived from direct sequencing of the ITS2 (Table 1).

1. One PCR reaction identifies *Anopheles oswaldoi* and an undescribed species (Species C) that we believe to be new to science and abundant in certain areas of Bolivia (Fig. 1).
2. A second PCR reaction identifies mosquitoes as belonging to a complex of four species (Albitarsis Complex), which includes *An.deanorum, An. albitarsis*A, *An. albitarsis* B, and *An. marajoara.* The resulting PCR product is then digested with a restriction enzyme that cuts the DNA at a specific site distinguishing *An. marajoara* (Fig. 1).

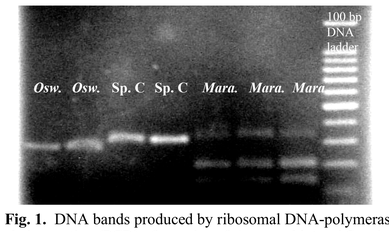


Fig. 1. DNA bands produced by ribosomal DNA-polymerase chain reaction (PCR) of samples of *An. oswaldoi* and Species C, and restriction enzyme digest of *An. marajoara.*

Fig. 2. Sequence alignment of *An. oswaldoi* and species C. Black letters indicate that the sequences are identical, and red indicates differences. Highlighted sections indicate species-specific primer sites.

Fig. 3. Sequence alignment Albitarsis complex. Black letters indicate that the sequences are identical, and red indicates differences. Highlighted section indicates Albitarsis complex unique primer and red rectangle represents Bfa-I (C/TAG) restriction enzyme cut site.

Conclusions:

- The two multiplex PCR developed can provide an unambiguous and relatively rapid identification of morphologically similar species. In addition, the multiplex PCR developed have proven to be highly specific, not only permitting the identification of *An. oswaldoi*, species C, and *An. marajoara*, but also differentiating them from other sympatric anopheline species in the subgenus *Nyssorhynchus.*
- The construction of two multiplex PCR that can diagnose three putative vectors of human *Plasmodium* in the Neotropics should make it more feasible to initiate studies on anopheline mosquito ecology and the dynamics of transmission of malaria, particularly since the primers can be used with a minute amount of sample material for species identification.

Table 1. Ribosomal DNA ITS2 species-specific primers and their melting temperatures (Tm)*

Primer name	Primer sequence (5'-3')	T_m (°C)
oswaldoi	CTT TGA GGG AGG ATA TGG	49.4
Species C.	CTA CCT TAT CTG CAC ATG	47.5
Albitarsis complex	TTT GAT AGA CCC CGT GTC	51.4

An. marajoara adult female.

Species C adult female.

An. oswaldoi adult female.

Methods:

Larval Collection

- Larvae were collected from 56 sites in the Carrasco/Chapare Valley, Bolivia in May 2003.
- Of these larvae, ~10 were reared to adults as voucher specimens from each site for identification with standard morphological keys.
- Sections of DNA that separate genes, but do not code for anything (known as "spacers"), evolve rapidly through time and, therefore, are good candidates for finding DNA differences between species. I chose a spacer known as the Internal Transcribed Spacer Two (ITS2) to develop species-specific primers.

Sequencing

- PCR of the ITS2 was done using primers that anneal to the flanking 5.8s and 28s rDNA genes (Porter and Collins 1991).
- Direct sequencing of the PCR product was done using a Beckman CEQ 2000 sequencer following manufactures' instructions.

Primer Choice

- Appropriate species specific primer sites were located by ITS2 alignment with CLUSTALW of all targeted species (Figs 2 and 3) along with those available on GenBank.
- The species specific primers were then combined in a multiplex PCR and tested with samples of DNA from the following species: *An. trinkae, An. triannulatus, An rangeli, An. strodei, An. aquasalis, An albimanus, An. darlingi, An evansae, An. oswaldoi, An. marajoara, An. albitarsis, An. nuneztovari, An. galveoi, An. deanorum, An. bennarochi, An. konderi, An. braziliensis, An. argrytarsis,* and Species C.

Acknowledgements:
We thank the Bolivian Ministerio de Salud, the Cochabamba Ministerio de Salud, Dr. Rene Mollinedo. This project was supported by the Council for Faculty Research, EIU and NIH Grant #1 R15 AI47796-01A1. Also this project was supported by a Charles B. Arzeni Tropical Biology Scholarship.

NATIONAL INSTITUTES OF HEALTH

EASTERN ILLINOIS UNIVERSITY