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

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

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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

Anopheles fluminensis was described by Root (1927) from the State of Rio de Janeiro, Brazil, and is a member of the Arribalzagia Series of the Laticorn Section in the subgenus *Anopheles* (Reid and Knight 1961). The Arribalzagia Series contains 24 species (Wilkerson and Peyton 1990). Within this series, *An. fluminensis* is morphologically very similar in the adult female to *Anopheles mediopunctatus* (Lutz) (Root 1927, Lounibos et al. 1997), which in turn has been shown recently to comprise a cryptic complex of three species (Sallum et al. 1999, Wilkerson and Sallum 1999). Although not currently considered an important vector of human *Plasmodium* spp. (Cerqueira 1961, Gorham et al. 1973), specimens identified as near *An. fluminensis* have been incriminated as vectors of human malaria in eastern Peru (Hayes et al. 1987, Rubio-Palis and Zimmerman 1997). In addition, *An. mediopunctatus*, which may be confused with *An. fluminensis*, has been shown to be vector competent for both *Plasmodium vivax* and *Plasmodium falciparum* and incriminated as a vector in Brazil (Klein et al. 1991a,b; Povóa et al. 2000).

An important first step in understanding the epidemiology of malaria in South America is accurate species identification, particularly to differentiate vector and nonvector species (Wilkerson et al. 1995, Rubio-Palis and Zimmerman 1997, Fritz 1998, Conn et al. 2002, Fritz et al. 2004). More than a decade ago, Oaks et al. (1991) opined that the single most important

laboratory technique that should be developed and transferred to field studies on malaria was simple and inexpensive methods to differentiate anopheline mosquitoes. Since then, several molecular protocols for identifying anopheline mosquitoes, particularly cryptic species, were described, most using the polymerase chain reaction (PCR) (Cooper et al. 1991, Porter and Collins 1991, Hill and Crampton 1994, Audtho et al. 1995, Fritz et al. 1995, Wilkerson et al. 1995, Cornel et al. 1996, Rutledge et al. 1996, West et al. 1997, Fritz et al. 2004). Recent reviews on molecular diagnostics for mosquitoes by Walton et al. (1999) and Krzywinski and Besansky (2003) have noted the continuing importance of developing quick and accurate means of species identification and have highlighted the usefulness of the rDNA internal transcribed spacers for this purpose (particularly the internal transcribed spacer 2 [ITS2]).

Although molecular identification techniques are now available for several Asian and African species of anopheline mosquitoes, few have been developed for the multitude of species found in South America. For example, the subgenus *Nysorrhynchus* includes the majority of species of anopheline mosquitoes considered important in vectoring human *Plasmodium* spp. in South America. At present, there are only two PCR-based methods generating single, species-specific amplicons identifying *Nysorrhynchus* spp.: primers that distinguish the four members of the *albitarsis* complex (Li and Wilkerson 2005) and a multiplex PCR for discriminating *Anopheles triannulatus* (Neiva & Pinto), *Anopheles trinkae* Faran, *Anopheles rangeli* Gabadon, Covia Garcia & Lopez, and *Anopheles strodei* Root (Fritz et al. 2004).

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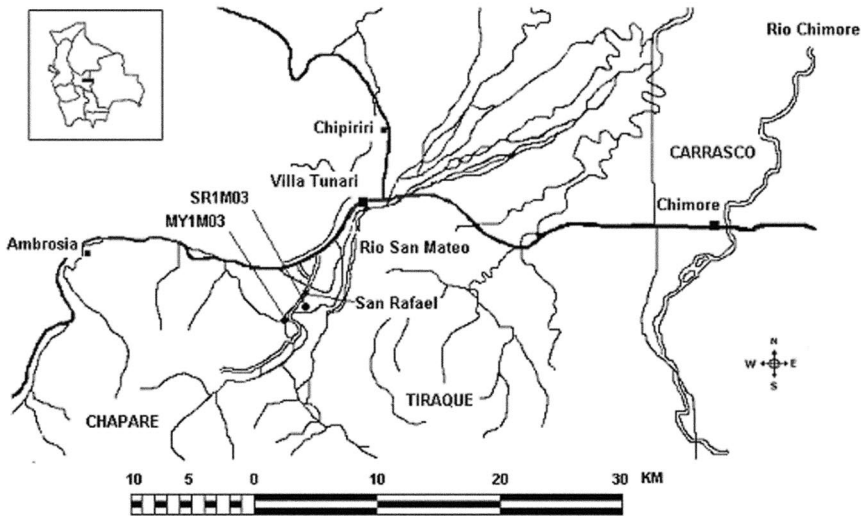


Fig. 1. Map of the Chapare/ Carrasco Valley. Solid circles with site codes SR1M03 and MY1M03 represent collection sites for *An. fluminensis* (see Table 1). Rectangles represent the towns of Ambrosia, San Rafael, Villa Tunari, Chipiriri, and Chimore.

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, Charlwood and Edoh 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-GAAGTGCAGGACACATG-3') and 28s (5'-ATGCT-TAAATTTAGGGGGTAGTC-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.

Site code	Location	Habitat	Elevation (m)	Coordinates
MY1M03	Muyurina	Partially shaded, rocky, mountain stream	500	S 17° 03.552' W 65° 29.924"
SR1M03	San Rafael River	Partially shaded, rocky, mountain stream	360	S 17° 03.88' W 65° 29.442"

Forward and reverse sequences were subsequently aligned using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI) and manually checked for optimal alignment. Two specimens of *An. fluminensis* were sequenced from site MY1M03 (Fig. 1; Table 1). A species-specific primer was designed by aligning available ITS2 sequences from a sequence database (GenBank) for members of the subgenus *Anopheles*, including *An. eiseni* Coquillett, *An. quadrimaculatus* Say, *An. mattogrossensis* Lutz & Neiva, *An. mediopunctatus*, and *An. peryassui* Dyar & Knab by using CLUSTALW (Thompson et al. 1994). The latter three species are in the same series (Arribalzaga) as *An. fluminensis*. In addition, the primer sequence was compared with the ITS2 sequence of *Anopheles* members of the subgenus *Nyssorhynchus*, including *An. dunhami* Causey, *An. strodei*, *An. trinkae*, *An. triannulatus*, *An. rangeli*, *An. galvaoui* Gabaldon, *An. darlingi* Root, *An. marajoara* Galvao & Damasceno, *An. albimanus* Wiedemann, *An. nuneztovari* Gabaldon, *An. evansae* Brethes, *An. konderi* Galvao & Damasceno, *An. argyritarsis* Robineau-Desvoidy, *An. braziliensis* (Chagas), *An. deaneorum* Rosa Freitas, *An. albitarsis* Lynch Arribalzaga, *An. albitarsis* B, and *An. rondoni* (Neiva & Pinto) and two members of the Oswaldoi species complex (C.L.B., unpublished data). Finally, the primer se-

quence was subjected to an automated search function (i.e., "blasted") to find homologous sequences on GenBank to confirm the primer's specificity for the targeted species. The primer and PCR conditions were optimized using standard protocols and chosen to give an easily resolved amplicon on 2% gels stained with ethidium bromide. The species-specific primer was paired with a primer that annealed to the 5.8s, and tested on its target species, and subsequently tested on samples of DNA from seven members of the subgenus *Anopheles* and 16 members of the subgenus *Nyssorhynchus* (Table 2). The sequence for *An. fluminensis* is in the GenBank database (DQ328638).

Restriction Enzyme Digest. Because the ITS2 sequence of *An. fluminensis* was found to contain three large, tandem repeats, we tested for the presence of these repeats in field samples of *An. fluminensis* by endonuclease digestion (Apek-I, New England Biolabs, Beverly, MA) of a unique restriction site found in each repeat. The complete ITS2 amplified by the PCR reaction outlined above was first purified using QIAquick PCR purification kits (QIAGEN) following manufacturer's instructions. However, in the last step of the QIAGEN kit, we suspending DNA in 40 µl of 1× #3 buffer supplied by New England Biolabs instead of buffer supplied with the QIAGEN PCR purification

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

Species	Collection location	Coordinates	No. tested
Subgenus <i>Nyssorhynchus</i> spp. tested			
<i>An. albimanus</i>	Laboratory colony, USDA, Gainesville, FL	Not known	1
<i>An. albitarsis</i>	Brazil, Iguape	24° 44' S, 47° 35' W	1
<i>An. aquasalis</i>	Suriname, Paramaibo	5° 50' N, 55° 11' W	1
	Brazil, Marajo Island	Not known	1
<i>An. benarrochi</i>	Brazil, Rondonia, Costa Marques	12° 25' S, 64° 18' W	1
<i>An. darlingi</i>	Bolivia, Beni, Guayaramirim	10° 51' N, 65° 21' W	1
<i>An. deaneorum</i>	Brazil, Rondonia, Costa Marques	12° 25' S, 64° 18' W	1
<i>An. evansae</i>	Brazil, Rio de Janeiro	23° 47' S, 43° 49' W	2
<i>An. konderi</i>	Brazil, Rondonia, Costa Marques	12° 25' S, 64° 18' W	1
<i>An. marajoara</i>	Venezuela, Cojedes	Not known	1
	Bolivia, Cochabamba, Chapare Valley	17° 58' S, 64° 49' W	2
<i>An. nuneztovari</i>	Brazil, Roraima, Boa Vista	2° 49' N, 60° 40' W	1
	Venezuela, Barinas, Boconoito	Not known	1
<i>An. oswaldoi</i>	Brazil, Pará, Uruçuri	Not known	1
	Bolivia, Cochabamba, Chapare Valley	16° 53' S, 65° 11' W	3
	Bolivia, Cochabamba, Chapare Valley	16° 59' S, 65° 37' W	2
<i>An. rangeli</i>	Bolivia, Cochabamba, Chapare Valley	16° 56' S, 65° 23' W	1
<i>An. strodei</i>	Bolivia, Cochabamba, Chapare Valley	17° 02' S, 64° 51' W	1
<i>An. triannulatus</i>	Bolivia, Cochabamba, Chapare Valley	17° 12' S, 64° 30' W	1
<i>An. trinkae</i>	Bolivia, Cochabamba, Chapare Valley	16° 57' S, 65° 19' W	1
Subgenus <i>Anopheles</i> spp. tested			
<i>An. earlei</i>	United States, Minnesota, Champlin, Elm Creek Park	45° 10' N, 93° 25' W	1
<i>An. franciscanus</i>	United States, California, El Dorado County, Cameron	39° 16' N, 123° 33' W	1
<i>An. freeborni</i>	United States, California, Yolo County, Knights Landing	38° 47' N, 121° 43' W	1
<i>An. hermsi</i>	United States, California, San Mateo County, Jasper Ridge Preserve	37° 24' N, 122° 13' W	1
<i>An. occidentalis</i>	United States, California, Alameda County, Coyote Hills	39° 58' N, 120° 26' W	1
<i>An. perplexens</i>	United States, Florida, Lake Panasofkee	Not known	1
<i>An. punctipennis</i>	United States, California, Lake Vera	39° 18' N, 121° 01' W	1

GCATATTGCGCATCGTGGACACAGCTCGATGCACATATCTTTGAGAGTCCATAC 55
 TTGACATAGTCAAACCTACGGTTGTCTGGGGCGCAAGCTCGGACACTACCGTGATA 110
 TTGGGGTGGCGGCCCTACTCGGGCTCGTAGCCCTTAAAATCCCTGTGGAGCGTG 165
 TTCACCGACTCTTTTGGTGGTCTCTCGTACCAAGTGG**GCAG**CGGTGGCCGGCAC 220
CCTCACCTCTTCACCGAGCCGTTGAGATGTGTTCCGGTCACACATACGCTGATCAC 275
GACGAGCGTCTTAAGGTGGTGGACTGCCCATGGAAGGGAGCTCTTGGTCTGCTC 330
TCGTACCAAAAAGTGG**GCAG**CGGTGGCCTGCACCTCTCACCTCTTCACCGAGCCG 385
TTGAGATGTGTACGGTCACGCATACGCTGACCACGACGAGCGATCATAAGGTGGT 440
 TGACTACCTCCTGATACGAGCTCTTGGTCTCTCGTACCAAAAAGTGG**GCAG** 495
GGTGGCCTGCACCTCTCACCTCTTCACCGAGCCGTTGAGATGTGTTCCGGTCACGC 550
ATACGCTGATCACGACGAGCGACCAAAAAGGTGGTACCAAGAATTATGTATGGGTA 605
 TGGTACCGAAACGAACGCTCCCTTCGGTGGAGTTTATGAGCAGT 649

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.

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

1 st	ACTCTTTTGGTGGTCTCTCGTACCAA . GTGGGCAGCGGTGGCCGGCA	219
2 nd	GAG--C-----C-----AA-----T---	362
3 rd	GAG--C-----C-----AA-----T---	506
1 st	CC . CTCACCTCTTCACCGAGCCGTTGAGATGTGTTCCGGTCACACATACG	267
2 nd	--T-----A-----G-----	411
3 rd	--T-----T-----G-----	555
1 st	CTGATCACGACGAGCGTCCCTTAAGGTGGT	296
2 nd	----C-----AT-AT-----	440
3 rd	----T-----AC-AA-----	584

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.

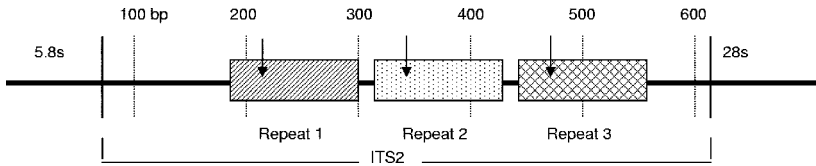


Fig. 4. Restriction map for Apek-I. The ITS2 is outlined on the bottom of the figure and flanked by the two conserved genes (5.8s and 28s). Repeat regions are represented by the rectangles with different shading patterns, labeled first repeat, second repeat, and third repeat, respectively, from left 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 ≈ 124 bp, whereas the second and third are 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: ≈ 143 , ≈ 144 , ≈ 223 , and ≈ 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-CAAAGAGTCGG-3') was chosen for amplifying a single amplicon in *An. fluminensis* of ≈ 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- μ l PCR reactions included 5 μ l of 10 \times buffer, 8 μ l of dNTP mix at 1.25 mM, 0.5 μ l of Taq

polymerase at 5 U/ μ l, 21.0 μ l of H₂O, 6 μ l of MgCl₂ at 25 mM, and 2.5 μ l of each of the two primers at 40 ng/ μ 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 (Quiñones 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 Cochabamba and perhaps Bolivia as a whole. Its presence in the eastern Andean piedmont is consistent with its ecoregional classification by Rubio-Palis 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; Wilkerson 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

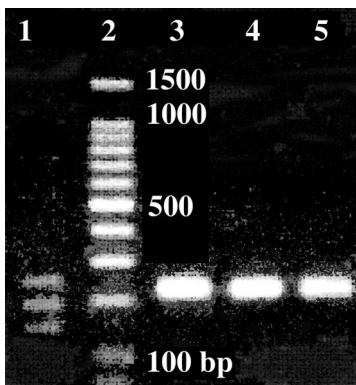


Fig. 5. Restriction enzyme digest with Apek-I of *An. fluminensis* ITS2 amplicon (lane 1) and species specific amplicon for *An. fluminensis* (lanes 3-5). Lane 2, 100-bp DNA ladder.

sequence through PCR, we cannot rule out the possibility that intraindividual variation exists for the number of repeats present in rDNA units. When amplifying the ITS2, the presence of two faint, additional amplicons of lower molecular weight suggests intraindividual 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*, *Rattus*, and *Xenopus* (Labhart 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. 2001) suggest 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 *Anopheles* 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 primer developed in this study anneals to other species in this Series. Furthermore, *An. mediopunctatus*, 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

- Audtho, M., A. Tassanakajon, V. Boosaeng, S. Tpiankijagum, and S. Panyim. 1995. Simple non-radioactive hybridization method for identification of sibling species of *Anopheles dirus* (Diptera: Culicidae) complex. *J. Med. Entomol.* 32: 107–111.
- Barbosa, O. C., U. Teodoro, A. L. Lozovei, V. La Salvia Filho, R. P. Spinosa, E. Maurício de Lima, and M.E.M.C. Ferreira. 1993. Nota sobre culicídeos adultos coletados na região sul do Brasil. *Rev. Saúde Pública* 27: 214–216.
- Cassidy, B. G., H. F. Yang-Yen, and L. I. Rothblum. 1986. Transcriptional role for the nontranscribed spacer of rat ribosomal DNA. *Mol. Cell. Biol.* 6: 2766–2773.
- Cerqueira, N. L. 1961. Distribuição geográfica dos mosquitos da Amazônia. *Rev. Bras. Entomol.* 10: 111–168.
- Charlwood, J. D., and D. Edoh. 1996. Polymerase chain reaction used to describe larval habitat use by *Anopheles gambiae* complex (Diptera: Culicidae) in the environs of Ifakara, Tanzania. *J. Med. Entomol.* 33: 202–204.
- Conn, J. E., R. C. Wilkerson, M.N.O. Segura, R.T.L. de Souza, C. D. Schlichting, R. A. Wirtz, and M. M. Póvoa. 2002. Emergence of a new Neotropical malaria vector facilitated by human migration and changes in land use. *Am. J. Med. Hyg.* 66: 18–22.
- Cooper, L., R. D. Cooper, and T. R. Burkot. 1991. The *Anopheles punctulatus* complex: DNA probes for identifying the Australian species using isotopic, chromogenic, and chemiluminescence detection systems. *Exp. Parasitol.* 73: 27–35.
- Cornel, A. J., C. H. Porter, and F. H. Collins. 1996. A polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. *J. Med. Entomol.* 33: 109–116.
- Crabtree, M. B., H. M. Savage, and B. R. Miller. 1995. Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. *Am. J. Trop. Med. Hyg.* 53: 105–109.
- Crease, T. J. 1993. Sequence of the intergenic spacer between the 28s and 18s rRNA encoding genes of the crustacean, *Daphnia pulex*. *Gene* 134: 245–249.
- Fairley, T. L., C. W. Kilpatrick, and J. E. Conn. 2005. Intra-genomic heterogeneity of internal transcribed spacer rDNA in Neotropical malaria vector *Anopheles aquasalis* (Diptera: Culicidae). *J. Med. Entomol.* 42: 795–800.
- Fritz, G. N. 1998. Sequence Analysis of the rDNA internal transcribed spacer 2 of five species of South American human malaria mosquitoes. *J. Seq. Map.* 84: 215–221.
- Fritz, G. N., H. Bermudez, and J. A. Seawright. 1995. Genetic differentiation and diagnostic loci of *Anopheles nuneztovari*, *An. trinkae*, and *An. rangeli* (Diptera: Culicidae). *J. Med. Entomol.* 32: 663–672.
- Fritz, G. N., J. Conn, A. Cockburn, and J. Seawright. 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Mol. Biol. Evol.* 11: 406–416.
- Fritz, G. N., Engman, S. Rodriguez, R., and Wilkerson, R. C. 2004. Identification of four vectors of human *Plasmodium* spp. by multiplex PCR: *Anopheles rangeli*, *An. strodei*, *An. triannulatus*, and *An. trinkae* (Diptera: Culicidae: *Nyssorhynchus*) *J. Med. Entomol.* 41: 1111–1115.
- Gorham, J. R., C. J. Stojanovich, and H. G. Scott. 1973. Illustrated key to the anopheline mosquitoes of western South America. *Mosq. Syst.* 5: 97–156.
- Guimarães, A. E., R. Pinto de Mello, C. M. Lopes, and C. Gentile. 2000. Ecology of mosquitoes (Diptera: Culici-

- dae) in areas of Serra do Mar State Park, State of São Paulo, Brazil. I. Monthly frequency and climatic factors. *Mem. Inst. Oswaldo Cruz* 95: 1–16.
- Hayes, J., G. Calderon, R. Falcon, and V. Zambrano. 1987. Newly incriminated anopheline vectors of human malaria, parasites in Junin Department, Peru. *J. Am. Mosq. Control Assoc.* 3: 418–422.
- Hill, S. M., and J. M. Crampton. 1994. Synthetic DNA probes to identify members of the *Anopheles gambiae* complex and to distinguish the two major vectors of malaria within the complex, *An. gambiae* s.s. and *An. arabiensis*. *Am. J. Trop. Med. Hyg.* 50: 312–321.
- Klein, T. A., J.B.P. Lima, M. S. Tada, and R. Miller. 1991a. Comparative susceptibility of anopheline mosquitoes in Rondonia, Brazil to infection by *Plasmodium vivax*. *Am. J. Trop. Med. Hyg.* 44: 463–470.
- Klein, T. A., J.B.P. Lima, and M. S. Tada. 1991b. Comparative susceptibility of anopheline mosquitoes to *Plasmodium falciparum* in Rondonia, Brazil. *Am. J. Trop. Med. Hyg.* 44: 598–603.
- Krzywinski, J., and N. J. Besansky. 2003. Molecular systematics of *Anopheles*: from subgenera to subpopulations. *Annu. Rev. Entomol.* 48: 111–139.
- Labhart, R., and R. Reeder. 1984. Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of *Xenopus laevis*. *Gene* 134: 245–249.
- Li, C., and R. Wilkerson. 2005. Identification of *Anopheles (Nyssorhynchus) albittarsis* complex species (Diptera: Culicidae) using rDNA internal transcribed spacer 2-based polymerase chain reaction primers. *Mem. Inst. Oswaldo Cruz* 100: 495–500.
- Lounibos, L. P., D. Duzak, J. R. Linley, and U. R. Lourenco-de-Oliveira. 1997. Egg Structures of *Anopheles fluminensis* and *Anopheles shannoni*. *Mem. Inst. Oswaldo Cruz* 92: 221–232.
- Miller, B. R., M. B. Crabtree, and H. M. Savage. 1996. Phylogeny of fourteen *Culex* mosquito species, including the *Culex pipiens* complex, inferred from the internal transcribed spacers of ribosomal DNA. *Insect Mol. Biol.* 5: 93–107.
- Milward de Andrade, R. 1962. Distribuição de Anophelinos pr município cidades e vilas do setado de Pernambuco Brasil. (Diptera: Culicidae). *Rev. Bras. Malariol. Doencas Trop.* 14: 145–58.
- Mitchell, C. J., and R. F. Darsie, Jr. 1985. Mosquitoes of Argentina. Part II geographic distribution and bibliography (Diptera: Culicidae). *Mosq. Syst.* 17: 279–286.
- Morales-Ayala, F. 1971. A list of the mosquitoes of Peru (Diptera: Culicidae). *Mosq. Syst. Newsl.* 3: 138–145.
- Murrell, A., N.J.H. Campbell, and S. C. Barker. 2001. Recurrent gains and losses of large (84–109 bp) repeats in the rDNA internal transcribed spacer 2 (ITS2) of rhipicephaline ticks. *Insect Mol. Biol.* 10: 587–596.
- Murtif, V. L., and P.M.M. Rae. 1985. In vivo transcription of rDNA spacers in *Drosophila*. *Nucleic Acids Res.* 13: 3221–3239.
- Oaks, S. C., V. S. Mitchell, G. W. Pearson, and C.C.J. Carpenter [eds.]. 1991. *Malaria. Obstacles and opportunities*. National Academy of Sciences Press, Washington, DC.
- Park, Y., and A. M. Fallon. 1990. Mosquito ribosomal RNA genes: characterization of gene structure and evidence for changes in copy number during development. *Insect Biochem.* 20: 1–11.
- Perera, O. P., A. F. Cockburn, S. E. Mitchell, J. Conn, and J. A. Seawright. 1998. Species-specific repeat units in the intergenic spacer of the ribosomal RNA cistron of *Anopheles aquasalis* Curry. *Am. J. Trop. Med. Hyg.* 59: 673–678.
- Porter, C. H., and F. H. Collins. 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles Hermsi* (Diptera: Culicidae). *Am. J. Trop. Med. Hyg.* 45: 271–279.
- Povóia, M. M., A.N.M. da Silva, C.C.B. dos Santos, M. N. de O. Segura., and R.L.D. Machado. 2000. Malaria transmission. *Cienc. Cult.* 52: 208–212.
- Quiñones, M. L., M. F. Suárez, and G. A. Fleming. 1987. Distribución y bionomía de los anofelinos de la costa pacífica de Colombia. *Colombia Med.* 18: 19–24.
- Reid, J. A., and K. L. Knight. 1961. Classification within the subgenus *Anopheles* (Diptera, Culicidae). *Ann. Trop. Med. Parasitol.* 55: 474–488.
- Root, F. M. 1927. Studies on Brazilian mosquitoes. IV. Notes on some Brazilian species of *Anopheles*. *Am. J. Hyg.* 7: 599–605.
- Rubio-Palis, Y., and R. H. Zimmerman. 1997. Ecoregional classification of malaria vectors in the Neotropics. *J. Med. Entomol.* 34: 499–510.
- Rutledge, R., A. J. Cornel, C. Lamar Meek, and F. H. Collins. 1996. Validation of a ribosomal DNA-polymerase chain reaction species diagnostic assay for the common malaria mosquito (Diptera: Culicidae) sibling species complex. *J. Med. Entomol.* 33: 952–954.
- Sallum, M. A., R. C. Wilkerson, and O. P. Forattini. 1999. Taxonomic study of species formerly identified as *Anopheles mediopunctatus* and resurrection of *An. costai* (Diptera: Culicidae). *J. Med. Entomol.* 36: 282–300.
- Simeone, A., A. La Volpe, and L. Boncinem. 1985. Nucleotide sequence of complete ribosomal spacer of *Drosophila melanogaster*. *Nucleic Acids Res.* 13: 1089–1101.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.
- Walton, C., R. G. Sharpe, S. J. Pritchard, N. J. Thelwell, and R. K. Butlin. 1999. Molecular identification of mosquito species. *Biol. J. Linn. Soc.* 68: 241–256.
- West, D. F., T. Payette, T. Mundy, and W. C. Black, IV. 1997. Regional molecular genetic key of thirteen snow pool *Aedes* species (Diptera: Culicidae) in northern Colorado. *J. Med. Entomol.* 34: 404–410.
- Wilkerson, R. C., and E. L. Peyton. 1990. Standardized nomenclature for the costal wing spots of the genus *Anopheles* and other spotted-wing mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 27: 207–224.
- Wilkerson, R. C., T. V. Gaffigan, and J. B. Lima. 1995. Identification of species related to *Anopheles (Nyssorhynchus) albittarsis* by random amplified polymorphic DNA-polymerase chain reaction (Diptera: Culicidae). *Mem. Inst. Oswaldo Cruz* 90: 721–732.
- Wilkerson, R. C., and M.A.M. Sallum. 1999. *Anopheles (Anopheles) forattinii*: a new species in series Arribalzagia (Diptera: Culicidae). *J. Med. Entomol.* 36: 345–354.
- Wilkerson, R. C., J. F. Reinert, and C. Li. 2004. Ribosomal DNA ITS2 sequences differentiate six species in the *Anopheles crucians* complex (Diptera: Culicidae). *J. Med. Entomol.* 41: 392–401.

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