Identification of Anopheles (Nyssorhynchus) marajoara (Diptera: Culicidae) in Bolivia Using Polymerase Chain Reaction and a Restriction Endonuclease

Gary Fritz, Eastern Illinois University
C. L. Brelsfoard, Eastern Illinois University
R. Rodriguez

Available at: https://works.bepress.com/gary_fritz/3/
Anopheles marajoara Galvão & Damasceno (Diptera: Culicidae) is a member of the Albitarsis cryptic species complex and is a primary vector of human Plasmodium in parts of Brazil. We report the first record of An. marajoara in the department of Cochabamba and confirm its presence in the department of Santa Cruz, Bolivia. We also describe a PCR protocol producing a single amplicon (≈340 bp) of a section of the internal transcribed spacer 2 specific for members of the Albitarsis species complex; the polymerase chain reaction protocol did not amplify the DNA of 17 other species in the subgenus Nyssorhynchus. Digestion of the amplicon with the restriction endonuclease BfaI produces two fragments specific to An. marajoara in Bolivia.

**KEY WORDS** Albitarsis Complex, taxonomy, ITS2, distribution, Anopheles marajoara

Anopheles marajoara Galvão & Damasceno (Diptera: Culicidae) is in the subgenus Nyssorhynchus, which includes most of the primary and secondary vectors of malaria in South America (de Arruda et al. 1986, Hayes et al. 1987, Gorup and Pull 1988, Haworth 1988, Brannerino et al. 1993, Lounibos and Conn 2000). Although historically thought to be unimportant in the transmission of malaria, An. marajoara has been in- criminated recently as a major vector in Amapá transmission of Plasmodium, though historically thought to be unimportant in the transmission of malaria (de Arruda et al. 1986, Hayes et al. 1987, Goriup and Pull 1988, Haworth 1988, Bra- mson et al. 1995a,b). The four species currently recognized in this complex can now be identified by a series of PCR reactions using different pairs of primers that anneal to the internal transcribed spacer (ITS) 2 (Li and Wilkerson 2005); species E has an ITS2 identical to that of An. marajoara.

An. marajoara was described in 1942 from specimens collected on the island of Marajo in Pará, Brazil (Galvão and Damasceno 1942). Subsequent reports of An. marajoara reported for Colombia, Panama, Venezuela, Costa Rica, Honduras, Trinidad, the Guianas, and Bolivia indicated this species had a very broad distribution (Linthicum 1988). In Bolivia, An. marajoara has been reported from the departments of Beni and Santa Cruz (Linthicum 1988). These records, and others reported for An. marajoara in South America, however, are questionable because they were obtained before the recognition of the Albitarsis Complex. In this study, we report the first record of An. marajoara in the department of Cochabamba and confirm its presence in the department of Santa Cruz, Bolivia. We also describe a PCR that identifies any life stage of an unknown anopheline mosquito as belonging to the Albitarsis Complex. Subsequent digestion of the amplicon with a restriction endonuclease resolves An. marajoara in Bolivia. This PCR protocol differs from the recently reported technique by Li and Wilkerson (2005) insofar as specimens do not require pre-identification to the Albitarsis Complex before PCR for specific diagnosis. We chose the ITS2 spacer sequence to develop a species diagnostic for An. marajoara for the following reasons: complete sequences of this spacer are available in GenBank for all four species in the Albitarsis Complex as well as for 17 other species in the subgenus Nyssorhynchus (of the 33 Nyssorhyn- chus species currently identified; Harbach 2004), the ITS2 exhibits rapid rates of sequence divergence between closely related species that otherwise show little genetic divergence (Fritz et al. 1994, 2004; Crabtree et al. 1995; Charlwood and Edoh 1996; Miller

**ABSTRACT** Anopheles (Nyssorhynchus) marajoara Galvão & Damasceno (Diptera: Culicidae) is a member of the Albitarsis cryptic species complex and is a primary vector of human Plasmodium in parts of Brazil. We report the first record of An. marajoara in the department of Cochabamba and confirm its presence in the department of Santa Cruz, Bolivia. We also describe a PCR protocol producing a single amplicon (≈340 bp) of a section of the internal transcribed spacer 2 specific for members of the Albitarsis species complex; the polymerase chain reaction protocol did not amplify the DNA of 17 other species in the subgenus Nyssorhynchus. Digestion of the amplicon with the restriction endonuclease BfaI produces two fragments specific to An. marajoara in Bolivia.
Materials and Methods

Collection and Preliminary Identification of Specimens. Mosquito larvae and pupae were collected from 56 sites in the Chapare and Carrasco valleys in the departments (states) of Cochabamba and Santa Cruz in May 2003. The collections were part of an ecological study on anopheline larvae in this region of Bolivia. Sites positive for An. albitarsis s.l. were identified from link-reared specimens from each location by using the key of Linthicum (1988). Specimens from each site also were stored in 90% ethanol for subsequent sequencing and PCR-based identification.

Sequencing and Primer Design. Whole mosquito DNA was isolated using DNeasy kits (QIAGEN, Valencia, CA) following manufacturer instructions for the isolation of DNA from animal tissue. The ITS2 was amplified by PCR using 2 µl of DNA, 0.5 µl of TaKaRa Ex TaqDNA polymerase, 5 µl of 10X Ex Taqbuffer, and 4 µl of dNTP mixture (2.5 mM each), all obtained from Takara Bio Inc. (Shiga, Japan); 33.5 µl of H2O, 2.5 µl of the 5.8s (5'-TGGTAACCTGAGGACACATG-3') and 28s (5'-ATGCTTAAATTAGGGGTACGT-3') primers (Porter and Collins 1991) conserved primers at 40 ng/µl; and a Hybaid PCR Express thermal cycler (Thermo Electron Corp., Waltham, MA). The thermocycler temperature profile was one cycle of 1 min at 94°C, 30 s at 65°C, and 30 s at 72°C; 28 cycles of 30 s at 94°C, 30 s at 65°C, and 30 s at 72°C; and one cycle of 30 s at 94°C, 30 s at 65°C, and 5 min at 72°C. Amplicons were subsequently purified using the Qia-Quick PCR purification kit (QIAGEN) according to manufacturer instructions for a microcentrifuge.

Forward and reverse cycle sequencing reactions of the ITS2 were done with a CEQ Dye Terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA) following the manufacturer’s recommendations for sequencing from a double-stranded template. Sequencing was completed using a CEQ 2000 dye terminator sequencer (Beckman Coulter). Forward and reverse sequences were aligned using Sequencher 3.0 (Gene Codes, Ann Arbor, MI) and saved as text format files.

A primer specific to the Albitarsis Complex was designed by aligning ITS2 sequences of all four members of the complex by using CLUSTALW (Thompson et al. 1994) along with other species of the subgenus Nyssorhynchus (available on GenBank), including: Anopheles dunhami Causey, Anopheles strodei Root, Anopheles trinkae Faran, Anopheles triannulatus (Neiva & Pinto), Anopheles rangeli Gavalond, Cova Garcia & Lopez, Anopheles galacoi Causey, Deane & Deane, Anopheles darlingi Root, Anopheles albimanus Wiedemann, Anopheles aquasalis Curry, Anopheles nuneztovari Gabaldon, Anopheles evansae (Brethes), Anopheles konderi Galvão & Damasceno, Anopheles argyritarsis Robineau-Desvoidy, Anopheles braziliensis (Chagas), Anopheles oswaldoi s.l., and Anopheles rondoni (Neiva & Pinto). CLUSTALW alignments were manually optimized, and a primer was chosen that annealed to a sequence in the ITS2 unique to the Albitarsis Complex. PCR reactants and thermocycler temperature profiles were optimized by standard protocols and chosen to give easily resolved amplicons on 2% gels. Optimized PCR reaction protocols for 50-µl amplifications included 5 µl of 10X buffer, 8 µl of 1.25 mM DNTPs, 0.5 µl of Taq polymerase at 5 U/µl, 21.0 µl of H2O, 6 µl of 25 mM MgCl2, and 2.5 µl of each of the two primers at 40 ng/µl. The Hybaid thermocycler (Thermo Electron Corp.) temperature profile was 30 cycles of 94°C for 1 min, 64°C for 30 s, and 72°C for 1 min. Amplification products were electrophoresed on 1.5% gels and visualized with ethidium bromide stain.


Restriction Enzyme Digest. A restriction endonuclease cut site, specific to the ITS2 of An. marajoara in Bolivia, was located by sequence alignment (by using CLUSTALW) with the other members of the Albitarsis Complex. Sequences of the other three members of the Albitarsis Complex, with confirmed identifications, were obtained from Richard Wilkerson, Walter Reed Biosystematics Unit, Smithsonian Institution, Washington, DC (Fig. 1).

Before restriction enzyme digestion, PCR amplifications were purified by addition of 7 µl of 8 M potassium acetate, centrifugation at 4°C for 15 min, supernatant transfer to a 0.5-ml Eppendorf tube, and incubated overnight at −15°C in 150 µl of 95% ethanol. After incubation, the DNA was centrifuged at 14,000 rpm for 15 min at 4°C, and the resulting pellet washed twice with 100 µl of 70% ethanol followed by two centrifugations at 20,000 × g for 15 min at 4°C; the resulting pellet was washed twice with 100 µl of 70% ethanol followed by two centrifugations at 20,000 × g for 2 min and resuspended in 40 µl of 1X #4 buffer (New England Biolabs, Beverly, MA). Twelve microliters of suspended DNA was then subjected to an overnight digestion at 37°C with 0.5 µl of BsaI (C/TAG) restriction endonuclease (New England Biolabs).

Results

Five specimens identified as An. albitarsis s.l. and collected at four locations in Bolivia (Table 2) were sequenced for the ITS2 (Fig. 1) and found to be An. marajoara when aligned with sequences available for the Albitarsis Complex. Sequence alignments of the
Albitaris Complex with those of all other available Nyssorhynchus species indicated a region (5'-TTT GAT AGA CCC CGT GTC GAT C-3') that was unique to species in the Albitarsis Complex (Fig. 1). As expected, a primer that anneals to this unique site, complemented with a conserved primer annealing to the 28s formally linked reared. All specimens produced the two diagnostic fragments indicative of An. marajoara. Digestion of the ITS2 of a single specimen of An. marajoara from Manaus, Brazil, however, did not produce the expected two-fragment pattern of DNA. Voucher specimens of An. marajoara from Bolivia as well as 40 samples of DNA were deposited in the Walter Reed Biosystematics Unit, Smithsonian Institution.

The five locations in the Chapare/Carrasco valley region (Fig. 3) that we chose for testing the diagnostic PCR protocol were sites that had relatively high densities of An. albittaris s.s. (as preliminarily identified using morphological characters of samples that were link reared). At all sites, larvae were found inhabiting sunlit, flooded grassland ponds or marshy areas in agricultural lands often including cattle ranches.

Discussion

Because An. marajoara has now been shown to be an important vector of human Plasmodium spp. in some parts of Brazil (Conn et al. 2002), its presence in the eastern valleys of Cochabamba and adjacent re-
region of Santa Cruz is noteworthy. Of the 16 provinces in Cochabamba, Chapare and Carrasco have historically had the highest malaria incidence rates (e.g., between 8.1 and 19 recorded infections per 1000 individuals in 1996–1997, Velasco and Soriano 1998). The Chapare/Carrasco valley system is also of particular interest to malariologists because it exemplifies the changing nature of tropical rain forest with concomitant changes in the epidemiology of malaria. Most of the Chapare/Carrasco valley region is now second-ary forest or cultivated lands interspersed by subsistence farms, large-scale agricultural monoculture, and a high proportion of migrant workers. Human disturbed rain forests such as that found in the Chapare/Carrasco valleys may exhibit a five-fold increase in anopheline densities over undisturbed forests (Tadei et al. 1998).

Rubio-Palis and Zimmerman (1997) reported An. albitarsis as a common anopheline in savannah ecoregions of South America, and Conn et al. (2002)

### Table 2. Collection data for samples of *A. marajoara* sequenced for the ITS2 from Bolivia

<table>
<thead>
<tr>
<th>Site and sample code</th>
<th>State, province</th>
<th>Locality</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>RL1M03-3</td>
<td>Cochabamba, Carrasco</td>
<td>Rio Lagrimas</td>
<td>17° 09' S, 064° 37' W</td>
</tr>
<tr>
<td>VIIM03-A</td>
<td>Cochabamba, Carrasco</td>
<td>Valle Ivirza</td>
<td>17° 07' S, 064° 55' W</td>
</tr>
<tr>
<td>IVSM03-15</td>
<td>Cochabamba, Carrasco</td>
<td>Ivirgarzama</td>
<td>17° 01' S, 064° 30' W</td>
</tr>
<tr>
<td>PG1M03-1 &amp; 40</td>
<td>Santa Cruz, Icilio</td>
<td>Pto. Grether</td>
<td>17° 10' S, 064° 16' W</td>
</tr>
</tbody>
</table>
found this species increasing in relative abundance where land was deforested and converted to agriculture. Consistent with these studies, the locations where we found high densities of *An. marajoara* were agricultural lands or grazing pastures cleared of forest. Larvae were collected in sunlit, marshy areas within the transition zone between the eastern piedmont of the Chapare/Carrasco valley system and the savannah grasslands of the department of Santa Cruz (Fig. 3). This region, devoid of cattle 10 yr ago, now has large tracts of grassland and a number of cattle ranches. Our data suggest that *An. marajoara* may be increasing in frequency in this rapidly changing region of Bolivia.

As part of a study on the ecology of anopheline larvae in the Chapare/Carrasco valleys of Cochabamba, we have been developing specific primers for several anopheline taxa collected in this region, thus enabling the identification of all life stages. Unlike the PCR protocol described by Li and Wilkerson (2005) for resolving species in the Albitarsis Complex, our PCR protocol does not require pre-identification to the complex. The Li and Wilkerson (2005) protocol was developed for adult specimens pre-identified to the Albitarsis Complex, but the primers have not been tested for possible amplification on species outside the complex. With the protocol described here, all stages of development of any unidentified anopheline can be assigned to the Albitarsis Complex. Our protocol, however, subsequently separates only *An. marajoara* from other members in the complex.

*An. marajoara* from Brazil exhibits substantial intragenomic sequence variation for the ITS2 (C. Li and R. C. Wilkerson, personal communication). Cloned ITS2 sequences of multiple specimens from Brazil show that the T-A transversion (underlined in Fig. 1) in the C/TAG restriction site is polymorphic in *An. marajoara*. If the polymorphism for this restriction site is present in all individuals, then our protocol should identify all members of this species. We cannot, however, rule out the possibility that there are individuals or populations fixed for an ITS2 sequence devoid of the BfaI cut site. The inability of BfaI to cut the ITS2 of a single specimen of *An. marajoara* from Manaus, Brazil (Table 1) suggests there may be such instances. Nevertheless, our data in Bolivia indicate that most, if not all, *An. marajoara* have the BfaI cut site; of 170 specimens analyzed in five locations, all had the BfaI cut site. Because no specimen identified as belonging to the Albitarsis Complex lacked the BfaI cut site, our data also suggest that other members of the Albitarsis Complex may be absent or rare from this region of Bolivia.
Accurate and quick molecular methods to identify anopheline mosquitoes, such as the method described in this study, have led to their recent use in field studies. Charlwood and Edoh (1996) used PCR to identify larvae in a study describing aquatic habitats used by species in the An. gambiae complex in Tanzania, and G.N.F. and Paudel (unpublished data) have described larval habitat and species distributions in the Chapare/Carrasco provinces for An. tricolor, An. triangulatus, An. rangeli, An. strodei, An. oswaldoi, and an undescribed species in the Oswaldoi Complex by using two separate multiplex PCRs (C.L.B. and G.N.F., unpublished data; Fritz et al. 2004). Combinations of several multiplex PCRs should allow quick and accurate identification of all or most species in any given area, enabling more comprehensive studies on the basic biology, behavior, and transmission dynamics of vectors of human Plasmodium spp. Because of the ecological complexity of the Neotropics, the rapidly changing environment because of human disturbance, and the high diversity of anopheline species, a number of authors have argued that the epidemiology and control of malaria is best understood and managed at the regional level (Rubio-Palis and Zimmerman 1997, Fritz 1998, 2000). The PCR diagnostic developed in this study, although not necessarily applicable to other regions of South America, is a taxonomic tool that has regional utility in an area of Bolivia with endemic malaria.

Acknowledgments

We thank Drs. Rene Mollinedo, Fidel Fernandes, Jhonny Bascopé, and Efrain Vallejo of the Bolivian Ministerio de Salud, the Cochabamba Ministerio de Salud, and SEDES, respectively. We thank the Escuela Tecnica de Salud Boliviano Japonesa and the Laboratorio de Entomologia Medica in Cochabamba. We thank Claudia Lopez for collecting assistance, Reema Paudel for technical assistance, and Richard Wilkerson and Cong Li for providing samples of multiple species of the subgenus Nyssorhynchus and advice on species identifications. This project was supported by the Council for faculty research, Eastern Indiana University, National Institutes of Health Grant 1 R15 AI47796-01A1, and the Charles B. Arzeni Tropical Biology Scholarship.

References Cited


Received 4 September 2005; accepted 8 March 2006.