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Corey L. Brelsfoard
Eastern Illinois University

Gary Fritz
Eastern Illinois University, gnfritz@eiu.edu

Roberto Rodriguez
Escuela Tecnica de Salud Boliviano Japonesa

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 RODRIGUEZ[2]

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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).

[1] Department of Biological Sciences, Eastern Illinois University, 600 Lincoln Ave., Charleston, IL 61920.

[2] Ministerio de Salud Y Prevision Social, Laboratorio de Entomologia Medica, Escuela Tecnica de Salud Boliviano Japonesa, Avenida Ancieto Arce No. 440, Cochabamba, Bolivia.

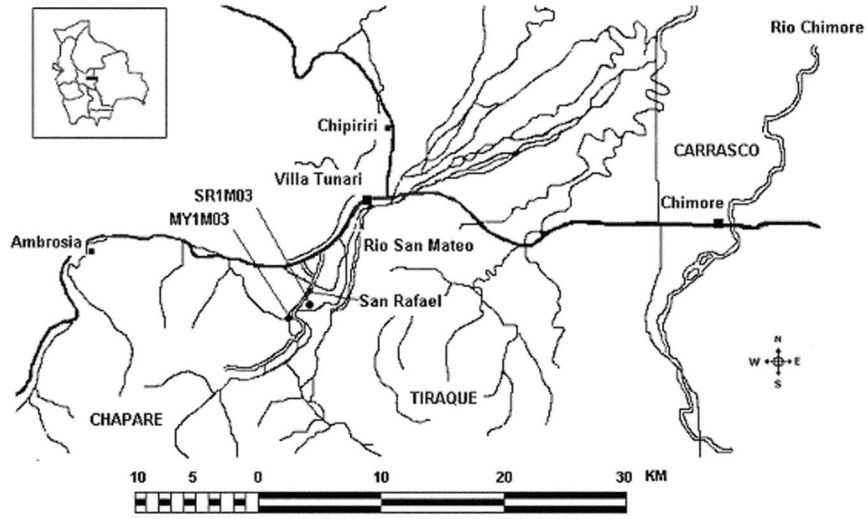

Fig. 1. Map of the Chapare/Carrasco Valley. Solid circles with site codes SR1M03 and MY1M03 represent collection sites for *An. fluminensis* (see Table 1). Rectangles represent the towns of Ambrosia, San Rafael, Villa Tunari, Chipiriri, and Chimore.

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′-TGTGAACTGCAGGACACATG-3′) and 28s (5′-ATGCTTAAATTTAGGGGGTAGTC-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 H_2O, 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 (Arribalzagia) 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. galvaoi* 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 sequence 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 *Nyssohynchus* spp. tested			
An. albimanus	Laboratory colony, USDA, Gainesville, FL	Not known	1
An. albitarsis	Brazil, Iguape	24° 44′ S, 47° 35 ′ W	1
An. aquasalis	Suriname, Paramaibo	5° 50 ′ N, 55° 11′ W	1
	Brazil, Marajo Island	Not known	1
An. bennarrochi	Brazil, Rondonia, Costa Marques	12° 25′ S, 64° 18′ W	1
An. darlingi	Bolivia, Beni, Guayaramirn	10° 51′ N, 65° 21′ W	1
An. deaneorum	Brazil, Rondonia, Costa Marques	12° 25′ S, 64° 18′ W	1
An. evansae	Brazil, Rio de Janeiro	23° 47′S, 43° 49′ W	2
An. konderi	Brazil, Rondonia, Costa Marques	12° 25′ S, 64° 18′ W	1
An. marajoara	Venezuela, Cojedes	Not known	1
	Bolivia, Cochabamba, Chapare Valley	17° 58′ S, 64° 49′ W	2
An. nuneztovari	Brazil, Roraima, Boa Vista	2° 49′ N, 60° 40′ W	1
	Venezuela, Barinas, Boconoito	Not known	1
An. oswaldoi	Brazil, Pará, Urucuri	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
An. rangeli	Bolivia, Cochabamba, Chapare Valley	16° 56′ S, 65° 23′ W	1
An. strodei	Bolivia, Cochabamba, Chapare Valley	17° 02′ S, 64° 51′ W	1
An. triannulatus	Bolivia, Cochabamba, Chapare Valley	17° 12′ S, 64° 30′ W	1
An. trinkae	Bolivia, Cochabamba, Chapare Valley	16° 57′ S, 65° 19′ W	1
Subgenus *Anopheles* spp. tested			
An. earlei	United States, Minnesota, Champlin, Elm Creek Park	45° 10′ N, 93° 25′ W	1
An. franciscanus	United States, California, El Dorado County, Cameron	39° 16′ N, 123° 33′ W	1
An. freeborni	United States, California, Yolo County, Knights Landing	38° 47′ N, 121° 43′ W	1
An. hermsi	United States, California, San Mateo County, Jasper Ridge Preserve	37° 24′ N, 122° 13′W	1
An. occidentalis	United States, California, Alameda County, Coyote Hills	39° 58′ N, 120° 26′ W	1
An. perplexens	United States, Florida, Lake Panasofkee	Not known	1
An. punctipennis	United States, California, Lake Vera	39° 18′ N, 121° 01′ W	1

GCATATTGCGCATCGTGCGACACAGCTCGATGCACATATCTTTGAGAGTCCATAC 55

TTGACATAGTCAAACTACGGTTGTCTGGGCGCAAGCTCGGACACTACCGTGCATA 110

TTGGGGTGGCGGCGCCTACTCGGCGTCGTAGCCCTTAAAATCCCTGTGGAGCGTG 165

TTCACCGACTCTTTTGGTGGTCTCTCGTCACCAAGTGG**GCAGC**GGTGGCCGGCAC 220

CCTCACCTCTTCACCGAGCCGTTGAGATGTGTTCGGTCACACATACGCTGATCAC 275

GACGAGCGTCCTTAAGGTGGTGGACTGCCCATGGAAGGGAGCTCTTGGTCGTCTC 330

TCGTCACCAAAAGTGG**GCAGC**GGTGGCCTGCACCTCTCACCTCTTCACCGAGCCG 385

TTGAGATGTGTACGGTCACGCATACGCTGACCACGACGAGCGATCATAAGGTGGT 440

TGACTACCTCCTGATACGAGCTCTTGGTCGTCTCTCGTCACCAAAAGTGG**GCAGC** 495

GGTGGCCTGCACCTCTCACCTCTTCACCGAGCCGTTGAGATGTGTTCGGTCACGC 550

ATACGCTGATCACGACGAGCGACCAAAAGGTGGTACCAAGAATTATGTATGGGTA 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

1st ACTCTTTTGGTGGTCTCTCGTCACCAA..GTGGGCAGCGGTGGCCGGCA 219
2nd GAG—-C-----C---------------AA----------------T--- 362
3rd GAG--C-----C---------------AA----------------T--- 506

1st CC.CTCACCTCTTCACCGAGCCGTTGAGATGTGTTCGGTCACACATACG 267
2nd --T-------------------------------A-------G------ 411
3rd --T-------------------------------T-------G------ 555

1st CTGATCACGACGAGCGTCCTTAAGGTGGT 296
2nd ----C-----------AT-AT-------- 440
3rd ----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.

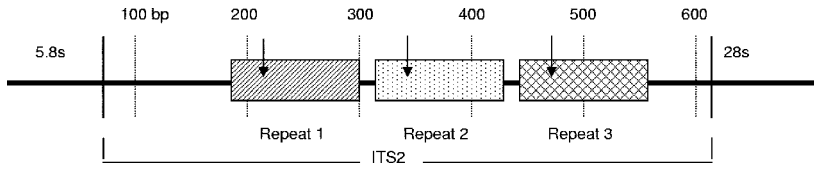

Fig. 4. Restriction map for Apek-I. The ITS2 is outlined on the bottom of the figure and flanked by the two conserved genes (5.8s and 28s). Repeat regions are represented by the rectangles with different shading patterns, labeled first repeat, second repeat, and third repeat, respectively, from left 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-CAAAAGAGTCGG-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-μl PCR reactions included 5 μl of 10× buffer, 8 μl of dNTP mix at 1.25 mM, 0.5 μl of Taq polymerase at 5 U/μl, 21.0 μl of H_2O, 6 μl of $MgCl_2$ at 25 mM, and 2.5 μl of each of the two primers at 40 ng/μl (*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.*

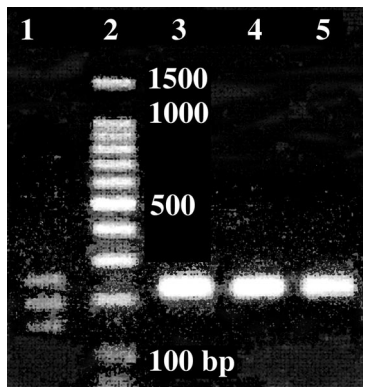

Fig. 5. Restriction enzyme digest with Apek-1 of *An. fluminensis* ITS2 amplicon (lane 1) and species specific amplicon for *An. fluminensis* (lanes 3–5). Lane 2, 100-bp DNA ladder.

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 Cochambamba 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

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 affect.

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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