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

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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.

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, Goriup and Pull 1988, Haworth 1988, Branquinho et al. 1993, Lounibos and Conn 2000). Although historically thought to be unimportant in the transmission of malaria, *An. marajoara* has been incriminated recently as a major vector in Amapá state (Conn et al. 2002).

Anopheles marajoara is one of at least four species belonging to a cryptic complex, (Albitarsis Complex), which also includes *Anopheles albitarsis* s.s. Lynch Arribalzaga, *Anopheles albitarsis* B, and *Anopheles deaneorum* Rosa-Freitas (Narang et al. 1993; Wilkerson et al. 1995a,b). A fifth species, species E, has been proposed based on cytochrome oxidase I (COI) mitochondrial DNA analysis (Lehr et al. 2005). Aside from the larvae of *An. deaneorum*, larvae, pupae, and adults of this complex are not distinguished reliably using morphological keys. Although initially resolved by rapid amplification of polymorphic DNA-polymerase chain reaction (PCR) by Wilkerson 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 preidentification 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 *Nyssorhynchus* 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

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et al. 1996; Fritz 1998), and multiple copies of the ITS2 increase PCR amplification efficiency.

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 *Taq*DNA polymerase, 5 μl of 10× Ex *Taq* buffer, and 4 μl of dNTP mixture (2.5 mM each), all obtained from Takara Bio Inc. (Shiga, Japan); 33.5 μl of H_2O, 2.5 μl of the 5.8s (5′-TGTGAACTGCAGGACACATG-3′) and 28s (5′-ATGCTTAAATTTAGGGGGTAGTC-3′) (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 QiaQuick 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* Gavaldon, Cova Garcia & Lopez, *Anopheles galvaoi* 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 10× buffer, 8 μl of 1.25 mM DNTPs, 0.5 μl of *Taq* polymerase at 5 U/μl, 21.0 μl of H_2O, 6 μl of 25 mM MgCl, 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.

The primer specific to the Albitarsis Complex was complexed with the 28s conserved primer (see above) and tested on the DNA from 19 species in the subgenus *Nyssorhynchus*, which included *An. trinkae*, *An. triannulatus*, *An. rangeli*, *An. strodei*, *An. aquasalis*, *An. albimanus*, *An. darlingi*, *An. evansae*, *An. oswaldoi*, *An. marajoara*, *An. albitarsis*, *An. nuneztovari*, *An. galvaoi*, *An. deaneorum*, *An. benarrochi*, *An. konderi*, *An. braziliensis*, *An. argrytarsis*, and species B (presumptive undescribed species in the Oswaldoi Complex found in the Chapare Valley; C.L.B. and G.N.F., unpublished data) (Table 1). These specimens had been link reared and identified using morphological keys (Faran 1980, Faran and Linthicum 1981, Linthicum 1988) or obtained from laboratory colonies.

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 amplicons 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 1× #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 BfaI (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

Table 1. Collection localities for specimens in the subgenus *Nyssorhynchus* tested with the complex specific primer for the ITS2 of the Albitarsis Complex

Species	Collection location	Coordinates	No. tested
A. albimanus	Lab Colony, USDA, Gainsville, FL	Unknown	1
A. albitarsis s.s.	Venezuela, Zulia, Rio Socuavo	8° 54′ N, 72° 38′ W	1
	Brazil, São Paulo, 6 km SW Registro,	24° 36′ S, 47° 53′ W	2
A. albitarsis B	Brazil, Bahia, Itaquara	Unknown	2
A. aquasalis	Suriname, Paramaibo	5° 50′ N, 55° 11′ W	1
A. argrytarsis	Brazil, Ceará, Ubjara	30° 53′ S, 40° 54′ W	1
A. benarrochi	Brazil, Rondônia, Costa Marques	12° 25′ S, 64° 18′ W	1
A. braziliensis	Brazil, Rondônia, Costa Marques	12° 25′ S, 64° 18′ W	1
A. darlingi	Bolivia, Beni, Guayarámirín	10° 51′ N, 65° 21′ W	1
A. deaneorum	Brazil, Rondônia, Costa Marques	12° 26′ S, 64° 18′ W	1
	Brazil, Rondônia, Ariquemes	Unknown	1
	Brazil, Guajará Mirim	10° 50′ S, 65° 20′ W	1
A. evansae	Brazil, Rio de Janeiro	23° 47′ S, 43° 49′ W	1
A. galvaoi	Brazil, São Paulo, Pariquera Açu	Not known	1
A. konderi	Brazil, Rondôndia, Costa Marques	12° 25′ S, 64° 18′ W	1
A. marajoara	Bolivia, Cochabamba, Chapare Valley	17° 10′ S, 64° 16′ W	2
	Bolivia, Cochabamba, Chapare Valley	17° 58′ S, 64° 49′ W	1
	Brazil, Mato Grosso, Peixoto de Azevedo	Unknown	1
	Brazil, Amazon, Manaus	2° 53′ S, 60° 15′ W	1
A. nuneztovari	Brazil Roraima, Boa Vista	2° 49′ N, 60° 40′ W	1
	Brazil Pará Belem	1° 24′ S, 48° 26′ W	1
A. oswaldoi s.l.	Bolivia,Cochabamba, Chapare Valley	16° 53′ S, 65° 11′ W	2
	Bolivia, Cochabamba, Chapare Valley	16° 59′ S, 65° 37′ W	1
	Bolivia, Cochabamba, Ichilo	17° 10′ S, 64° 16′ W	2
	Brazil, Pará, Urucuri	Unknown	1
A. rangeli	Bolivia, Cochabamba, Chapare Valley	17° 14′ S, 64° 23′ W	1
	Bolivia, Cochabamba, Chapare Valley	16° 55′ S, 65° 23′ W	1
A. strodei	Bolivia, Cochabamba, Chapare Valley	17° 01′ S, 64° 53′ W	1
	Bolivia, Cochabamba, Chapare Valley	17° 02′ S, 64° 51′ W	1
A. triannulatus	Bolivia, Cochabamba, Chapare Valley	17° 14′ S, 64° 23′ W	1
	Bolivia, Cochabamba, Chapare Valley	17° 12′ S, 64° 30′ W	1
A. trinkae	Bolivia, Cochabamba, Chapare Valley	16° 58′ S, 65° 22′ W	1
	Bolivia, Cochabamba, Chapare Valley	16° 57′ S, 65° 19′ W	1

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, complexed with a conserved primer annealing to the 28s (see Materials and Methods), produced a single amplicon in species of the Albitarsis Complex only (Fig. 2). The PCR produced a single (≈340 bp), easily resolved amplicon, subsequently confirmed on 170 specimens of *An. marajoara* from five sites in Bolivia (Fig. 3), one specimen of *An. marajoara* from Brazil, and other members of the Albitarsis Complex (Tables 1 and 3).

A restriction enzyme cut site was identified as unique to *An. marajoara* because of a single nucleotide transversion (T-A) (Fig. 1). Digestion of amplicons with the restriction enzyme BfaI cut those of *An. marajoara* at the species-specific sequence (Fig. 1), producing two expected fragments of ≈202 and ≈138 bp (Fig. 2). These two fragments were not observed when amplicons of the three other members of the species complex were subjected to digestion with BfaI; these specimens included *An. deaneorum* from Ariquemes, Rondônia, Brazil; *An. albitarsis* s.s. from São Paulo, Brazil; and *An. albitarsis* B from Itaquara, Bahia, Brazil (Table 1). Clones of *An. marajoara* from Brazil (C. Li and R. C. Wilkerson, personal communication) indicate that the point mutation creating the species specific cut site (Fig. 1) may be polymorphic in at least some populations of this species. We amplified and BfaI digested the DNA of 170 *An. marajoara* collected from five collection sites in the departments of Cochabamba and Santa Cruz, Bolivia, and of one specimen from the Matto Grosso, Brazil (Table 3; Fig. 3). 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. albitarsis* s.l. (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-

Fig. 1. Sequence alignment of the ITS2 and partial flanking regions of the 5.8s and 28s rRNA genes of *An. albitarsis* s.s., *An. albitarsis* B, *An. deaneorum*, and *An. marajoara* (consensus sequence from three sites in Bolivia). The ITS2 begins at position 21 and ends 30 bp from the end of the sequence. A dash indicates identity with the sequence given for *An. marjoara* and a period indicates a gap introduced to maintain alignment. *An. marajoara* sequence has been deposited in the GenBank database (accession no. DQ364141). Underlined regions signify the single BfaI (C/TAG) restriction endonuclease cut site and Albitarsis Complex specific primer annealing site.

gion 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 secondary 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* s.l. 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

Site and sample code	State, province	Locality	Coordinates
RL1M03-3	Cochabamba, Carrasco	Rio Lagrimas	17° 09′ S, 064° 37′ W
VI1M03-A	Cochabamba, Carrasco	Valle Ivirza	17° 07′ S, 064° 55′ W
IV5M03-15	Cochabamba, Carrasco	Ivirgarzama	17° 01′ S, 064° 50′ W
PG1M03-1 & 40	Santa Cruz, Ichilo	Pto. Grether	17° 10′ S, 064° 16′ W

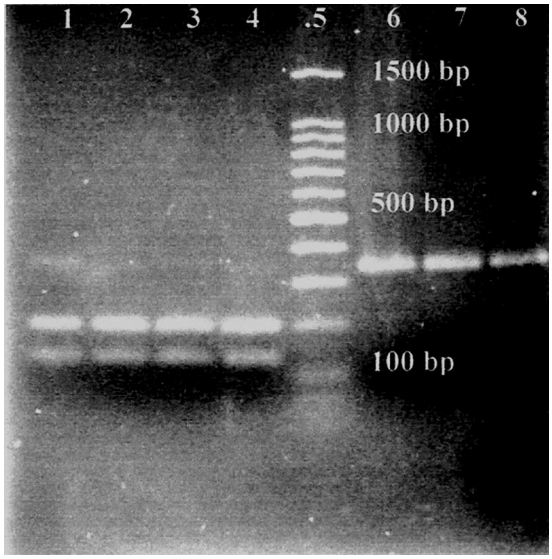

Fig. 2. Restriction enzyme digest with BfaI of Albitarsis Complex amplicon distinguishing *An. marajoara* (lanes 1–4). Lane 5, 100-bp ladder; lanes 6–8, other three members of the Albitarsis Complex.

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/T<u>A</u>G 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.

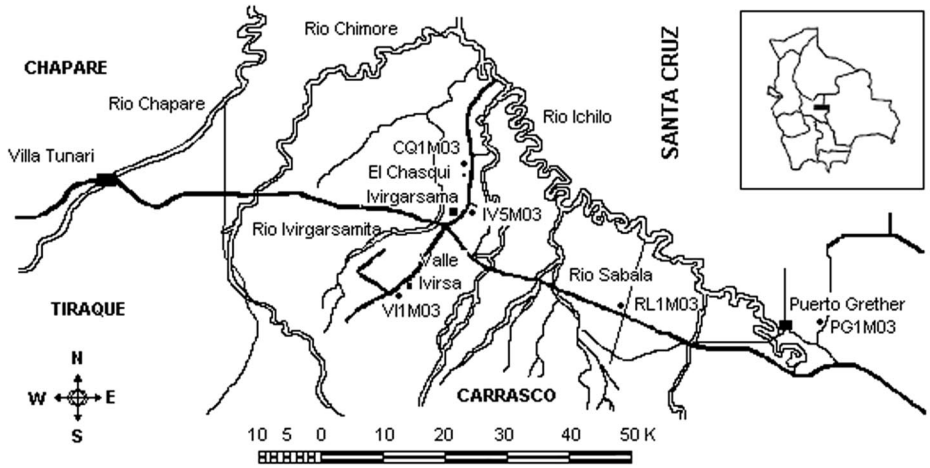

Fig. 3. Locations in the Chapare/Carrasco valley region where specimens of *An. marajoara* were collected and tested with diagnostic PCR protocol. Small closed circles with site codes (see Table 3) represent collection sites. Labeled rectangles represent towns.

Table 3. Collection localities and no. of *A. marajoara* specimens identified by using Albitarsis Complex specific primer and Bfa-I restriction enzyme

Country, state, province	Locality	Site code	Coordinates	No. tested
Bolivia				
Cochabamba,Carrasco	Chasqui	CQ1M03	17° 58′ S, 64° 4′ W	37
Cochabamba, Carrasco	Ivirgarzama	IV5M03	17° 0′ S, 64° 5′ W	40
Cochabamba, Ichilo	Puerto Grether	PG1M03	17° 1′ S, 64° 1′ W	47
Cochabamba, Carrasco	Rio Lagrimas	RL1M03	17° 0′ S, 64° 3′ W	6
Cochabamba, Carrasco	Valle Ivirza	VI1M03	17° 0′ S, 64° 5′ W	40
Brazil				
Mato Grosso	Peixoto de Azevedo	Unknown	Unknown	1

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. trinkae*, *An. triannulatus*, *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, Tadei et al. 1998, Lounibos and Conn 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.

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