Sequence Analysis of the rDNA Internal Transcribed Spacer 2 and Polymerase Chain Reaction Identification of Anopheles fluminensis (Diptera: Culicidae: Anopheles) in Bolivia

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COREY L. BRELSFOARD,¹ GARY N. FRITZ,¹ AND ROBERTO RODRIGUEZ²


**ABSTRACT** *Anopheles fluminensis* Root is a member of the Arribalzagia Series in the subgenus *Anopheles*. We report the first record of this species in the department of Cochabamba, Bolivia. This species was sampled from two locations in the foothills of the eastern Andes Mountains within the Chapare Valley. Larvae were collected in fast-flowing, shaded streams at the edges of rocky pools. We provide the first sequence data for the rDNA of *An. fluminensis*, a partial sequence of the 5.8S and the internal transcribed spacer 2 (ITS2). The ITS2 of *An. fluminensis*, sequenced from two individuals at one site, was at least 596 bp, had 56.5% GC, and included three large repeats (~125 bp each). We describe a polymerase chain reaction protocol and species-specific primers for identifying this species in the Chapare Valley, Bolivia.

**KEY WORDS** *Anopheles fluminensis*, PCR identification, ITS2, repeat units
Currently, the only way to differentiate species of the Arribalzagia Series is by examining morphology of various life stages. For example, Wilkerson and Peyton (1990) emphasized the use of costal wing spots to develop standardized nomenclature for the Arribalzagia Series. Other studies have used morphological characteristics of male genitalia, and immature life stages (i.e., egg, larva, and pupa) (Lounibos et al. 1997, Sallum et al. 1999, Wilkerson and Sallum 1999), all of which require time-intensive sample preparation.

In this study, we provide the sequence of the rDNA ITS2 of An. fluminensis, we provide the first report of this species in the Department of Cochabamba, Bolivia, and we describe a PCR protocol for identifying any stage of this species in this region of Bolivia. A diagnostic primer was developed from sequences of the ITS2 because rDNA spacers diverge rapidly, exhibit homogenization and concerted evolution and, therefore, are useful for differentiating closely related species that otherwise exhibit little genetic divergence (Fritz et al. 1994, Crabtree et al. 1995, Charwood and Eodh 1996, Miller et al. 1996, Fritz et al. 2004).

Materials and Methods

Collection and Identification of Specimens. Specimens of An. fluminensis examined in this study were collected in May 2003 and May 1996 from two sites in Cochabamba, Bolivia (Table 1). Collection sites were located in the Chapare Valley near the San Rafael River in the piedmont ecoregion of the eastern Andes foothills (Fig. 1). Several individuals were link-reared from each site to obtain larval and pupal exuviae, and pinned adults. Specimen identifications were done by Richard C. Wilkerson (Walter Reed Biosystematics Unit, Smithsonian Institution, Washington, DC); identification of samples to the species An. fluminensis was only possible with characters of the male genitalia correlated with presence of upper mesepimeral scales and an unpublished key character concerning the nature of light and dark spots surrounding the subcostal dark spot. Larval and pupal samples were stored in 90% ethanol until used for morphological identifications, sequencing, and the development and testing of PCR diagnostics.

Sequencing and Primer Design. Single larval or adult mosquito DNA was isolated using DNeasy kits (QIAGEN, Valencia, CA) following the instructions for the isolation of DNA from animal tissues. The ITS2 region was amplified by the PCR by using primers that annealed to conserved regions of the 5.8s (5'-TGT-GAAGTCAGGACACATG-3') and 28s (5'-ATGCT-TAATTTAGGGGTAGTGC-3') (Porter and Collins 1991) with the following thermocycler temperature profile: 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. Amplifications were found to be sufficient for use in cycle sequencing by using 2 μl of DNA after isolation with DNeasy kits in a 50-μl reaction, containing the following reagents: 0.5 μl of TaKaRA Ex Taq, 5 μl of 10× Ex Taq buffer, 4 μl of dNTP mixture (2.5 mM each) (all obtained from Takara Bio Inc., Shiga, Japan), 33.5 μl of H2O, and 2.5 μl of each of the two primers at 40 ng/μl (5.8s and 28s). Amplicons were subsequently purified using QIAquick PCR purification kits (QIAGEN) according to manufacturer’s instructions for a microcentrifuge. Forward and reverse consensus sequences of the ITS2 were obtained by using primers that anneal to conserved regions of the 5.8s and 28s. Sequencing reactions were accomplished using a CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter, Fullerton, CA) following manufacturer’s recommendations for sequencing from a double-stranded template. All sequencing was completed using a Beckman Coulter 2000 sequencer.

**Table 1. Larval collection data for *An. fluminensis* specimens collected in the Chapare Valley, Cochabamba, Bolivia.**

<table>
<thead>
<tr>
<th>Site code</th>
<th>Location</th>
<th>Partially shaded, rocky, mountain stream</th>
<th>Elevation (m)</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY1M03</td>
<td>Muyurina</td>
<td>500</td>
<td>S 17° 03’55” W 65° 29’04”</td>
<td></td>
</tr>
<tr>
<td>SR1M03</td>
<td>San Rafael River</td>
<td>360</td>
<td>S 17° 03’58” W 65° 29’44”</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection location</th>
<th>Coordinates</th>
<th>No. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. albimanus</em></td>
<td>Laboratory colony, USDA, Gainesville, FL</td>
<td>Not known</td>
<td>1</td>
</tr>
<tr>
<td><em>An. albimanus</em></td>
<td>Brazil, Iguape</td>
<td>24° 44’ S, 47° 35’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. aquasalis</em></td>
<td>Suriname, Paramaribo</td>
<td>5° 50’ N, 55° 11’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. aquasalis</em></td>
<td>Brazil, Marajo Island</td>
<td>Not known</td>
<td>1</td>
</tr>
<tr>
<td><em>An. bennarrochi</em></td>
<td>Brazil, Rondonia, Costa Marques</td>
<td>12° 25’ S, 64° 18’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. darlingtoni</em></td>
<td>Bolivia, Beni, Guayaramirn</td>
<td>10° 51’ N, 65° 21’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. darlingtoni</em></td>
<td>Brazil, Rondonia, Costa Marques</td>
<td>12° 25’ S, 64° 18’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. DecimalFormat</em></td>
<td>Brazil, Rio de Janeiro</td>
<td>23° 47’ S, 43° 49’ W</td>
<td>2</td>
</tr>
<tr>
<td><em>An. kwendleri</em></td>
<td>Brazil, Rondonia, Costa Marques</td>
<td>12° 25’ S, 64° 18’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. marajoara</em></td>
<td>Venezuela, Céjedez</td>
<td>Not known</td>
<td>1</td>
</tr>
<tr>
<td><em>An. moskevskii</em></td>
<td>Brazil, Boraima, Boa Vista</td>
<td>2° 49’ N, 60° 40’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. moskevskii</em></td>
<td>Venezuela, Barinas, Boronato</td>
<td>Not known</td>
<td>1</td>
</tr>
<tr>
<td><em>An. osceola</em></td>
<td>Brazil, Pará, Urucuri</td>
<td>Not known</td>
<td>1</td>
</tr>
<tr>
<td><em>An. rangelii</em></td>
<td>Bolivia, Cochabamba, Chapare Valley</td>
<td>16° 53’ S, 65° 11’ W</td>
<td>3</td>
</tr>
<tr>
<td><em>An. rangelii</em></td>
<td>Bolivia, Cochabamba, Chapare Valley</td>
<td>16° 59’ S, 65° 37’ W</td>
<td>2</td>
</tr>
<tr>
<td><em>An. rangelii</em></td>
<td>Bolivia, Cochabamba, Chapare Valley</td>
<td>16° 56’ S, 65° 23’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. rangelii</em></td>
<td>Bolivia, Cochabamba, Chapare Valley</td>
<td>17° 02’ S, 64° 51’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. triannulatus</em></td>
<td>Bolivia, Cochabamba, Chapare Valley</td>
<td>17° 12’ S, 64° 30’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. trinkae</em></td>
<td>Bolivia, Cochabamba, Chapare Valley</td>
<td>16° 57’ S, 65° 19’ W</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2. Collection localities for specimens in the subgenera *Nyssorhynchus* and *Anopheles* tested with species-specific primer.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection location</th>
<th>Coordinates</th>
<th>No. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. albimanus</em></td>
<td>United States, Minnesota, Champlin, Elm Creek Park</td>
<td>45° 10’ N, 97° 25’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. franciscanus</em></td>
<td>United States, California, El Dorado County, Cameron</td>
<td>39° 16’ N, 123° 33’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. freeborni</em></td>
<td>United States, California, Yolo County, Knights Landing</td>
<td>38° 47’ N, 121° 43’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. hermsi</em></td>
<td>United States, California, San Mateo County, Jasper Ridge Preserve</td>
<td>37° 24’ N, 122° 13’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. occidentalis</em></td>
<td>United States, California, Alameda County, Coyote Hills</td>
<td>39° 58’ N, 120° 26’ W</td>
<td>1</td>
</tr>
<tr>
<td><em>An. perplexus</em></td>
<td>United States, Florida, Lake Panasofkeef</td>
<td>Not known</td>
<td>1</td>
</tr>
<tr>
<td><em>An. punctipennis</em></td>
<td>United States, California, Lake Vera</td>
<td>39° 18’ N, 121° 01’ W</td>
<td>1</td>
</tr>
</tbody>
</table>
kit. Twelve microliters of the purified DNA was subjected to an overnight digest at 75°C with 0.5 µl of Apek-I, followed by electrophoresis of 10 µl of cut DNA on a 3% agarose gel stained with ethidium bromide. Field samples tested with Apek-I included nine individuals from MY1M03 and six from SR1M03 (Fig. 1; Table 1).

Results

Larvae of *An. fluminensis* were found at two sites on the eastern foothills (piedmont ecoregion) of the Andes Mountains that lead into the Chapare Valley (Fig. 1). The aquatic sites were fast-flowing, shaded streams, and larvae were collected at the edges of rocky pools, often in the vegetation, debris, or among roots that border these pools.

ITS2 sequence was obtained from within 4–12 bp of the primers that annealed to the 5.8s and 28s regions flanking the ITS2. The boundary of the ITS2 with its flanking, conserved rDNA genes was estimated by comparison with those determined for the ITS2 sequences of *An. mediopunctatus* and *An. quadrimaculatus* available on Genbank (accession no. AF462379 and U32550, respectively). The ITS2 begins at approximately position 53 (Fig. 2) and encompasses a region of at least 596 bp. The ITS2 of *An. fluminensis* was 56.5% GC and contained three large repeats (Figs. 2–4), each containing a single unique restriction endonuclease (Apek-I) cut site (Figs. 2 and 4). The first repeat

![Fig. 2. ITS2 sequence and flanking regions of the 5.8s and 28s rRNA genes of *An. fluminensis*. Underlined regions represent repeat units. Bolded sections of sequence represent Apek-I digest sites.](image)

![Fig. 3. Sequence alignment of repeat units within the ITS2 of *An. fluminensis*. Dashes represent identical sequence with the first repeat unit and dots represent gaps introduced to maintain alignment. The numbering scale for the nucleotides to the right of the sequences coincides with the scale in Fig. 2.](image)
Fig. 4. Restriction map for Apek-I. The ITS2 is outlined on the bottom of the figure and flanked by the two conserved genes (5.8s and 28s). Repeat regions are represented by the rectangles with different shading patterns, labeled first repeat, second repeat, and third repeat, respectively, from left to right. Dotted lines with numbers above them separate 100-bp segments that coincide with the numbering in Fig. 2. Arrows represent approximate Apek-I cut sites unique to each repeat unit.

(from the 5' end of the ITS2) is \( \sim 124 \) bp, whereas the second and third are \( \sim 127 \) bp (Fig. 3). Digestion of the ITS2 amplicon with Apek-I was expected to produce four fragments of DNA of the following approximate sizes: \( \sim 143, \sim 144, \sim 223, \) and \( \sim 170 \) bp. As expected, agarose gel electrophoresis resolved three bands (Fig. 5). The conserved region of the 28s rDNA gene that flanks the 3' end of the ITS2 was not observed in the sequence generated for An. fluminensis; thus, the ITS2 reported here is most likely partial.

A single species-specific primer (5' - GACCAC-CAAAAAGAGTCGG-3') was chosen for amplifying a single amplicon in An. fluminensis of \( \sim 225 \) bp (Fig. 5) when combined in a PCR with the 5.8s primer found in the conserved 5.8s rDNA gene. The primer sequence was not present in its presumptive close relative An. mediopunctatus nor in any of the other species tested in the subgenera Anopheles or Nyssorhynchus. The location for the primer was chosen so that it did not anneal within any of the repeats. The rationale for this choice was to prevent ambiguous amplicons of different sizes in individuals of An. fluminensis that may have variable numbers of repeats in the ITS2. Nevertheless, Apek-I digestion of all field-collected samples produced the expected three-banded pattern of the correct sizes in gel electrophoresis (Fig. 5).

Optimized 50-\( \mu \)l PCR reactions included 5 \( \mu \)l of 10\( \times \) buffer, 8 \( \mu \)l of dNTP mix at 1.25 mM, 0.5 \( \mu \)l of Taq polymerase at 5 U/\( \mu \)l, 21.0 \( \mu \)l of H\(_2\)O, 6 \( \mu \)l of MgCl\(_2\) at 25 mM, and 2.5 \( \mu \)l of each of the two primers at 40 ng/\( \mu \)l (An. fluminensis species-specific and 5.8s). We examined various program parameters, especially the annealing temperature to optimize amplifications. A Hybaid PCR express thermocycler (Thermo Electron, Waltham, MA) was used for all PCR with the following program: 29 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C and once cycle of 30 s at 94°C, 30 s at 60°C, with a final extension step of 2 min at 72°C. Amplification products were electrophoresed on 1.5% gels and visualized with ethidium bromide stain. PCR reactions with An. fluminensis species-specific and 5.8s primers produced no unexpected amplicons when tested on other members of the subgenus Anopheles and Nyssorhynchus.

Discussion

Since its description, An. fluminensis has been reported in Colombia (Quinones et al. 1987), Peru (Morales-Ayala 1971), Argentina (Mitchell and Darsie 1985), and various locations in Brazil (Milward de Andrade 1962, Barbosa et al. 1993, Lounibos et al. 1997, Guimarães et al. 2000). Although Gorham et al. (1973) list An. fluminensis as present in Bolivia, no reference is provided. The Mosquito Catalog of the World, managed by the Walter Reed Biosystematic Unit, also lists An. fluminensis as present in Bolivia, but none of the references provided for its known distribution mention this species with regard to Bolivia. Consequently, we know of no published records of An. fluminensis in Bolivia. We assume, therefore, that this is the first confirmed report of An. fluminensis in Cochambamba and perhaps Bolivia as a whole. Its presence in the eastern Andean piedmont is consistent with its ecoregional classification by Rubio-Falis and Zimmerman (1997).

The ITS2 of An. fluminensis is unusually large, because anopheline mosquitoes, with some exceptions (e.g., two members of the An. crucians complex; Wilkinson et al. 2004), have this spacer in the range of 200 to 400 bp in length (sequences recorded in GenBank). The three repeats in the ITS2 suggest that one or two duplications led to its unusual size. A BLAST search of this repeat in GenBank did not show similarity to any other sequences; suggesting the origin of the repeat is within the ITS2 itself. Because we did not sequence clones of the ITS2, but rather obtained the consensus
sequence through PCR, we cannot rule out the possibility that intraindividual variation exists for the number of repeats present in rDNA units. When am- plifying the ITS2, the presence of two faint, additional amplicons of lower molecular weight suggests intra- individual variation in repeat number, but this possibility will need to be confirmed with the sequencing of clones.

The ITS2 of An. fluminensis is also unusual for having large repeats. Large repeats of varying size have been reported in the rDNA spacers of species in the genera Daphnia, Aedes, Drosophila, Battus, and Xenopus (Lab- hart and Reeder 1984, Simeone et al. 1985, Murtif and Rae 1985, Cassidy et al. 1986, Park and Fallon 1990, Crease 1993). Perera et al. (1998) also reported a 36-nucleotide repeat in the intergenic spacer of Anopheles aquasalis Curry. The size of the repeats found in this study, however, has not hitherto been reported in any species of Anopheles for the rDNA internal transcribed spacers. Relatively short repeats, often dinucleotides associated with microsatellite regions, have been reported in the internal spacers of some anophelines (Park and Fallon 1990, Porter and Collins 1991, Fritz et al. 1994, Fritz 1998, Wilkerson et al. 2004, Fairley et al. 2005). Whether the large repeats found in An. fluminensis affect transcription or RNA processing is not known, but conformational studies on large repeats in the ITS2 of ticks (Murrell et al. 2004, Fairley et al. 2005) have suggested no effect.

Our species-specific primer for An. fluminensis exhibits no significant sequence similarity to three other species in the Series Arribalzagia and did not amplify the DNA of any species tested in the subgenus Anoph- eles and Nyssorhynchus. Additionally, when blasted on GenBank, the primer sequence was not similar to any mosquito DNA sequenced to date. Of the 24 species in the Arribalzagia Series, however, only four have now been sequenced for their ITS2. Thus, it is possible that the species developed in this study awaits to other species in this Series. Furthermore, An. mediorunca- tus, with which An. fluminensis has sometimes been confused, is thought to be restricted to the coastal regions of São Paulo, Rio de Janeiro and Paraná, Brazil (Wilkerson and Sallum 1999). We think, therefore, that the PCR diagnostic developed in this study has, at least, regional utility and will facilitate studies that aim to elucidate the basic biology, ecology, and behavior of An. fluminensis in Bolivia.

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References Cited


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