Incrimination of *Anopheles (Nyssorhynchus) rangeli* and *An.* (*Nys.*) *oswaldoi* as natural vectors of *Plasmodium vivax* in Southern Colombia

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Malaria transmission in the Southern Colombian state of Putumayo continues despite the absence of traditional vector species, except for the presence of Anopheles darlingi *near the southeastern border with the state of Amazonas. In order to facilitate malaria vector incrimination in Putumayo, 2445 morphologically identified* Anopheles *females were tested for natural infection of* Plasmodium vivax *by ELISA. Specimens tested included* An. apicimacula *(n = 2),* An. benarrochi *B (n = 1617),* An. darlingi *(n = 29),* An. mattogrossensis *(n = 7),* An. neomaculipalpus *(n = 7),* An. oswaldoi *(n = 362),* An. peryassui *(n = 1),* An. punctimacula *(n = 1),* An. rangeli *(n = 413), and* An. triannulatus *(n = 6). Despite being overwhelmingly the most anthropophilic species in the region and comprising 66.1% of the mosquitoes tested,* An. benarrochi *B was not shown to be a vector. Thirty-five* An. rangeli *and one* An. oswaldoi *were naturally infected with* P. vivax *VK210. Sequence data were generated for the nuclear second internal transcriber space region of 31 of these 36 vivax positive mosquitoes (86.1%) to confirm their morphological identification.*

An. oswaldoi *is known to be a species complex in Latin America, but its internal taxonomy remains unresolved. Herein we show that the* An. oswaldoi *found in the state of Putumayo is genetically similar to specimens from the state of Amapá in Brazil and from the Ocama region in the state of Amazonas in Venezuela, and that this form harbors natural infections of* P. vivax. *That* An. rangeli *and this member of the* An. oswaldoi *complex are incriminated as malaria vectors in Putumayo, is a novel finding of significance for malaria control in Southern Colombia, and possibly in other areas of Latin America.*

Key words: *Anopheles rangeli* - *Anopheles oswaldoi* - *Anopheles benarrochi* B - ELISA - Colombia

Anopheles (*Nyssorhynchus*) *albimanus* Wiedemann, *An.* (*Nys.*) *darlingi* Root, and *An.* (*N.*) *nuneztovari* Gabaldón are considered to be the major malaria vectors in Colombia (Faran 1980, Herrera et al. 1987, Olano et al. 2001, Sierra et al. 2004). Other species considered to be of local or secondary vector importance include *An.* (*Kertezia*) *lepidotus* Zavortink, *An.* (*K.*) *neivai* Howard, Dyar & Knab, *An.* (*Anopheles) neomaculipalpus* Curry, *An.* (*Ano.*) *pseudopunctipennis* Theobald, and *An.* (*Ano.*) *punctimacula* Dyar & Knab (Ferro 1979, Carvajal et al. 1989, Olano et al. 2001, Moreno et al. 2005). In the Southern Colombian state of Putumayo, malaria cases due to *Plamodium vivax* are high (API 21-60 in last decade) yet neither *An. albimanus* nor *An. nuneztovari* are present. *An. darlingi* is present only as a limited population in the municipality of Puerto Leguízamo bordering the Colombian Amazonas (Fig. 1), where it is believed to be the vector of a unique focus of *P. falciparum* in the region (OPS 2003). Of the known secondary vectors, only *An. punctimacula* has been detected, but it is present in such low numbers that it is not thought likely to be involved in malaria transmission in Putumayo. The most anthropophilic species is reported to be *An. benarrochi* B (Ruiz et al. 2005), followed by *An. rangeli* Gabaldón, Cova García & López and *An. oswaldoi* (Peryassú) (Quiñones et al. 2000, 2001), and thus it seems most likely that one or more of these three species may be involved in the transmission of *P. vivax* in Putumayo.

Previously *An. evansae* (Brèthes) (as *An. noroestensis* Galvao & Lane) was reported from Putumayo and as it was highly anthropophilic and a vector in other areas of Latin America, it was suspected to be the main vector of malaria in Southern Colombia (Ferro 1979). However, recent studies by our team have shown that the species misidentified as *An. evansae* in Putumayo corresponds to a morphological variant of *An. benarrochi* (Quiñones et al. 2001, Calle et al. 2002, Estrada et al. 2003), which was designated *An. benarrochi* B by Ruiz et al. (2005). Although the Colombian *An. benarrochi* is morphologically similar to that found in Peru, it differs morphologically and behaviorally from the nominotypical zoophilic *An. benarrochi* found in Venezuela (Quiñones et al. 2001, Calle et al. 2002, Estrada et al. 2003, Ruiz et al. 2005).

An. benarrochi B is the most anthropophilic species in Putumayo and, therefore, is highly suspected to be the

Financial support: The Wellcome Trust (grant 053401), Colciencias (grant 1115-04-460-98)
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Received 15 February 2006
Accepted 28 June 2006

principal vector in this state (Quiñones et al. 2000, 2001). Recently, *An. benarrochi s.l.* was reported to be the dominant vector in the west of Loreto Province in Peru, which borders Putumayo (Aramburú et al. 1999, Schloeler et al. 2003), and recently Flores-Mendoza et al. (2004) reported that wild-caught *An. benarrochi* were vectors of both *P. falciparum* and *P. vivax* in Eastern Peru, with 0.14% (9 in 6323 pools containing 1-10 mosquitoes) ELISA positive. Barring one T insertion, Ruiz et al. (2005) showed that the second internal transcribed spacer (ITS2) sequences of Colombian *An. benarrochi* were identical to the GenBank entry AF055071 from Yurimaguas in Peru (misidentified as *An. oswaldoi* in Marrelli et al. 1999b), suggesting that these two highly anthropophilic populations comprise one species. The only other *An. benarrochi* sequences available in GenBank are from the state of Rondônia in Brazil (AF462383, AF462384, Marrelli et al. direct submissions 2001) and showed hugely distinct sequences from *An. benarrochi* B (15.4-16.3%, ungapped). Close analysis showed these sequences are most similar to members of the *An. nuneztovari* complex. The male genitalia of *An. benarrochi* B are morphologically distinct from those of *An. benarrochi* sensu Faran in the slide collections of the Smithsonian Institute (R Wilkerson & Y-M Linton, unpublished). The discovery that *An. benarrochi* is a species complex of at least two species clarifies the conflicting reports of behavioral differences between the zoophilic concept of *An. benarrochi s.s.* and the anthropophilic profile of *An. benarrochi* B (Faran 1980, Rubio-Palis 2000). A *P. vivax* susceptibility trial with *An. benarrochi* specimens from Rondônia, Brazil proved negative (Klein et al. 1991).

An. oswaldoi is reported to be a species complex of at least four species in Latin America based on DNA sequences of the nuclear ITS2 (Marrelli et al. 1999b). However, the component species of the *An. oswaldoi* complex were not delineated by Marrelli et al. and subsequently one of these was shown to correspond to *An. benarrochi* B (Ruiz et al. 2005). In the Brazilian state of Acre, *An. oswaldoi* is reportedly the most anthropophilic species and acts as an efficient vector (Branquinho et al. 1993, 1996, Marrelli et al. 1999a). More than 7% (190/2610) of specimens tested by ELISA were positive: 3.41% for *P. falciparum*, 2.26% for *P. vivax* VK210, 1.22% for *P. vivax* VK247, and 0.42% for *P. malariae* (Branquinho et al. 1993). In a later study in the same area, 29% of specimens (1/34) were found positive by dissection of guts and salivary glands (Branquinho et al. 1996), suggesting that *An. oswaldoi* is the principal vector of malaria in Acre. The species has also been found naturally infected in Peru (Hayes et al. 1987, Flores-Mendoza et al. 2004) and Venezuela (Rubio-Palis et al. 1992), but it is not considered to be an important vector in these countries, or in Colombia, due to its low densities.

An. rangeli is the third species of interest in Putumayo because of its apparent high densities and anthropophilic behaviour. Although this species is not thought to play a significant role in malaria transmission anywhere in Latin America (Faran 1980, Rubio-Palis 2000), ELISA detection studies carried out on specimens captured in Caqueta-Putumayo between 1987-88 showed that 6.2% of 419 tested positive for *P. vivax* VK210 circumsporozoite proteins by ELISA (Suárez et al. 1990). However, these results were never formally published, and no attempts have been undertaken to verify these results.

Given their high levels of anthropophily, it seems likely that *An. benarrochi B, An. oswaldoi,* and/or *An. rangeli* could be involved in malaria transmission in Putumayo. Morphologically, *Anopheles* mosquitoes of the subgenus *Nyssorhynchus* are notoriously difficult to identify as adult females, and yet this is the stage most commonly collected in epidemiological studies. Although adult females of *An. rangeli* are easy to identify, it was difficult to reliably separate the morphological variant *An. benarrochi* B from those of *An. oswaldoi* in Colombia (Quiñones et al. 2001), except on the basis of egg morphology (Estrada et al. 2003). To facilitate rapid and accurate differentiation of these three species, a PCR-RFLP assay was designed in our laboratories for use in the present study (Ruiz et al. 2005), the objective of which was to incriminate the species of *Anopheles* mosquitoes likely to be responsible for the transmission of *P. vivax* in Putumayo. Identification of vector species, combined with ecological and behavioural data, will facilitate targeted malaria control strategies in the region.

MATERIALS AND METHODS

Mosquito collections - The Southern Colombian state of Putumayo is typified by humid tropical forest with an annual average temperature of 25.9°C, relative humidity of 90% and annual average continuous rainfall of 4521 mm. The state borders Ecuador and Peru in the south and the Colombian Amazon in the east (Fig. 1).

Human landing collections were carried out over 33 nights between 16 March 2000 and 11 October 2001. Human landing collections were carried according to the recommendations of the National Institute for Health (Colombia). Ethical clearance was obtained through the ethics committees of The Wellcome Trust and Colciencias, who both funded this study. Collections were carried out

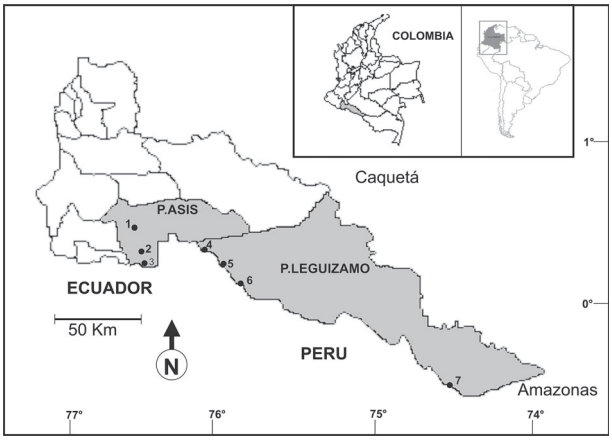

Fig. 1: map of Putumayo showing the seven localities sampled in the municipalities of Puerto Asís (1-3) and Puerto Leguízamo (4-7): 1. El Amaron, 2. La Manuela, 3. Toaya Abajo, 4. Piñuña Blanco, 5. Piñuña Negro, 6. La Concepción, 7. Puntales.

in seven villages across two municipalities (Puerto Asís and Puerto Leguízamo), but due to civil unrest in the region, collections were sporadic and sampling was heavily biased towards the village of La Manuela, Puerto Asís (Table). Collections were carried out on the following dates: Puerto Asís, El Amaron (n = 2), 16,17.vii.01; La Manuela (n = 22), 16-22.iii.00, 4,9-14.v.00, 13-17.vi.00, 26,29.i.01, 17,20.ii.01; Toaya Abajo (n = 1), 15.vii.01: Puerto Leguízamo, La Concepción (n = 1), 9.v.01; Piñuña Blanco (n = 3), 28,29.iv.01, 14.vii.01; Piñuña Negro (n = 3), 1.v.01, 13,15.vii.01; Puntales (n = 1), 11.x.01 (Fig. 1, Table).

ELISA methods - Prior to ELISA detection of *P. vivax* (Wirtz et al. 1985, 1987), females were identified using the morphological keys of Faran (1980), Faran and Linthicum (1981), and Rubio-Palis (2000). Molecular confirmation of specimens identified as *An. benarrochi* and *An. oswaldoi* was carried out using the ITS2 PCR-RFLP described in Ruiz et al. (2005). Prior to ELISA, the head and thorax of each specimen were separated from the remaining body parts (wings, legs, and abdomens), which were stored as voucher specimens. Mosquito head/thorax sections were individually macerated and ELISA carried out following the standard protocol distributed with the ELISA kits (Centre for Disease Control, Atlanta, GA, US).

Mosquitoes were assayed in a 96-well ELISA plate, which also included seven negative controls consisting of colony *An. albimanus* and two positive mosquito samples. Results were read in an ELISA reader with a 415 nm filter, and rechecked after 1 h. A value equivalent to twice the average of the negatives was used as a cut-off point as this was found to be most dependable in field evaluations (Beier et al. 1988). Confidence limits of the positive proportion were calculated under the assumption of a binomial distribution using the Epistat program (Gustafson 1989). To reduce the chance of reading false positives, all ELISA-positive individuals were retested at a later date. Stored abdomens of ELISA positive mosquitoes were subsequently used for molecular identification. Following the initial screening of 608 samples for both *P. vivax* VK210 and *P. vivax* VK247, no *P. vivax* VK247 was detected, thus all remaining specimens were tested for *P. vivax* VK210 only.

Molecular methods - Template DNA was acquired from the abdomens of mosquitoes using either the phenol-chloroform extraction protocol of Linton et al. (2001) or by placing a single leg directly into the PCR reaction. Amplification of the ITS2 was achieved using the 5.8SF and 28SR primers listed in Collins and Paskewitz (1996). PCR products were amplified using the reaction and thermocycler parameters described in Linton et al. (2001), and cleaned using the QIAgen PCR Purification Kit (QIAgen Ltd, Sussex, England), following the manufacturers instructions. Sequencing reactions were carried out in both directions using the Big Dye Terminator Kit (PE Applied Biosystems, Warrington, England) and sequence chromatograms were read by an ABI 377 automated sequencer (PE Applied Biosystems). Sequences were edited using Sequencher^{TM} version 3.1.1 (Genes Codes Corporation, Ann Arbor, Michigan) and aligned in CLUSTAL X (Thompson et al. 1997). Similarity of the ITS2 sequences with those available in GenBank was compared using the Internet based FASTA search available at http://www.ebi.ac.uk/fasta33/.

RESULTS

Wild-caught mosquitoes (n = 2445) comprising 10 *Anopheles* species (Table) were tested for the presence of *P. vivax* circumsporozoite proteins. Thirty-six of the specimens (1.5%) were found positive for *P. vivax* VK210, including *An. oswaldoi* (n = 1) and *An. rangeli* (n = 35) (Table). A total of 8.47% (35/413) of the *An. rangeli* and 0.27% (1/362) of the *An. oswaldoi* specimens were found to be naturally infected (Table). All 36 naturally infected

TABLE

Results of ELISA detection of *Plasmodium vivax* circumsporozoite proteins in 2445 wild-caught female mosquitoes captured landing on human bait in Putumayo between 16 March 2000 and 11 October 2001. Localities are numbered as follow: Puerto Asís: 1, El Amaron; 2, La Manuela; 3, Toaya Abajo; Puerto Leguizámo: 4, Piñuña Blanco; 5, Piñuña Negro; 6, La Concepción; 7, Puntales. 95% confidence intervals (CI) are shown for the percentages of infected specimens

			ELISA positives	Minimum prevalence	
Species	Localities	n	(VK210)	Value	CI (95%)
Anopheles apicimacula [a]	2	2	-	-	-
An. benarrochi [b]	1, 2, 3, 4, 5, 6	1617	-	-	-
An. darlingi [a]	7	29	-	-	-
An. mattogrosensis	4	7	-	-	-
An. neomaculipalpus [b]	2, 4	7	-	-	-
An. oswaldoi [b]	1, 2, 3, 4, 5, 6, 7	362	1	2.76%	0.1-21.9
An. peryassui	7	1	-	-	-
An. punctimacula [a]	4	1	-	-	-
An. rangeli	1, 2, 3, 4, 5, 6	413	35	8.47%	5.6-11.2
An. triannulatus	2, 5	6	-	-	-
Total		2445	36		

a: species reported or suspected to act as primary or secondary malaria vectors in Colombia; *b*: species incriminated in malaria transmission in other regions of Latin America.

specimens were collected in the village of La Manuela in the municipality of Puerto Asís, Putumayo from 16-22 March 2000. To verify the morphological identification, nuclear ITS2 rDNA sequences were generated for 31 of the 36 specimens.

The ITS2 sequence generated for the positive specimen of *An. oswaldoi s.l.* (GenBank accession AY679155) was 531 bp long (Fig. 2). The sequence was identical to those previously reported for *An. oswaldoi* from Putumayo (AY679149-154, Ruiz et al. 2005) and shared 99.2% similarity with those of *An. oswaldoi* from Santana, Amapá, Brazil (AF056318) and Ocama, state of Amazonas, Venezuela (AF055070) (Marrelli et al. 1999a,b). Pairwise sequence alignment of *An. rangeli* and *An. oswaldoi* was 539 bp and interspecific variation was 88.9% (92.2% ungapped) (Fig. 2).

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oswaldoi(1) gagcgggcta tccagtcaca atccccagcg acatgtgc— aca-gatagc cccgatgtgg
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oswaldoi(1) ag—gaccat cctccctcaa agccagccca tgtgatac-a cacaaacgga gcgagaccaa
rangeli(30) ..aa...t.. t......... .......t.. .....c..c. ...c..a... .a........

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oswaldoi(1) acgtaccctg aagcaacgta tgcgcacacg cgtgcagctc attgaagcgc gcacgatcga
rangeli(30) .......... .........-g .ca.tg.... a.....a... ..cc.c.tt. ...t.-c.tt

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           8888888889 9999999990 0000000001 1111111112 2222222223 333333333
           1234567890 1234567890 1234567890 1234567890 1234567890 123456789
oswaldoi(1) aagagaaccg at-caagtgg gcctcaaata atgtgtgact acccctaaa tttaagcat
rangeli(30) ctc..cg.ga .ca....... .......... .......... .......... .........
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Fig. 2: a 539 bp alignment of the nuclear ITS2 region of 31 of the 36 specimens found to be positive for *Plasmodium vivax* VK210 by ELISA. The alignment includes *Anopheles oswaldoi* (n = 1) and *Anopheles rangeli* (n = 30). Primer sequences are in boldface and are underlined.

No intraspecific variation was noted in the 30 specimens of ELISA positive *An. rangeli* (529 bp) and another 27 specimens of these species sequenced from progeny broods from Putumayo (DQ666854-DQ666910). This ITS2 sequence was compared to others for *An. rangeli* available in GenBank: U92329 (Danoff-Burg & Conn, direct submission 1997) of unknown origin, Y09239 (Fritz 1998 which is a consensus sequence of nine *An. rangeli* specimens from Bolivia (San Ramon, Beni State, n = 3), Brazil (Senador Guiomard, Acre, n = 1), Ecuador (Coca, Napo, n = 4) and Venezuela (Veguita, Barinas, n = 1), as well as AF462381 & AF462382 from Acre, Brazil (Marrelli et al. direct submission 2002). Because some of these sequences are considerably shorter than ours, an alignment corresponding to the shortest sequence (U92329, 348 bp) was created that corresponded to bases 145-501 in Fig. 2 (Fig. 3). This alignment revealed that our 57 *An. rangeli* sequences from Putumayo share 100% identity with Y09239 and U92329 from Bolivia, Northern Brazil, Ecuador, and Venezuela. These sequences exhibit four fixed differences from the two *An. rangeli* sequences from Acre, Brazil (AF462381, AF462382) at base 457 (A/T), base 491 (A/G) and a 2-bp indel event (CG) at bases 488 and 489. In addition, an indel (A) is unique to sequence AF462382 between bases 444-445.

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                  23333
                  90333
                  14568
rangeli (60)      -acga
Y09239            -....
U92329            -....
AF462382          at--g
AF462381          -t--g
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Fig. 3: a 348 bp alignment of all *Anopheles rangeli* sequences generated from Putumayo (n = 57) and those available in GenBank: U92329 of unknown origin (Danoff-Burg & Conn, direct submission 1997), Y09239 (Fritz 1998) – a consensus sequence of nine *An. rangeli* specimens from Bolivia (San Ramon, Beni State, n = 3), Brazil (Senador Guiomard, Acre, n = 1), Ecuador (Coca, Napo State, n = 4), Venezuela (Veguita, Barinas State, n = 1), and AF462381 & AF462382 from Acre, Brazil (Marrelli et al., direct submission 2002). Due to differing lengths of GenBank entries and our amplified fragment, this alignment corresponds to bases 145-501 of Fig. 2.

DISCUSSION

In this study, 35 *An. rangeli* and 1 *An. oswaldoi* were found naturally infected with *P. vivax* VK210, supporting the incrimination of two novel malaria vectors in Colombia. All positive specimens were collected in the space of a single week (16-22 March 2000) in La Manuela, Puerto Asís. Although this may seem curious at first, the raw data confirm that these 36 positive mosquitoes were detected in six of the 31 ELISA plates processed, on four separate days. All positive individuals were subsequently retested to discount contamination. Careful analysis of the raw data showed that 551 mosquitoes (22.5% of those tested) were captured during the same week, thus the data are heavily biased towards this weeks collection. Due to civil unrest, collections were heavily skewed towards La Manuela in Puerto Asís and two-thirds of all night biting collections in this study took place in this village. Although little is known about the distribution and seasonality of malaria in Putumayo, the main transmission season does coincide with early spring, when all the *P. vivax* positive mosquitoes were found.

Of the 413 specimens of *An. rangeli* tested, 8.47% were positive for *P. vivax* VK210. That *An. rangeli* appears to be a malaria vector in Putumayo confirms the unpublished findings of Suarez et al. (1990). They reported 6.2% of *An. rangeli* from Caqueta-Putumayo to be ELISA positive for *P. vivax* – a similar rate to that found in this study. Among specimens of *An. rangeli* from Peru, Hayes et al. (1987) reported that 0.4% (2/480) were sporozoite-positive in the dissected salivary glands. Circumsporozoite proteins of *P. malariae* have also been reported in *An. rangeli* from Amapá, Brazil (Povoa et al. 2001), but because of its low density and predominantly zoophilic behaviour, the species is not considered to be of vector significance in Brazil. In contrast, blood meal determination of *An. rangeli* in western Venezuela revealed a human blood index of 30.8-40%, which was significantly higher than for *An. nuneztovari*, the principle vector (Rubio-Palis et al. 1994). That *An. rangeli* appears to be the principal local malaria vector in Putumayo, despite its relatively low abundance, suggests that its vectorial importance across its range of distribution could perhaps be masked by the presence of better-known vectors. The importance of *An. rangeli* in the natural transmission of malaria needs now to be fully assessed in other regions of Colombia and across Latin America.

One specimen of *An. oswaldoi* was found to be positive for *P. vivax* VK210 in this study. Comparisons of the ITS2 sequence of this specimen with ITS2 sequences in GenBank showed 100% identity to other *An. oswaldoi* from Putumayo (AY679149-AY679154) (Ruiz et al. 2005), 99.2% identity to AF056318 from Amapá, Brazil and AF055070 from Ocamo, Amazonas, Venezuela (Marrelli et al. 1999b). This study shows that this genetically identifiable species of the *An. oswaldoi* complex are likely to be involved in *P. vivax* transmission and may therefore be of importance elsewhere within its range of distribution.

Susceptibility trials of *An. benarrochi* from Rondônia, Brazil to *P. vivax* proved negative (Klein et al. 1991), contrasting with reports of a highly anthropophilic *An. benarrochi* acting as a vector in Peru (Aramburú et al. 1999, Schloeler et al. 2003, Flores-Mendoza et al. 2004). Given the morphological similarities between Colombian *An. benarrochi* B and specimens identified as *An. benarrochi* that vectors malaria in Peru (R Wilkerson & C Flores-Mendoza, pers. commun.), we assumed these highly anthropophilic populations comprised the same species. Comparison of ITS2 sequence with dissected male genitalia of voucher specimens, showed that *An. benarrochi* from Peru comprises two morphological forms, one that matches the original description of the species (i.e. *An. benarrochi s.s.*) and another that corresponds to the Southern Colombian *An. benarrochi* B of Ruiz et al. (2005) (Wilkerson, Flores-Mendoza & Linton, unpublished). Despite being the most prevalent anthropophilic species captured in Putumayo, comprising 66.1% of all

mosquitoes tested, *An. benarrochi* B was not found naturally infected in this study (Table). Efforts are now underway in our laboratory to formally describe and name *An. benarrochi* B, and it is now prudent to use molecular methods to examine populations of *An. benarrochi s.l.* across Latin America to ascertain their taxonomic identity.

Given the natural infection of *An. oswaldoi* reported herein, and the contrasting vector incrimination results of the highly anthropophilic, morphological variant of *An. benarrochi* in Putumayo and neighboring Peru with those elsewhere, it its important to correlate vector incrimination with the taxonomic and genetic identity of these two species in future studies to avoid further confusion. The taxonomic identity of *An. rangeli* is also now under some question, with two very different ITS2 sequences detected in Colombia and Brazil. Incorrect species identification hampers malaria control efforts, and it is clear from this study that efforts must be made to understand the biology and behaviour of genetically identified vectors as a prerequisite to effective malaria control.

ACKNOWLEDGEMENTS

To Dr Ivan Dario Vélez and Dr William Galarza and the entomology teams at PECET and DASALUD.

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