Short Report: Molecular Taxonomy of *Anopheles (Nyssorhynchus) benarrochi* (Diptera: Culicidae) and Malaria Epidemiology in Southern Amazonian Peru

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Abstract. Anopheline specimens were collected in 2011 by human landing catch, Shannon and CDC traps from the malaria endemic localities of Santa Rosa and San Pedro in Madre de Dios Department, Peru. Most specimens were either *Anopheles (Nyssorhynchus) benarrochi* B or *An. (Nys.) rangeli*, confirmed by polymerase chain reaction-restriction fragment length polymorphism-internal transcribed spacer 2 (PCR-RFLP-ITS2) and, for selected individuals, ITS2 sequences. A few specimens from Lupuna, Loreto Department, northern Amazonian Peru, were also identified as *An. benarrochi* B. A statistical parsimony network using ITS2 sequences confirmed that all Peruvian *An. benarrochi* B analyzed were identical to those in GenBank from Putumayo, southern Colombia. Sequences of the mtDNA *COI* BOLD region of specimens from all three Peruvian localities were connected using a statistical parsimony network, although there were multiple mutation steps between northern and southern Peruvian sequences. A Bayesian inference of concatenated Peruvian sequences of ITS2+*COI* detected a single clade with very high support for all *An. benarrochi* B except one individual from Lupuna that was excluded. No samples were positive for *Plasmodium* by *CytB*-PCR.

INTRODUCTION

The majority of malaria cases in Peru are from the Amazonian departments of Loreto and Madre de Dios (MdD). MdD is located between 9°54'S, 72°29'W, and 13°22'S, 68°36'W and is part of the Southern Peruvian Amazon Basin (Figure 1). Of the 112,814 human inhabitants, 73% live in urban areas such as Puerto Maldonado (PM), the largest city¹ (Figure 1). The main economic driver in MdD is gold mining, primarily in Bajo Pukiri (Delta-1) and Huepetuhe.² According to the most recent census, these communities have 9,404 and 6,978 inhabitants, respectively, with 40% of the population involved in mining.¹ Santa Rosa (SRA), along the Peru-Brazil Inter-Oceanic Highway 3 hours west of PM, is considered a new mining site (since 2009). The newly constructed highway, connecting Amazonian Brazil to coastal Peru, is also a source of significant migration and likely contributes to endemic malaria in MdD.² SRA had 2,000 inhabitants in 2007 and at that time 55% of the population lived in rural areas. In general, mining areas lack electricity, potable water, and public sewage.¹ Many of the workers who flock to this area are vulnerable migrants from other parts of Peru with no previous exposure to malaria. There are also small foci of *Plasmodium vivax* cases not far from the Bolivian border, in eastern MdD, near San Pedro (SPD), one of our research localities.

MdD ranks fourth in number of malaria cases in Peru (25,677 between 2005 and 2010) and all the cases during this time were *P. vivax*.^{3,4} According to the Regional Health Directorate, Delta-1 accounts for ~70% of all cases and the remaining ones are mostly from Huepetuhe.⁵ SRA reported 113 malaria cases in 2011, a 47% increase in a single year. In general in MdD, malaria cases increase during the rainy season (December to February) however transmission takes place yearlong. According to data from the Dirección Generalde

Epidemiologia-Ministerio de Salud de Peru (DGE-MINSA), the number of *P. vivax* cases declined from 2008 (3,700) to 2011 (1,750). No *Plasmodium falciparum* cases have been registered in MdD since 2007 (Table 1). Some of this reduction may be attributed to the distribution of insecticide-treated bed nets through the Global Fund-supported PAMAFRO from 2006 to 2011. With the recent termination of the Global Fund, funding for malaria control measures is threatened, therefore monitoring high malaria risk regions of Peru remains important.

The port city of Iquitos in Loreto was the focus of a remarkable surge of malaria cases in the 1990s.^{6,7} This trend, blamed on ecological changes that facilitated the westward spread of the primary malaria vector, *Anopheles (Nyssorhynchus) darlingi*,^{8,9} has continued as frequent outbreaks, mostly in the surrounding rural villages. Here, most people who become ill with malaria are involved in agriculture, fishing, or timber extraction.² *Plasmodium vivax* cases in Loreto have decreased overall during the most recent 4-year period, from 20,565 (2008) to 9,198 (2011). Similarly, *P. falciparum* cases have declined from 4,598 (2008) to 0 (2011) (Table 1). The Pan American Health Organization (PAHO) 2008 map of malaria transmission in endemic areas of South America (<http://new.paho.org/hq/index.php?option=com.content&task=view&id=2459&Itemid=2049>) shows that parts of MdD range from very high (where our research site, SRA, is located) to low (where SPD is located), whereas Loreto is an area of intermediate risk.

In MdD, near the Peru-Bolivia border, both *An. darlingi* and *Anopheles benarrochi* have been shown to be abundant,¹⁰ and *An. benarrochi* has been hypothesized to be an important regional vector.¹¹ Despite being more frequently collected in western Loreto and Ucayali than *An. darlingi* (71% versus 24%), *An. benarrochi* was found to be infected with either *P. falciparum* or *P. vivax* at much lower rates.¹¹ These data are consistent with a study in southern Colombia that found despite *An. benarrochi* B's anthropophily and high prevalence (66.1% of 2,445 anophelines tested), no individual specimen was positive for *Plasmodium* by ELISA.^{12,13} Researchers have

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FIGURE 1. Map of southern Amazonian Peru. The main city is Puerto Maldonado. Research sites are San Pedro, north of Puerto Maldonado, and Santa Rosa to the southwest. Major river systems are also depicted. Many of the gold mining workers come from the nearby departments of Cusco and Puno.

investigated seasonal species diversity in a distinctive ecological setting near Iquitos, collecting mosquitoes using a human landing catch (HLC) method. Not surprisingly, *An. darlingi* was the most abundant species in all site types, however, interestingly, *An. benarrochi* was quite abundant in a rural area, but not in the forest, forest village, or periurban sites.¹⁴ It has also been reported that both *An. benarrochi* s.s. and *An. benarrochi* B occur in Peru, and that *Anopheles rangeli*, a species in the Oswaldoi Group of subgenus *Nyssorhynchus*, is a important local or regional vector in southern Colombia.¹³ The major objective of this study was to verify the molecular identity of selected species, and then focus on *An. benarrochi* samples collected in Mdd, compare them with those from San José de Lupuna

(LUP) in Loreto, Peru and from other South American localities (by GenBank), and test them for infection with *Plasmodium* using *CytB*-polymerase chain reaction (PCR).

Mosquitoes were collected in SRA, using HLC, Shannon traps, and Centers for Disease Control and Prevention (CDC) light traps, alternating 12 h (18 h–6 h) and 4 h (18 h–22 h) nights. In SPD, collections were done using only Shannon traps for logistical reasons. In Loreto, mosquitoes were collected from LUP on the Nanay River with HLC and Shannon traps, using the same collection schedule (Table 1). In this report, we present only the samples of *An. benarrochi* that were collected in LUP February 24, April 4, and April 6, 2011 for comparative purposes. Identifications and biological details of other anopheline species from northern Amazonian

TABLE 1
Confirmed and suspected (=total) malaria cases from Madre de Dios and Loreto Departments, Peru, 2008–2011*

	2008			2009			2010			2011		
	TOTAL	<i>P. vivax</i>	<i>P. falciparum</i>	TOTAL	<i>P. vivax</i>	<i>P. falciparum</i>	TOTAL	<i>P. vivax</i>	<i>P. falciparum</i>	TOTAL	<i>P. vivax</i>	<i>P. falciparum</i>
Loreto	25,163	20,565	4,598	26,006	22,031	3,975	11,504	9,208	2,296	11,663	9,198	2,465
Mdd	4,489	4,489	0	2,151	2,151	0	3,041	3,041	0	1,750	1,750	0

*Data from <http://www.minsa.gob.pe/>.

TABLE 2
Identities of anopheline specimens from San Pedro and Santa Rosa, Madre de Dios, 2011*

Site	Date	No.	Trap	Morph. Id.	PCR-RFLP Id.	ITS2	COI
SPD	2/1	5	SHA	<i>nuneztovari</i>	<i>benarrochi</i> B	<i>benarrochi</i> B	<i>benarrochi</i> B
SPD	2/1	4	SHA	<i>benarrochi</i>	<i>benarrochi</i> B	<i>benarrochi</i> B	<i>benarrochi</i> B
SPD	2/2	2	SHA	<i>benarrochi</i>	<i>benarrochi</i> B	<i>benarrochi</i> B	<i>benarrochi</i> B
SPD	2/2	2	SHA	<i>rangeli</i>	<i>rangeli</i>		
SRA	1/24	1	CDC	<i>oswaldoi</i>	<i>rangeli</i>		
SRA	1/24	3	SHA	<i>oswaldoi</i>	<i>rangeli</i>		
SRA	1/27	1	SHA	<i>rangeli</i>	<i>benarrochi</i> B	<i>benarrochi</i> B	<i>benarrochi</i> B
SRA	1/27	1	HLC	<i>rangeli</i>	<i>benarrochi</i> B	<i>benarrochi</i> B	<i>benarrochi</i> B
SRA	3/22	1	SHA	<i>Ano.</i>	NT		
SRA	5/27	1	SHA	<i>benarrochi</i>	NT		
SRA	9/27	5	SHA	<i>rangeli</i>	<i>rangeli</i>		
SRA	9/27	1	SHA	<i>trinkae</i>	<i>rangeli</i>		
SRA	9/27	1	SHA	<i>benarrochi</i>	NT		
SRA	9/27	2	SHA	<i>triannulatus</i>	NT		
SRA	9/27	1	SHA	<i>Nys.</i>	NT		
SRA	9/28	1	SHA	<i>trinkae</i>	<i>rangeli</i>		
SRA	9/28	1	SHA	<i>rangeli</i>	<i>rangeli</i>		
SRA	11/15	1	SHA	<i>Nys.</i>	<i>rangeli</i>		

*SPD = San Pedro; SRA = Santa Rosa; No. = number of specimens; SHA = Shannon trap; CDC = CDC light trap; HLC = human landing catch; Morph. Id. = morphological identification; PCR-RFLP Id. = PCR-RFLP-ITS2, see text; ITS2 = rDNA internal transcribed spacer 2 sequence; COI = mtDNA COI sequence; *Ano.* = *Anopheles*; *Nys.* = *Nyssorhynchus*; NT = not tested.

Peru will be reported elsewhere. Mosquitoes were identified in the field,^{15–17} and then stored in individual tubes with silica gel. The DNA extraction was performed at the UCSD-PRISMA Laboratory, Iquitos, from the head+thorax portion of each specimen following the manufacturer's instructions (Qiagen DNA blood tissue extraction kit, Valencia, CA). Aliquots of the head+thorax DNA were used for the *Plasmodium* Cyt-B-PCR protocol¹⁸ for detection of parasites in Iquitos, and for the molecular identification of the anopheline species at the Wadsworth Center in Albany, New York. The latter procedure was undertaken with the PCR-fragment length polymorphism-internal transcribed spacer 2 (RFLP-ITS2) using the restriction endonu-

cleases *Alu I* and *BsrB I* as previously described.¹⁹ Amplification of the ITS2 followed the published protocol.²⁰ After standard PCR reactions, both strands were sequenced at the Applied Genomic Technologies Core (Wadsworth Center) on an ABI PRISM 3700 automated DNA sequencer (Life Technologies, Applied Biosystems, Carlsbad, CA). The BOLD (Barcode of Life Database) region of the COI gene was amplified using the universal primers.²¹ Sequencher 4.1 (Gene Codes Corp., Ann Arbor, MI) was used for automatic sequence alignment into contigs and proofreading sequences files; subsequently, all sequences were aligned with Clustal W and then compiled in MEGA5.05²² Unique sequences were submitted to

FIGURE 2. TCS 90% Statistical Parsimony Network: *Anopheles benarrochi* B ITS 2 (448 bp). All samples from SRA, SPD, and LUP, Peru group as one haplotype with *An. benarrochi* B samples from Colombia. Samples from Acre state, Brazil grouped 12 mutation steps away as a single haplotype. Samples from Rondonia, Brazil are not connected to the network. Details in Table 3.

TABLE 3

Source of 2011 Peruvian *Anopheles benarrochi* field specimens and GenBank ITS2 and *COI* sequences analyzed for molecular confirmation of *An. benarrochi*

Locality	Department/state	Country	N		Coordinates	<i>COI</i> accession no.	Source
			ITS2*	<i>COI</i>			
SPD	Madre de Dios	Peru	11	11	11°43.220'S, 69°10.588'W	JX170907-08	This study
SRA	Madre de Dios	Peru	2	2	12°55.457'S, 70°18.201'W	JX170909	This study
LUP	Loreto	Peru	2	3	03°44.591'S, 73°19.615'W	JX170904-06	This study
Granada†	Acre	Brazil	4	—	09°41'03.5"S, 67°08'05.3"W	EU636797-EU636800	31
Puerto Asís	Putumayo	Colombia	9	—	00°31'N, 66°31'W	AY684976-AY684983	12
São Miguel	Rondônia	Brazil	2	—	08°49'S, 63°54'W	AF462383, AF462384	27

* All ITS2 sequences were identical (a single haplotype), GenBank accession no. JX173490.

† Acrelandia.

ITS2 = internal transcribed spacer 2; *COI* = mtDNA *COI* sequence; SPD = San Pedro; SRA = Santa Rosa; LUP = Lupuna.

GenBank using Sequin v12.30 (<http://www.ncbi.nlm.nih.gov/Sequin/>).

To infer the haplotype relationships within the ITS2 and BOLD data sets, we performed the median-joining network algorithm,²³ available in NETWORK v 4.5.1.0 (www.fluxus-engineering.com), which combines the topology of a minimum spanning tree with a parsimonious search for the missing haplotypes. Because this algorithm was designed for non-recombining molecules (i.e., mitochondrial DNA [mtDNA]), we analyzed ITS2 using a statistical parsimony network²⁴ at the 90% confidence level with TCS v 1.21 software.²⁵ We also conducted a Bayesian inference of the concatenated (ITS2+*COI*) sequences using MrBayes 3.0.4²⁶ with *An. rangeli* as the outgroup.²⁷ The settings were two simultaneous runs of the Markov Chain Monte Carlo for 20 million generations, sampling every 2,000 and discarding the first 25% as burn-in.

Although there was only a single Shannon trap collection (two nights) in SPD, *An. benarrochi* B was relatively abundant (11 of 13 specimens). Two individuals of *An. rangeli* were also confirmed molecularly (Table 2). In SRA, despite low densities, the most commonly collected species was *An. rangeli* (13 specimens confirmed by PCR-RFLP-ITS2 of 15 tested). There were two confirmed *An. benarrochi* B specimens collected in January 2011. Generally, for *An. triannulatus*, morphological identification is not problematic, therefore we did not molecularly confirm the two specimens collected in September (Table 2). Four specimens of *An. rangeli*, collected in January, were initially identified as *Anopheles oswaldoi*. Misidentification based on adult female morphology is common among *An. nuneztovari* s.l., *An. rangeli*, *An. oswaldoi* s.l., and *An. benarrochi* s.l. and other closely related species and can compromise vector control strategies^{12,13,19,28,29}; in contrast, ITS2 is generally reliable.^{27,30,31} All specimens collected were tested for the presence of *Plasmodium* using *CytB*-PCR but none were positive. However, the sample sizes were small, and as the overall average prevalence of *Plasmodium* in neotropical vectors is around 1%, this was not an unexpected result. Overall, because of anthropophily and relative abundance, it appears that *An. benarrochi* B and (or) *An. rangeli* have the potential to be involved in malaria transmission in our study sites in MdD, but additional collections are needed to investigate this further.

The ITS2 sequences of *An. benarrochi* from LUP, SPD, and SRA, Peru (GenBank accession no. JX173490) were all 448 bp in length, and the statistical parsimony network (SPN) at 90%, grouped them as a single haplotype (Figure 2), together with nine *An. benarrochi* B sequences from southern Colombia (GenBank; Table 3). They were 12 mutation steps away from *An. benarrochi* from Acre state, Brazil,³¹ and could not be

connected with the GenBank samples from Rondonia, Brazil,²⁷ suggesting that these Rondonia sequences may represent a distinct species. However, the TCS program would not run at 95% for the ITS2 sequences because the 24 sequences from Colombia and Peru were all a single haplotype. The SPN and the median-joining network of the mtDNA *COI* BOLD region of *An. benarrochi* B (632 bp) were identical; only the SPN is shown (Figure 3) (GenBank accession nos. JX170904–909). In this case, the three southern Peruvian haplotypes are single mutation steps apart, and form a compact group, whereas the three northern Peruvian haplotypes are separated from each other by 13–14 steps, and differ from the southern haplotypes by 11–12 steps. Of note is that for both network analyses of *COI* sequences at 95% the three LUP samples were not connected to those from MdD. We could not compare the Peruvian samples with any from GenBank because none are publically available. We were able to sequence only one specimen of *An. benarrochi* for both ITS2 and the BOLD region from LUP, sample 1069 (Figures 2 and 3). Because of the relatively large number of mutation steps separating this specimen from the southern *An. benarrochi* for the BOLD region

FIGURE 3. 90% SPN: *An. benarrochi* Folmer *COI* (632 bp).

FIGURE 4. Bayesian inference of concatenated ITS2+COI Peruvian *An. benarrochi* B sequences. *An. rangeli* is the outgroup. 1 = Bayesian posterior probability for the clade including all *An. benarrochi* B sequences except LUP1069.

(Figure 3), and because the three LUP sequences did not connect at 95%, we conducted a Bayesian inference using the concatenated data set (ITS2+COI) to test the hypothesis that the northern and southern Peruvian *An. benarrochi* are in the same clade. These results (Figure 4) show that the single LUP (northern) specimen does not group with the southern ones. Clearly, this warrants additional investigation. It is possible that the northern Peruvian *An. benarrochi* represent a distinctive lineage but this requires additional collections to increase the sample sizes, and further analysis.

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