Molecular differentiation of *Anopheles (Nyssorhynchus) benarrochi* and *An. (N.) oswaldoi* from Southern Colombia

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Anopheles (Nyssorhynchus) benarrochi, An. (N.) oswaldoi, *and* An. (N.) rangeli *are the most common anthropophilic mosquitoes in the southern Colombian state of Putumayo. Adult females are most commonly collected in epidemiological studies, and this stage poses significant problems for correct identification, due to overlapping inter-specific morphological characters. Although* An. rangeli *is easy to identify, the morphological variant of* An. benarrochi *found in the region and An. oswaldoi are not always easy to separate. Herein we provide a rapid molecular method to distinguish these two species in Southern Colombia.*

Sequence data for the second internal transcribed spacer (ITS2) region of rDNA was generated for link-reared progeny of An. benarrochi *and* An. oswaldoi, *that had been identified using all life stages. ITS2 sequences were 540 bp in length in* An. benarrochi *(n = 9) and 531 bp in* An. oswaldoi *(n = 7). Sequences showed no intra-specific variation and ungapped inter-specific sequence divergence was 6.4%. Species diagnostic banding patterns were recovered following digestion of the ITS2 amplicons with the enzyme* Hae *III as follows:* An. benarrochi *(365, 137, and 38 bp) and* An. oswaldoi *(493 and 38 bp). This polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay provides rapid, accurate, and inexpensive species diagnosis of adult females. This will benefit future epidemiological studies and, as PCR amplification can be achieved using a single mosquito leg, the remaining specimen can be either retained as a morphological voucher or further used in vector incrimination studies. That* An. benarrochi *comprises a complex of at least two species across Latin America is discussed.*

Key words: *Anopheles benarrochi* B - *An. oswaldoi - Nyssorhynchus* - internal transcribed spacer 2 - diagnostic assay - Colombia

Malaria transmission in Colombia is mainly attributed to *Anopheles (Nyssorhynchus) albimanus* Wiedemann, *An. (N.) darlingi* Root or *An. (N.) nuneztovari* Gabaldón (Faran 1980, Herrera et al. 1987, Olano et al. 2001). Despite the absence of these major vectors, reported cases of malaria in the southern Colombian state of Putumayo averaged 1894 cases per year between 1999-2003. Human malaria in Putumayo is exclusively due to *Plasmodium vivax*, except in the municipality of Puerto Leguízamo where cases of *P. falciparum* are also reported (OPAS 2003).

Early studies in Putumayo, including internal reports of the now-defunct Malaria Eradication Service, showed that *An. evansae* (Brèthes) (as *An. noroestensis* Galvão and Lane) was highly anthropophilic, and thus it was circumstantially incriminated in the transmission of malaria in Southern Colombia (Ferro 1979). However a 1988 survey detected no *An. evansae* in Putumayo, and, most unusually, reported natural infection of *P. vivax* in 6.2% of 419 *An. rangeli* Gabaldón, Cova García and López tested by ELISA (Suárez et al. 1990).

Integrated molecular and morphological studies of progeny material reared from wild-caught females captured on human bait in Putumayo were undertaken in our laboratories. These showed that the highly anthropophilic specimens found in Putumayo, previously believed to be *An. evansae,* were infact a morphological variant of *An. benarrochi* Gabaldón, Cova García and López (Quiñones et al. 2000, 2001). *An. benarrochi* is believed to be predominantly zoophilic and therefore not of importance in malaria transmission (Faran 1980). A susceptibility trial of *P. vivax* with *An. benarrochi* from Rondônia, Brazil also proved negative (Klein et al. 1991), and in Venezuela the species is zoophilic, present in low densities, and therefore not believed to be involved in malaria transmission (Rubio-Palis, pers. comm.). Conversely, and more in line with our findings in Southern Colombia, *An. benarrochi* was reported to be the dominant vector in the west of Loreto Province in Perú, which shares a border with Putumayo (Aramburú et al. 1999, Schloeler et al. 2003). Flores-Mendoza et al. (2004) reported that *An. benarrochi* was indeed a vector of both *P. falciparum* and *P. vivax* in Eastern Perú with 0.14% (9 of 6323 pools containing 1-10 mosquitoes each) ELISA positive for either *Plasmodium* species.

An. oswaldoi (Peryassú) is regarded as an efficient vector in Brazil (Branquinho et al. 1996), and a secondary vector in Venezuela where it was found ELISA positive for *P. vivax* (Rubio-Palis et al. 1992). The species has been

reported biting man in Putumayo, where it is suspected to be involved in malaria transmission (Quiñones et al. 2000). On the basis of sequence data for the nuclear second internal transcribed spacer (ITS2), Marrelli et al. (1999) suggested that *An. oswaldoi* comprised a complex of at least four species.

Thus, it appeared that three species, *An. rangeli, An. benarrochi,* and *An. oswaldoi,* could prove important in vector incrimination studies in Putumayo. *An. rangeli* is morphologically distinct and easy to identify, but overlapping characters in the adult females made the correct identification of *An. oswaldoi* and *An. benarrochi* nearly impossible (Quiñones et al. 2001, Calle et al. 2002), and hampered our vector incrimination studies. Eggs of *An. benarrochi* can be differentiated from all other species of the subgenus *Nyssorhynchus* by the presence of a small, short-walled deck at the anterior end and this finding led to the development of an indirect method of identifying wild-caught females based on the morphology of their induced eggs (Estrada et al. 2003). This method is extremely effective but proved difficult to implement on a large scale under field conditions, hence a more rapid approach to facilitate the identification of adult females of *An. oswaldoi* and *An. benarrochi* was needed.

Molecular techniques that can differentiate closely related species on the basis of their ITS2 are well documented in *Anopheles,* and the low level of intra-specific sequence variation has proven useful as the basis for species-specific PCR assays, including the African *An. (Cellia) gambiae* Giles complex (Scott et al. 1993) and the Holarctic *An. (Anopheles) maculipennis* Meigen group (Cornel et al. 1996, Proft et al. 1999). ITS2 sequences have also been used in taxonomic studies of closely related species in the subgenus *Nyssoryhnchus,* including *An. nuneztovari* Gabaldón (Fritz et al. 1994, Sierra et al. 2004) and *An. oswaldoi* (Marrelli et al. 1999), for species characterisation (Lounibos et al. 1998), and in phylogenetic reconstruction (Manguin et al. 1999, Sallum et al. 2002).

In this study we obtained ITS2 sequence data for specimens of *An. benarrochi* and *An. oswaldoi* from Colombia's southern state of Putumayo. These sequences were used as a basis for designing a PCR-RFLP assay that would allow the rapid identification of *An. benarrochi* and *An. oswaldoi* adult females to facilitate vector incrimination studies in the region. Similar PCR-RFLP assays have proven useful in the differentiation of closely related members of species complexes, for example, in the *An. (N.) albitarsis* Lynch-Arribalzaga species complex (Narang et al. 1993).

MATERIALS AND METHODS

Mosquito specimens - Mosquitoes were collected using human landing catches (both indoors and out) between 18-21 h. Specimens were collected from three localities in two towns (Puerto Asís and Puerto Leguízamo) in the Department of Putumayo, Southern Colombia (Fig. 1). Efforts to collect from wider localities were thwarted by civil unrest in the area. Progeny of wild-caught females were individually reared to obtain adults with associated fourth-instar larval and pupal exuviae (see procedures of Belkin et al. 1965 and Reinert et al. 1997). Only those isofamilies positively identified as *An. benarrochi* and *An. oswaldoi* in all life stages, using the existing morphological keys (Faran 1980, Faran & Linthicum 1981, Suárez et al. 1988, Rubio-Palis 2000) and the original descriptions (Peryassú 1922, Gabaldón et al. 1941), were used to generate the DNA sequences. Their siblings, housed at the PECET laboratory (Programa de Estudio y Control de Enfermedades Tropicales, Universidad de Antioquia, Medellín), serve as morphological voucher specimens for this work.

Fig. 1: map showing the locations of Puerto Asís and Puerto Leguízamo in the state of Putumayo in Southern Colombia where mosquitoes used in this study were collected. The relative position of Putumayo in Colombia and in South America is also indicated.

Associated wild-caught adults were killed using ethyl acetate, and individually transferred to clean, labelled 1.5 ml Eppendorf® tubes. Tubes were capped, pierced with a mounted needle to allow the escape of moisture, and placed in plastic bags containing silica gel to ensure best DNA preservation. Those specimens morphologically grouped as either *An. benarrochi* or *An. oswaldoi* were identified to species using the PCR-RFLP assay developed herein.

Molecular methods - Template DNA was acquired using either the phenol-chloroform extraction protocol listed in Linton et al. (2001), or from a single leg placed directly into the PCR reaction (after Scott et al. 1993). Amplification of the nuclear ribosomal spacer (ITS2) was achieved using 5.8SF and 28SR primers listed in Collins and Paskewitz (1996). PCR products were amplified using the reaction and thermocycler parameters described by Linton et al. (2001), and cleaned using a commercially available PCR purification kit (QIAgen Ltd, Sussex, England), prior to direct sequencing. Sequencing reactions were carried out in both directions using the Big Dye Terminator Kit (PE Applied Biosystems, Warrington, England) and

chromatograms were read on an ABI 377 automated sequencer (PE Applied Biosystems).

Sequences were edited using Sequencher™ version 3.1.1 (Genes Codes Corporation, Ann Arbor, MI) and aligned in CLUSTAL X (Thompson et al. 1997) software packages. Sequence similarity of the *An. benarrochi* and *An. oswaldoi* ITS2 genotypes generated in this study were compared with those available in GenBank using FASTA (http://www.ebi.ac.uk/fasta33/). Intra-specific variation was calculated using MEGA2 (Kumar et al. 2001). Most of the template DNA was dried and retained at –70ºC in the Molecular Systematics Laboratory, Department of Entomology (BMNH) for future reference. Sequences generated in this study are available in GenBank, under the following accession numbers: *An. benarrochi* (AY684976-AY684984) and *An. oswaldoi* (AY679149-AY679155). All sequenced individuals were collected in La Manuela, Puerto Asís, except two *An. benarrochi* specimens (AY684979 and AY684980) that were collected in Lisberia, Puerto Asís (Table).

Species-diagnostic banding patterns following digestion of the ITS2 fragments with different restriction enzymes were assessed using the program Webcutter 2.0 (http://www.ccsi.com/firstmarket/cutter/cut2). The enzymes *Hae* III and *Avi* II both showed clear species diagnostic bands, but *Hae* III was employed in this assay, as it was significantly cheaper. RFLP digestions of the ITS2 PCR products were carried out in 20 µl reaction volumes. Four µl of ITS2 PCR product were added to clean 200 µl PCR tubes containing 0.5U of *Hae* III (GG↓CC) (PROMEGA), 2 µl of the manufacturers incubation buffer (PROMEGA- Y+/Tango ™) and 13.5 µl ddH$_2$O. Tubes were incubated at 37ºC overnight to ensure full digest of the ITS2 fragments. The digestion products were visualised on 2% agarose gels, containing 1% ethidium bromide.

RESULTS

In order to obtain baseline ITS2 sequences, DNA was extracted and sequenced from morphologically verified progeny brood specimens collected in Puerto Asís (Table). Sequences were obtained for 9 *An. benarrochi* (2 from Lisberia, Puerto Asís, and 7 from La Manuela, Puerto Asís), and 7 *An. oswaldoi* from La Manuela, Puerto Asís (Table). Amplified fragment lengths (including primers, 43 bp) were 540 bp in *An. benarrochi* and 531 bp in *An. oswaldoi*. No intra-specific differences were noted in the ITS2 sequences of either species (Fig. 2) and the two species shared 93.6% sequence similarity. Inter-specific variation manifested itself in the ITS2 region, not in the flanking sequences of the 5.8S and 28S genes (Fig. 2).

Intra-specific homogeneity in the ITS2 sequences of these species allowed this region to be exploited in an PCR-RFLP assay. The banding pattern after *Hae* III digestion was as follows: *An. oswaldoi* (493 and 38 bp) and *An. benarrochi* (365, 137, and 38 bp) (Fig. 3). Although fragments of less than 50 bp were difficult to observe on the gel, large ones were clearly differentiable for each species and permitted their clear identification (Fig. 3).

In addition to the sixteen sequences obtained from link-reared *An. benarrochi* (n = 9) and *An. oswaldoi* (n = 7) specimens, a further 440 *Nyssorhynchus* females captured landing on humans in Putumayo were identified as *An. oswaldoi* or *An. benarrochi* using the PCR-RFLP assay designed in this study. The majority of specimens came from La Manuela in Puerto Asís (441), with an additional 2 specimens from Lisberia, Puerto Asís and 13 more from La Concepción, Puerto Leguízamo. Using both sequencing and PCR-RFLP of the ITS2 region, all 456 specimens tested were successfully identified to species, with 418 *An. benarrochi* (91.7%) and 38 *An. oswaldoi* (8.3%) (Table).

DISCUSSION

Difficulties in morphological identification of adult female *Nyssorhynchus* mosquitoes from the state of Putumayo in southern Colombia prompted this study. Overlapping morphological characters in adult females resulted in some specimens keying out unsatisfactorily to either *An. evansae* or *An. oswaldoi* using available morphological keys (Faran 1980, Faran & Linthicum 1981, Rubio-Palis 2000). However, detailed studies in all the life stages noted that these "odd" specimens were actually a morphological variant of *An. benarrochi* (Quiñones et al. 2001, Calle et al. 2002, Estrada et al. 2003). Specimens identical to the morphological variant of *An. benarrochi* from Putumayo have also been detected in Perú (R Wilkerson and C Flores-Mendoza, pers. comm.).

According to the morphological keys of Faran (1980), and Faran and Linthicum (1981), *An. oswaldoi* and *An.*

TABLE

Identification of 456 female *Anopheles (Nyssorhynchus)* specimens collected by human landing catches in Putumayo, Southern Colombia. Specimens were identified by morphology, by sequence comparison of the second internal transcribed spacer (ITS2) of the nuclear rDNA, or the ITS2 PCR-RFLP assay designed in this study. Exact localities of collection sites within the towns of Puerto Asís (00'31"N, 66'31"W) and Puerto Leguízamo (00'14"S, 74'45"W) are given

Town	Exact locality	Morphology	PCR-RFLP		Sequenced	
		BEN + OSW	BEN	OSW	BEN	OSW
Puerto Asís	La Manuela	441	396	31	7	7
	Lisberia	2	-	-	2	-
Puerto Leguízamo	La Concepción	13	13	-	-	-
Total		456	409	31	9	7

BEN: *Anopheles benarrochi*; OSW: *An. oswaldoi*

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oswaldoi    ATCACTCGGCTCGTGGATCGATGAAGACCGCAGCTAAATGCGCGTCAGAATGTGAACTGC 60
benarrochi  ........................................................... 60

oswaldoi    AGGACACATGAACACCGACACGTTGAACGCATATTGCGCATTGCACGACTCAGTGCGATG 120
benarrochi  ........................................................... 120

oswaldoi    TACACATTTTTGAGTGCCCACATTCACCGCAGAACCAACTAGCATAGCCGTCGAAAGCTT 180
benarrochi  ...................................................AG.T..----.G.... 176

oswaldoi    TGCTGCGTACTGATGATT-GGTTGACCATGTGCCAACCAAGCATTGAAGGACTGTGGCGT 239
benarrochi  ......C...........T.A.......C.......T........................ 236

oswaldoi    GGTGGGTGCACCGTGTGTGTGTCGTTGCTTAATACGACTCATTCTCTGGTATCACATCTG 299
benarrochi  ...................-...........--........................... 292

oswaldoi    GAGCGGGCTATCCAGTCACAATCCCCAGCGACATGTGCACA---GATAGCCCCGATGTGG 356
benarrochi  ...............................A.......A.TAA............... 352

oswaldoi    AGGACCA------TCCTCCCTCAAAGCCAGC---------------CCATGTGATACAC 394
benarrochi  ...T...AACGGGC...............TGTGATACACTTCATCA...CAC.G.GA.G 412

oswaldoi    ACAAACGGAGCGAGACCAAACGTACCCTGAAGCAACGTATGCGCACACGCGTGCAGCTCA 454
benarrochi  .G.GCGA...A......T.G.................GT..----...A.....A.... 468

oswaldoi    TTGAAGCGCGCACGATCGAAAGAGAACCGATCAAGTGGGCCTCAAATAATGTGTGACTAC 514
benarrochi  .C..---.............C..--.............................. 523

oswaldoi    CCCCTAAATTTAAGCAT 531
benarrochi  ................. 540
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Fig. 2: a 577 bp alignment of the DNA sequences of the second internal transcribed spacer (ITS2) of the nuclear rDNA of *Anopheles benarrochi* (n = 9) (540 bp) and *An. oswaldoi* (n = 7) (531 bp). Primer sequences are underlined and in bold. Dashes (–) indicate insertion/deletion sites and dots (•) indicate identical bases to the lead sequence. *Hae* III restriction sites are indicated by grey shading.
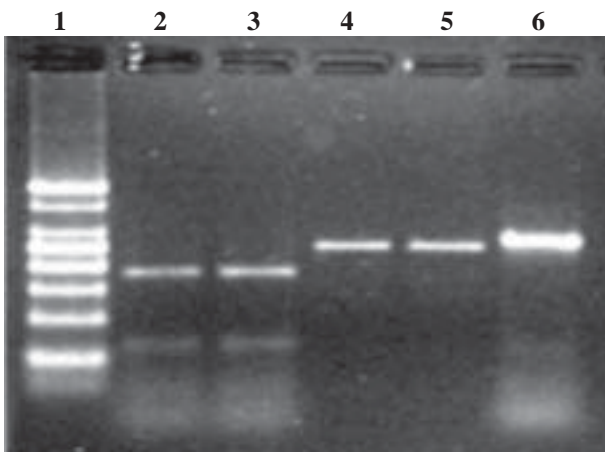

Fig. 3: gel picture showing the species diagnostic banding patterns obtained following the digestion of the internal transcribed spacer 2 (ITS2) PCR products from *An. benarrochi* (365, 137, and 38 bp) and *An. oswaldoi* (493 and 38 bp) with the restriction enzyme *Hae* III. Note that products under 50 bp are generally not visible on the gel. Lanes - 1: DNA ladder; 2 and 3: *An. benarrochi*; 4 and 5: *An. oswaldoi;* 6: undigested *An. oswaldoi* ITS2 product.

benarrochi are easily differentiated on the relative lengths of the basal dark band on hindtarsomere 2. The basal dark band usually occupies less than 25% of hindtarsomere 2 in *An. oswaldoi,* whereas it is significantly longer (40-55%) in *An. benarrochi*. This was echoed the keys of Rubio-Palis (2000), where the length of the dark band on hindtarsomere 2 in Venezuelan *An. benarrochi* was reported to be 45%. Quinoñes et al. (2001) reported in the specimens of morphologically variant *An. benarrochi* from Putumayo, the dark band ranged between 17-33%, overlapping with the range of *An. oswaldoi*, thus accounting for mistaken identification of these two species using the existing morphological keys. Barring one T insertion, the ITS2 sequences of the Colombian *An. benarrochi* generated in this study were identical to the GenBank entry AF055071 from Yurimaguas in Perú. This entry, labelled *An. oswaldoi,* was mistakenly included in Marrelli et al.'s (1999) characterization of the sibling species in the Oswaldoi Complex. This shows that the correct identification of the morphological variant of *An. benarrochi* is problematic using existing taxonomic keys and implies that the species could be commonly misidentified across Latin America.

Morphological and behavioural differences serve to support the presence of at least two cryptic species within *An. benarrochi*. It appears zoophilic in Rondônia, Brazil (Klein et al. 1991) and Venezuela (Rubio-Palis 2000), and highly anthrophophilic in Colombia (Quiñones et al. 2000, 2001, herein) and Perú (Aramburú et al. 1999, Marrelli et al. 1999, Schloeler et al. 2003, Flores-Mendoza et al. 2004). Additionally sequences generated from the morphologically and behaviourally distinct *An. benarrochi* from Putumayo, Colombia (and Yurimaguas, Perú) are genetically distinct (15.4-16.3%, ungapped) from two other *An.*

benarrochi sequences entered in GenBank (AF462383 and AF462384) from Rondônia in Brazil. A FASTA search with a third sequence labelled *An. benarrochi* (U92325 Danoff-Burg and Conn, direct submission 1997) was found to be *An. albimanus* (99.47% identity with L78065 [Teco Strain, Colony] and U92323, Danoff-Burg and Conn).

Ecological, morphological and molecular evidence seem to suggest that the specimens conforming to the original description of *An. benarrochi* are present in Venezuela and Brazil, whereas a second, highly anthrophophilic species, herein designated *An. benarrochi* B, is present in Southern Colombia and Western Perú. Given that the type specimen of *An. benarrochi* is missing (R Wilkerson, pers. comm.), it is now prudent to obtain material from the type locality of La Ceiba, Trujillo State in Venezuela and to designate a neotype to fix the true identity of *An. benarrochi*. This neotype description should include both a revised morphological study and molecular sequence data, as was done for *An. (Cellia) sundaicus* (Rodenwaldt) in Southeast Asia (Linton et al. 2001). Fixing the identity of *An. benarrochi s.s.* will serve as a foundation for further systematic and vector incrimination studies on the species in Latin America, and only then can *An. benarrochi* B and any other component members in the Benarrochi Complex be clearly defined.

The *An. oswaldoi* specimens sequenced from Putumayo showed highest similarity with those from the state of Amapá in Brazil (AF056318) and Ocamo in Venezuela (AF055070) (Marrelli et al. 1999). AF056318 and AF055070 are identical (Marrelli et al. 1999) but differ from our sequences by three variable bases and a 2-bp insertion (98.76% gapped; 99.2% ungapped). Following the work of Marrelli et al. (1999), *An. oswaldoi* was regarded as a species complex of at least four species. One of these is believed to represent *An. konderi*, then a junior synonym of *An. oswaldoi* but which has now been re-elevated to separate species status (Flores-Mendoza et al. 2004). It remains unclear which of Marrelli et al.'s four forms this corresponds to, and another has been shown in this study to correspond to Colombia specimens of *An. benarrochi* B, thus leaving two remaining species in the Oswaldoi Complex. It is now essential to establish the genetic identity of *An. konderi* and of *An. oswaldoi* from its type localities of Valle do Rio Doce in the state of Espirito Santo and Baixada Fluminense in the state of Rio de Janeiro, both in Brazil, to facilitate our understanding of the Oswaldoi Complex. Efforts are underway in our laboratories to resolve these issues.

The ITS2 PCR-RFLP assay designed in this study rapidly and reliably identified all mosquito specimens tested, indicating that *An. benarrochi* B is significantly more prevalent than *An. oswaldoi* in Putumayo and thus could act as a potential vector in the region, as in Perú (Aramburú et al. 1999). The assay worked equally effectively on PCR products generated from template DNA extracted using the phenol-chloroform method of Linton et al. (2001), or for those products generated by placing a single leg directly into the PCR reaction (Scott et al. 1993). This method is extremely valuable in vector incrimination studies as the remainder of the specimen can be utilised for ELISA or PCR detection of *Plasmodium*. The assay as it stands has proven invaluable in our efforts to separate and identify female *An. benarrochi* B and *An. oswaldoi* in Southern Colombia and has facilitated vector incrimination studies in the region (data presented elsewhere). However, comparison of our sequence data with that in GenBank has brought to light the taxonomic complexity of these two species across their range, and we acknowledge that this assay may have to be modified in other regions, depending on which members of the complexes are present. It is now necessary to obtain specimens of both species across Latin America to facilitate the design of a robust identification tool for all component members of the Benarrochi and Oswaldoi Complexes in all regions in future.

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