

## Identification of Four Vectors of Human *Plasmodium* spp. by Multiplex PCR: *Anopheles rangeli*, *An. strodei*, *An. triannulatus*, and *An. trinkae* (Diptera: Culicidae: *Nyssorhynchus*)

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J. Med. Entomol. 41(6): 1111-1115 (2004)

**ABSTRACT** One of the major obstacles for studies of the biology, ecology, and behavior of Neotropical vectors of human *Plasmodium* has been the lack of reliable and efficient means of identifying many species. Although the subgenus *Nyssorhynchus* includes most species responsible for human transmission in South America, there are no polymerase chain reaction (PCR)-based techniques for identifying members of this taxon. We describe the first multiplex PCR for identifying four species in the subgenus *Nyssorhynchus* that are vectors of human *Plasmodium* spp. Four species specific primers, together with a universal primer that anneals to the 5.8S rDNA region, produce amplicons of the internal transcribed spacer two with base pair sizes of 131, 308, 371, and 441 for *An. triannulatus*, *An. trinkae*, *An. strodei*, and *An. rangeli*, respectively.

**KEY WORDS** *An. rangeli*, *An. strodei*, *An. triannulatus*, *An. trinkae*, polymerase chain reaction

ONE OF THE MAJOR OBSTACLES in studies attempting to determine the basic biology, behavior, and ecology of vectors of human *Plasmodium* spp. in the Neotropics has been the difficulty of species identification. Species within the subgenus *Nyssorhynchus* (Faran 1980, Faran and Linthicum 1981, Linthicum 1988, Peyton et al. 1992, Lounibos et al. 1998) are responsible for most cases of malaria in South America (Arruda et al. 1986, Hayes et al. 1987, Goriup and Pull 1988, Haworth 1988, Branquinho et al. 1993, Lounibos and Conn 2000), and many of the species implicated in pathogen transmission are difficult to distinguish using morphological keys; some species can only be correctly identified by examining a particular sex or developmental stage. Studies on immature stages are particularly onerous because identification requires time-intensive procedures of clearing and mounting specimens (which sometimes compromise characters). Because the correct identification of vector species is an important first step in understanding the entomological aspects of the epidemiology of malaria, the authors of a critical review of malaria research concluded that the development of simple and inexpensive methods to differentiate *Anopheles* species was the single, most important laboratory technique that should be transferred to

field studies (Oaks et al. 1991). Subsequent to this review, a variety of molecular methods for identifying anopheline species have appeared in the literature, offering more accurate and efficient alternatives (Walton et al. 1999). These alternatives include the use of the polymerase chain reaction (PCR) with species-specific primers, diagnostic allozymes, randomly amplified polymorphic markers (RAPDs), and probes (Cooper et al. 1991, Porter and Collins 1991, Hill and Crampton 1994, Audho et al. 1995, Fritz et al. 1995, Wilkerson et al. 1995, Cornel et al. 1996, Rutledge et al. 1996, West et al. 1997).

The subgenus *Nyssorhynchus* includes at least 27 species (Faran 1980, Peyton et al. 1992, Harbach 1994, Lounibos et al. 1998). Although Fritz et al. (1995) described diagnostic enzyme loci for three putative vector species in the genus *Nyssorhynchus*, and Wilkerson et al. (1995) identified four cryptic species by RAPD, there are, otherwise, no reports of molecular methods for screening species in this subgenus. The purpose of this investigation was to develop a set of primers that could be used in a single multiplex PCR reaction to identify any life stage of a number of species in the subgenus *Nyssorhynchus* that are considered primary or secondary vectors of human *Plasmodium* in parts of South America.

### Materials and Methods

The species for which a diagnostic multiplex PCR reaction was developed included *An. triannulatus* (Neiva and Pinto), *An. trinkae* Faran, *An. rangeli* Galdon, Covia Garcia and Lopez, and *An. strodei* Root. Each of these species has been incriminated or sus-

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pected as a primary or secondary vector of human *Plasmodium* in areas of South America (e.g., Corrêa 1938, Elliot 1968, Elliot 1972, Hayes et al. 1987). Mosquito DNA samples used for testing all primers were obtained from the link-reared offspring (egg, larvae, pupae, and adults from a single female) of blood-fed females collected in the field (Table 1). The specific identity of a link-reared series was determined by examination of the exuviae of larvae and pupae and characters from pinned adults of both sexes using the keys of Faran (1980), Faran and Linthicum (1981), and Linthicum (1988). Mosquitoes were stored at  $-80^{\circ}\text{C}$  until used in the PCR. The region of the mosquito genome chosen for the elaboration of species-specific primers was the ribosomal internal transcribed spacer two (ITS2). This spacer was chosen for the following reasons: (1) the complete sequence of this spacer was known for 19 of the 27 species in the subgenus *Nyssorhynchus* (Fritz 1998; GenBank); (2) substantial interspecific divergence; and (3) the ITS2 occurs in multiple copies, thus increasing the sensitivity of amplification.

The aim of this study was to develop multiplex PCR using a species-specific primer for each species of mosquito along with a primer that was common to all species and annealed to a conserved region in the 5.8s rRNA gene flanking one end of the ITS2 (Porter and Collins 1991). Appropriate species-specific primer sites were located by ITS2 alignment of all targeted species (see Fritz 1998) along with those available in GenBank. Thus, selected primer sequences were compared with the complete ITS2 sequences of other species in the subgenus *Nyssorhynchus*, including *An. albimanus*, *An. aquasalis*, *An. dunhami*, *An. konderi*, *An. evansae*, *An. nuneztovari*, *An. oswaldoi*, *An. benarrochi*, *An. rondoni*, *An. albitarsis*, *An. marajoara*, *An. braziliensis*, *An. argyritarsis*, *An. darlingi*, *An. galvaoui*, *An. strodei*, *An. triannulatus*, *An. rangeli*, and *An. trinkae*. Primers were optimized by standard protocols and chosen to give easily resolved bands on 1.5% agarose gels.

Prospective pairs of species-specific primers (where one primer was species specific and the other was in the conserved region of the 5.8s rDNA) were tested with genomic DNA of the target species as well as DNA from 16 other species of *Nyssorhynchus* (see list below) and three species in the subgenus *Anopheles* (*An. pseudopunctipennis*, *An. hermsi*, and *An. freeborni*). Some species were represented by samples collected from as many as two or more different countries in South America (Table 1). Finally, multiplex PCR containing all five primers was tested with samples of DNA from the following species: *An. albimanus*, *An. dunhami*, *An. rangeli*, *An. nuneztovari*, *An. strodei*, *An. evansae*, *An. trinkae*, *An. oswaldoi*, *An. benarrochi*, *An. aquasalis*, *An. dunhami*, *An. konderi*, *An. darlingi*, *An. deaneorum*, *An. albitarsis*, *An. triannulatus*, *An. marajoara*, *An. pseudopunctipennis*, *An. hermsi*, and *An. freeborni*.

## Results

Four species-specific primers (Table 2) were chosen that produced single amplicons in their targeted species. Four of these primers anneal to regions within the ITS2, whereas the sixth primer (5'-TGTGAACTGCAGGACACATG-3') anneals to a conserved region in the 5.8S rRNA gene (Porter and Collins 1991).

Minimal mosquito DNA preparation was found to be sufficient for producing species-specific amplicons. Single larval or adult mosquitoes were ground (in a microcentrifuge tube with a manual pestle) in 100  $\mu\text{l}$  of a cold buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 50 mM NaCl, at pH 8.2. Samples were boiled for 5 min and centrifuged for 5 min at 14,000 rpm, and 2  $\mu\text{l}$  of the supernatant was used in each 50- $\mu\text{l}$  PCR reaction. This protocol worked well even for samples stored in 100% alcohol for 9 yr. The PCR amplification conditions that produced optimal species-specific fragments in 50- $\mu\text{l}$  reactions were the following: each reaction tube contained 2  $\mu\text{l}$  of DNA sample, 5  $\mu\text{l}$  of 10 $\times$  buffer, 2.5  $\mu\text{l}$  of each of five primers at 40 ng/ $\mu\text{l}$  (for the 5.8s region, *An. strodei*, *An. rangeli*, and *An. triannulatus*, *An. trinkae*), 8.5  $\mu\text{l}$  of deionized water, 8  $\mu\text{l}$  of a dNTP mix at 1.25 mM, 0.5  $\mu\text{l}$  of taq polymerase at 5 U/ $\mu\text{l}$ , and 6  $\mu\text{l}$  of 25 mM  $\text{MgCl}_2$ . Thermocycler parameters were 30 cycles of 94 $^{\circ}\text{C}$  for 30 s, 65 $^{\circ}\text{C}$  for 30 s, and 72 $^{\circ}\text{C}$  for 30 s; the last cycle had an extension time of 5 min. Amplification products were electrophoresed on 1.5% agarose gels.

The multiplex PCR produced single, easily resolved amplicons when tested on the four targeted species (Fig. 1) and produced no reaction or faint, multiple, nonspecific bands for other species of *Nyssorhynchus* tested. Identical results were obtained for samples of species collected from various geographic locations (Table 1). In addition, voucher samples (including larvae) of all four targeted species collected from various locations in the Chapare and Carrasco Valleys, Bolivia, produced the expected single amplicons.

## Discussion

Although the ITS2 sequence exhibits substantial divergence between species in the subgenus *Nyssorhynchus*, the size of this spacer has not changed considerably; 16 of the 19 species sequenced to date have an ITS2 between 351 and 406 bp long. For this reason, it is not feasible to distinguish these species solely on the size of the complete ITS2 amplified by primers in conserved regions flanking the spacer. Rather, it was necessary to use primers elaborated from species-specific sequences within the ITS2.

The ITS2 occurs in multiple copies within the mosquito genome, and there is the possibility of intra-individual as well as intra-specific variation in the sequence. Although previous reports of cloned or enzyme-restricted ITS2 sequences suggested that intra-individual/intraspecific variation was minimal or absent in anophelins (Fritz et al. 1991, Porter and Collins 1991, Paskewitz et al. 1993), Onyabe and Conn (1999) have shown recently that some intra-individual

**Table 1.** Collection localities for specimens in the subgenus *Nyssorhynchus* tested with species-specific primers for the ITS2 of *An. rangeli*, *An. strodei*, *An. triannulatus*, and *An. trinkae*

Species	Collection location	Coordinates	No. Tested
<i>An. albimanus</i>	United States Laboratory colony, USDA, Gainesville, FL		2
<i>An. albitarsis</i>	Brazil Pará, Capanema	1°17' S, 47°34' W	2
	Venezuela Zulia, Rio Socuavo	8°54' N, 72°38' W	2
<i>An. aquasalis</i>	Brazil Rio de Janeiro, Magé	23°47' S, 43°49' W	2
	Venezuela Carabobo, Moron	10°29' N, 68°1' W	2
	Suriname Paramaibo	5°50' N, 55°11' W	2
<i>An. argyritarsis</i>	Bolivia Cochabamba, Chapare Valley	17°10' S, 64°16' W	2
<i>An. benarrochi</i>	Bolivia Cochabamba, Pto. Villarroel	16°50' S, 64°48' W	1
	Venezuela El Juval, Trujillo	9°33' N, 70°36' W	2
<i>An. darlingi</i>	Brazil Pará, Capanema	1°17' S, 47°34' W	2
	Bolivia Beni, Guayaramirín	10°51' N, 65°21' W	1
<i>An. deaneorum</i>	Brazil Rondonia, Costa Marques	12°26' S, 64°18' W	1
<i>An. dunhami</i>	Brazil Tabatinga	4°13' S, 69°55' W	1
<i>An. Evansae</i>	Brazil Rio de Janeiro	23°47' S, 43°49' W	2
<i>An. konleri</i>	Brazil Rondonia, Costa Marques	12°26' S, 64°18' W	1
<i>An. marajoara</i>	Brazil Marajo Island	0°49' S, 48°51' W	2
<i>An. nuneztovari</i>	Bolivia Beni, Guayaramirín	10°51' N, 65°21' W	1
	Brazil Roraima, Boa Vista	2°49' N, 60°40' W	2
	Pará, Belem	1°24' S, 48°26' W	2
	Surinam Brokopondo, Victoria	5°5' N, 54°58' W	2
	Venezuela Barinas, Anima	8°20' N, 72°4' W	2
	Zulia, Rio Socuavó	8°54' N, 72°38' W	2
	Colombia Valle, Sitronela	3°49' N, 77°4' W	2
<i>An. oswaldoi</i>	Bolivia Cochabamba, Chapare Valley	17°6' S, 64°47' W	2
	Suriname Brokopondo, Victoria	5°5' N, 54°58' W	2
<i>An. rangeli</i>	Bolivia Beni, San Ramón	3°49' N, 77°4' W	2
	Cochabamba, Chapare Valley	16°58' S, 65°22' W	2
	Brazil Acre, SEM Guiomard	10°15' S, 68°0' W	1
	Rondonia, Costa Marques	12°26' S, 64°18' W	1
	Ecuador Napó, Coca	0°28' S, 76°58' W	2
<i>An. strodei</i>	Brazil Bahia, Ita Quara	20°24' S, 44°30' W	1
	Bolivia Cochabamba, Carrasco Valley	17°2' S, 64°51' W	2
<i>An. triannulatus</i>	Bolivia Cochabamba, Chapare Valley	16°50' S, 64°48' W	2
<i>An. trinkae</i>	Ecuador Napó, Sardina Yacu	0°5' S, 77°5' W	2
	Bolivia Cochabamba, Chapare Valley	17°1' S, 65°26' W	2

variation occurs, primarily in repetitive regions of the sequence. Nevertheless, the assumption, in this study, that a particular pair of primers will diagnose a species

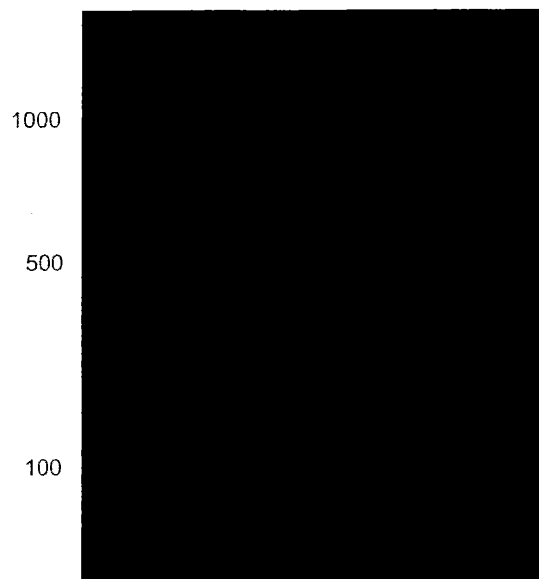
across its range is consistent with data on the consensus sequence of the ITS2 (obtained by PCR sequencing) of broadly distributed anophelines (Fritz et al.

**Table 2.** Species-specific primers annealing to the ITS2 of four species of South American anopheline mosquitoes in the subgenus *Nyssorhynchus*

Species	ITS2 primer sequence (5' to 3')	Amplification product (bp)
<i>An. triannulatus</i>	CCGCGTGGGGACCCAGC	131
<i>An. trinkae</i>	ATCGGGGCTACCCTTTGTA	308
<i>An. strodei</i>	TCTACGCACTGACTACGCGC	371
<i>An. rangeli</i>	CCCCTTGTGTTCGCGCTGA	441

1991, 1994, Fritz and Washino 1993, Fritz 1998). This assumption is also corroborated by the generation of identical amplicons from multiple specimens of a species collected from geographically distant locations of South America (e.g., the samples of *An. strodei*, *An. rangeli*, and *An. trinkae*).

The advent of accurate and easily used molecular methods for identifying species of *Anopheles* has led to their recent use in field studies. Charlwood and Edoh (1996), for example, used PCR to describe the larval habitat used by the *An. gambiae* complex in Tanzania, and Fritz and Washino (1993) used PCR to describe the distribution of *An. hermsi* in New Mexico. The elaboration of multiplex PCR that can, in a single reaction, diagnose four putative vectors of human *Plasmodium* in the Neotropics should now make it more feasible to initiate studies on their ecology and the dynamics of transmission, particularly because the primers can be used to identify any life stage. Because any particular region in the Neotropics will probably have only a subset of the species in the *Nyssorhynchus* subgenus, two or possibly three successive multiplex PCRs would be sufficient to identify all or most species in any given area.



**Fig. 1.** Species specific amplicons for *An. rangeli* (lane 2), *An. strodei* (lane 3), *An. trinkae* (lane 4), and *An. triannulatus* (lane 5). Lane 1 = 100-bp ladder.

## Acknowledgments

We thank P. Lounibos, J. Conn, and E. L. Peyton for support in making this study possible. We are also grateful for the cooperation of various persons associated with state and federal health institutions concerned with malaria control and research programs in Bolivia, Colombia, Surinam, Ecuador, Brazil, and Venezuela, including R. Mollinedo, A. Anselmi, R. Sifontes, J. Berti, E. Borges, C. Moreno, R. Alvarado, N. Castillo, P. Morel, H. Pérez, C. Brancho, M. de la Rosa, J. Scorza, E. Rojas, E. Brown, M. Jimenez, H. Bermudez, E. Vallejo, S. Villarroel, J. Alarcon, and "Shiguango." This research was supported in part by National Institutes of Health Grant AI-31034.

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Received 23 December 2003; accepted 29 July 2004.