A new mtDNA COI gene lineage closely related to *Anopheles janconnae* of the Albitarsis complex in the Caribbean region of Colombia

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An understanding of the taxonomic status and vector distribution of anophelines is crucial in controlling malaria. Previous phylogenetic analyses have supported the description of six species of the Neotropical malaria vector Anopheles (Nyssorhynchus) albitarsis *s.l. (Diptera: Culicidae):* An. albitarsis, Anopheles deaneorum, Anopheles marajoara, Anopheles oryzalimnetes, Anopheles janconnae *and* An. albitarsis *F. To evaluate the taxonomic status of* An. albitarsis *s.l. mosquitoes collected in various localities in the Colombian Caribbean region, specimens were analyzed using the complete mitochondrial DNA cytochrome oxidase I (COI) gene, the ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) region and partial nuclear DNA* white *gene sequences. Phylogenetic analyses of the COI gene sequences detected a new lineage closely related to* An. janconnae *in the Caribbean region of Colombia and determined its position relative to the other members of the complex. However, the ITS2 and* white *gene sequences lacked sufficient resolution to support a new lineage closely related to* An. janconnae *or the* An. janconnae *clade. The possible involvement of this new lineage in malaria transmission in Colombia remains unknown, but its phylogenetic closeness to* An. janconnae*, which has been implicated in local malaria transmission in Brazil, is intriguing.*

Key words: Colombia - Albitarsis complex - *Anopheles janconnae* - malaria - COI - *white* gene - ITS2

By 2006, an estimated 9.4% of the total Colombian population was living in moderate or high-risk areas for malaria transmission; the Annual Parasite Index, the number of confirmed malaria cases per 1,000 inhabitants, was an average of 26.2 (PAHO 2007). In 2007, the National Health Institute (INS) of Colombia reported 110,480 cases of malaria caused by *Plasmodium vivax* Grassi & Felleti and *Plasmodium falciparum* Welch (Coccidia: Plasmodiidae).

Because many important malaria vectors are in cryptic species complexes, it is essential to precisely identify species and to clarify their phylogenetic status to better understand patterns of malaria transmission. Several studies have defined the taxonomic status and phylogenetic relationships among members of the Albitarsis complex using nuclear and mitochondrial markers (Lehr et al. 2005, Merritt et al. 2005, Wilkerson et al. 2005, Brochero et al. 2007, Li & Wilkerson 2007) and researchers have shown that the Albitarsis complex consists of at least six species, including *Anopheles albitarsis* Lynch-Arribálzaga, *Anopheles deaneorum* Rosa-Freitas, 1989, *Anopheles marajoara* Galvão & Damasceno, *An. albitarsis* B (Wilkerson et al. 1995a, b, 2005), *An. albitarsis* E (Lehr et al. 2005) and *An. albitarsis* F (Brochero et al. 2007). Recently, Motoki et al. (2009) presented morphological descriptions of adult males and females, fourth-instar larvae and pupae of *An. albitarsis*, *An. marajoara, An. deaneorum* and *An. albitarsis* B and adult males and females of *An. albitarsis* E. These authors also validated and named species B and E as *Anopheles oryzalimnetes* Wilkerson & Motoki and *Anopheles janconnae* Wilkerson & Sallum, respectively. However, species F has yet to be formally described. Furthermore, a new study has determined that *An. deaneorum* is a complex (Bourke et al. 2010) and thus the number of species in the Albitarsis complex is no doubt more than six.

Species of the Albitarsis complex have been reported in several South American countries (Lehr et al. 2005, Li & Wilkerson 2005, Brelsfoard et al. 2006, Brochero et al. 2007, Motoki et al. 2009). Some of these species seem to be allopatric. However, sympatric distributions have been recorded for *An. marajoara* and *An. deaneorum*, *An. oryzalimnetes* (as *An. albitarsis* B) and *An. albitarsis*, in Brazil (Wilkerson et al. 1995a, b, Lehr et al. 2005) as well as *An. albitarsis* F and *An. marajoara* in Colombia (Brochero et al. 2007). Based primarily on morphological identification, *An. marajoara* has been recorded in 25 of the 32 Colombian departments (González & Carrejo 2007) and in a study in Villavicencio, eastern Colombia, *An. marajoara* was highly abundant and widely distributed in urban areas, suggesting its efficient adaptation to human environments (Brochero et al. 2005). *An. albitarsis* F is suspected as a malaria vector in the Department of Vichada, eastern Colombia (Brochero et al. 2007).

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Among members of the Albitarsis complex, *An. marajoara* (Rubio-Palis & Zimmerman 1997, Conn et al. 2002) and *An. janconnae* (as *An. albitarsis* E) (Póvoa et al. 2006) have been incriminated as malaria vectors, *An. deaneorum* is suspected as a vector (Klein et al. 1991a, b) and *An. albitarsis*, *An. oryzalimnetes* and *An. albitarsis* F do not have a defined role in transmission. In Colombia, a recent study on the natural *Plasmodium* infectivity of *Anopheles* species from the Caribbean and Pacific regions reported the presence of *An. albitarsis* s.l. in three of the seven localities investigated: Santa Rosa de Lima (SRL), Moñitos (MON) and Tumaco (Gutiérrez et al. 2008). However, all of the specimens analyzed were uninfected, probably due to the small sample size. To further define the distributions of species of the Albitarsis complex in Colombia, we used three molecular markers, including the complete mitochondrial cytochrome oxidase I (COI) gene sequence, the internal transcribed spacer 2 (ITS2) region of the ribosomal DNA (rDNA) sequence and a sequence fragment of the single copy nuclear *white* gene, to identify the species of the Albitarsis complex in several localities in the Colombian Caribbean region. Phylogenetic analyses based on the COI sequences identified the specimens as a new lineage closely related to *An. janconnae*.

MATERIALS AND METHODS

Mosquito collection - Adult mosquitoes were wild-caught. Collection data and species identification for the mosquitoes from SRL and MON have previously been published in Gutiérrez et al. (2008). *An. albitarsis* s.l. specimens previously collected in Puerto Libertador (PLT), Córdoba Department (Gutiérrez et al. 2009), were also included in this study. Species-specific ITS2 regions were amplified from mosquitoes morphologically identified as *An. albitarsis* s.l. following the scheme proposed by Li and Wilkerson (2005) (Table I). The collection locations of the putative and described species of the Albitarsis complex, which are based on the COI and ITS2 sequences available in GenBank and those tested in this study, were depicted using DIVA-GIS software version 5.2.0.2 (Fig. 1) (Hijmans et al. 2001).

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing - Total DNA was extracted from individual abdomens of *An. albitarsis* s.l. mosquitoes using a salt precipitation protocol (Birungi & Munstermann 2002) or DNeasy® Blood & Tissue Kit (QIAGEN, Duesseldorf, Germany). Three molecular markers were amplified, sequenced and

Fig. 1: location of putative and described species of the Albitarsis complex based on cytochrome oxidase I (COI) and internal transcribed spacer 2 sequences available in GenBank and obtained in this study. Based on the COI gene phylogenetic analyses the three populations from the Caribbean region of Colombia are in the same clade as the sample of *Anopheles albitarsis* E from Venezuela and closely related to *Anopheles janconnae* from the type locality in Northern Brazil. MON: Moñitos; PLT: Puerto Libertador; SRL: Santa Rosa de Lima.

TABLE I

Collection and molecular data for *Anopheles albitarsis* s.l.

Department	Locality	Mosquitoes collected (n)	PCR ITS2[a]	COI[b]	ITS2[b]	*white* gene[b]	Coordinates
Bolívar	SRL	73	73	6	5	1	10°26'N 75°21'W
Córdoba	MON	2	2	1	2	*	9°15'N 76°06'W
	PLT	10	10	7	5	1	7°54'N 75°40'W

a: number of specimens identified using the internal transcribed spacer 2 (ITS2) polymerase chain reaction (PCR) strategy in Li and Wilkerson (2005); *b*: number of specimens from each locality with DNA sequences for the respective marker; COI: cytochrome oxidase I gene; MON: Moñitos; PLT: Puerto Libertador; SRL: Santa Rosa de Lima. Asterisk means that the *white* gene PCR amplification failed for specimens from MON.

analyzed: (i) the ITS2 of the rDNA using primers and PCR conditions previously described (Li & Wilkerson et al. 2005, Zapata et al. 2007), (ii) the complete COI gene amplified using two sets of conserved primer pairs described in Lunt et al. (1996) and (iii) the partial sequence of the single copy nuclear *white* gene amplified using the PCR conditions recommended by Mirabello and Conn (2008). The COI and *white* gene PCR reactions were performed using PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare, UK) containing 0.4 µM of each primer in 25-µL volumes. The ITS2 PCR reactions were performed using BIOTAQ™ DNA Polymerase (Bioline, London, UK). The PCR products were purified using CentriSpin 40 columns (Princeton Separations, Adelphia, NJ) or the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). Fragments were sequenced in both directions at the Applied Genomic Technologies Core facility of the Wadsworth Center, New York State Department of Health, Albany, NY. The nucleotide sequences were compiled and edited using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI).

Multiple sequence alignment - Using CLUSTALX 1.83 (Thompson et al. 1997), 12 ITS2 and two *white* gene sequences from the *An. albitarsis* s.l. specimens obtained in this study (Table I) were aligned with the available ITS2 and *white* gene sequences from GenBank (Table II), which correspond to *An. marajoara*, *An. albitarsis*, *An. deaneorum*, *An. oryzalimnetes* and *An. albitarsis* F (Thompson et al. 1997). All the positions that contained alignment gaps and missing data were eliminated for pairwise sequence comparisons (pairwise deletion option). Neighbour-joining (NJ) analyses were conducted in MEGA4 (Tamura et al. 2007). Additionally, the COI DNA sequences from 14 specimens, representing the three collection sites (Table I) were aligned with DNA sequences available in GenBank for *An. albitarsis*, *An. marajoara*, *An. oryzalimnetes*, *An. deaneorum* and *An. janconnae* (Table II). In most of the phylogenetic analyses conducted in this study, *Anopheles darlingi* Root and *Anopheles braziliensis* Chagas were included as the outgroup. The COI, ITS2 and *white* gene sequences from the present study are deposited in GenBank under the following accessions: ITS2, GQ153583-GQ153594, *white* gene, GQ153595-GQ153596 and COI, GQ153597-GQ153610.

TABLE II

Sequences for *Anopheles albitarsis* s.l. from GenBank used in the alignments

Species	ITS2	*white* gene	COI
An. albitarsis	DQ077807, AY828334-AY828336 AY828320-AY828323 AF462385-AF462387, U92332	AF318198, AY956300, AY956299	DQ076204-DQ076209
Anopheles oryzalimnetes	AY828324-AY828327, AY828337, AY828338 U92333	AY956297, AY956298	DQ076210-DQ076215
Anopheles janconnae	*	*	DQ076231-DQ076234
An. albitarsis F	DQ228315	DQ228314	*
Anopheles deaneorum	AF461751, AF461752, AY828330-AY828333, AY828341-AY828343, U92335	AY956301- AY956302	DQ076226-DQ076230
Anopheles marajoara	DQ364141, AY028127, DQ077808, DQ848153, AY828328, AY828329, AY828339-AY828341, AY828344-AY828354, U92334	DQ906919, DQ906920, DQ848154, AY956295, AY956296	DQ076216-DQ076221
Anopheles darlingi	AF462389	GQ285644	DQ076235, DQ076236
Anopheles braziliensis	AF461753	*	DQ076237, DQ076238
Anopheles albimanus	*	L76302	*

Anopheles darlingi, *Anopheles braziliensis* and *Anopheles albimanus* are outgroup. COI: cytochrome oxidase I gene; ITS2: internal transcribed spacer 2. Asterisks mean no data available in GenBank at time analyses were conducted.

Phylogenetic analyses based on the complete mitochondrial DNA (mtDNA) COI *gene* - Both maximum parsimony (MP) and maximum likelihood (ML) analyses were implemented in PAUP version 4.0b10 (Swofford 2000). For the MP analysis, optimal trees were generated using the heuristic search option with the tree bisection-reconnection (TBR) branch-swapping algorithm. Multiple trees were saved for each run. Bootstrap support values were generated from 1,000 pseudoreplicates. For the ML analysis, an appropriate model of nucleotide substitutions was determined using the program Modeltest 3.8 (Posada 2006); we chose to use the Akaike information criterion (AIC). Bootstrap support values with the heuristic search option and the TBR algorithm chosen for branch-swapping were generated from 662 pseudoreplicates. Bayesian analysis was performed using Mr Bayes version 3.1.2 (Ronquist & Huelsenbeck 2003). Bayesian inference was conducted using the program default values for the prior probabilities, performing two runs with four independents chains. The Markov chain Monte Carlo algorithm was allowed to run 10,000,000 generations and to sample every 100 generations after a burn-in of 2,500,000 generations (25,000 trees). Convergence was assessed based on results from three different parameters (i) the decrease in the average standard deviations of split frequencies ranging from 1-0, (ii) the estimated parameters using the sump command and (iii) the plot of generation vs. the log probability of observing the data.

RESULTS

ITS2 and white gene analyses - The ITS2-PCR analyses showed that the *An. albitarsis* s.l. mosquitoes collected from the Caribbean region corresponded to the molecular pattern reported for *An. marajoara* (Fig. 2). However, it is important to note that the ITS2-PCR analyses (Li & Wilkerson 2005) only identify the species *An. marajoara*, *An. deaneorum*, *An. albitarsis* and *An. oryzalimnetes* and subsequent analysis added species F to the previous scheme (Brochero et al. 2007). Therefore, further analyses using additional markers were performed to determine the identity of the *An. albitarsis* s.l. specimens from the three Colombian localities.

The ITS2 sequences obtained from specimens collected in SRL, MON and PLT showed 100% identity among them and *white* gene sequences from SRL and PLT were also identical. A BLASTN search (GenBank) based on ITS2 and *white* gene sequences of samples from the Caribbean region detected 99% identity with an ITS2 sequence, which corresponds to *An. marajoara* from Chocó, Colombia (accession AY028127) and shares a 98% identity with a *white* gene sequence reported as *An. marajoara* from Puerto Carreño, Colombia (accession DQ906920). In this study, NJ analyses based on ITS2 sequences basically clustered all sequences into two groups. One group included all the species of the Albitarsis complex and the other group included sequences from both *An. darlingi* and *An. braziliensis*. The NJ tree based on *white* gene sequences showed individual clustering for *An. albitarsis*, *An. deaneorum* and *An. albitarsis* F as well as one sequence of *An. oryzalimnetes* clustered with *An. albitarsis*. Samples from this study, collected in the Caribbean region, clustered with sequences of *An. marajoara* (Supplementary data).

COI *gene analyses* - COI sequences from *An. albitarsis* s.l. collected in the Caribbean localities produced an alignment without indels, with 31 variable and four parsimony informative sites, all at the third nucleotide position. Alignment of all the COI sequences from the Albitarsis complex specimens from this work and GenBank detected 209 (14.2%) variable and 155 (10.5%) parsimony informative sites distributed at the first and third nucleotide positions in the alignment. The strict consensus of the 14 most parsimonious trees was estimated (Supplementary data). A ML tree (Supplementary data). was estimated considering the best-fit DNA substitution model selected using the AIC criterion (general time reversible model: GTR + I + G) for 1,470 sites of the COI dataset (Tavaré 1986), with invariable sites (I = 0.7261) and a gamma distribution shape parameter (G = 1.1297). The Bayesian analysis was visualized using the majority rule consensus tree employing the GTR + I + G model and six categories of rates (Fig. 3). Results for the ML (662 replicates), MP (1,000 replicates) and Bayesian inference trees based on COI sequence analyses, including *An. darlingi* and *An. braziliensis* as outgroups, illustrated a similar topological conformation. Three sequences of *An. janconnae* from the type locality of Roraima (RR), Brazil, consistently grouped together with high bootstrap support (96 for each of MP and ML, 1.00 for Bayesian inference), whereas the specimens from the Colombian Caribbean localities consistently grouped with one sequence of purported *An. janconnae* from Portuguesa, Venezuela (Fig. 1). In summary, monophyly of the *An. albitarsis*, *An. oryzalimnetes* and *An. deaneorum* clades was observed under MP, ML and Bayesian analyses. *An. marajoara* was consistently more closely related to *An. deaneorum* and *An. oryzalimnetes* to *An. albitarsis*. *An. janconnae* (as *An. albitarsis* E) (Fig. 3 and Supplementary data) is polyphyletic. The mean genetic distance (GTR and Kimura 2-parameter models) computed using pairwise comparisons was 0.028 (range 0.024-0.031) for the different specimens of *An. janconnae* from Venezuela and Brazil and were comparable with specimens from the Caribbean region of Colombia.

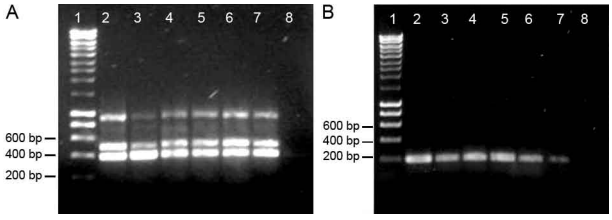

Fig. 2: polymerase chain reaction (PCR) products from internal transcribed spacer 2 (ITS2) analyses. A: PCR amplification using specific ITS2 primers albCD, ITS2F and ITS2R, observed patterns are diagnostic for *Anopheles marajoara* and *Anopheles deaneorum*; B: PCR amplification using specific ITS2 primers albC, ITS2F and ITS2R, observed patterns are diagnostic for *An. marajoara* (~194 bp); Lane 1: DNA ladder; 2-7: amplicons corresponding to *Anopheles albitarsis* s.l. specimens from Santa Rosa de Lima (SRL); 2: SRL55; 3: SRL56; 4: SRL57; 5: SRL58; 6: SRL59; 7: SRL60; 8: PCR negative control.

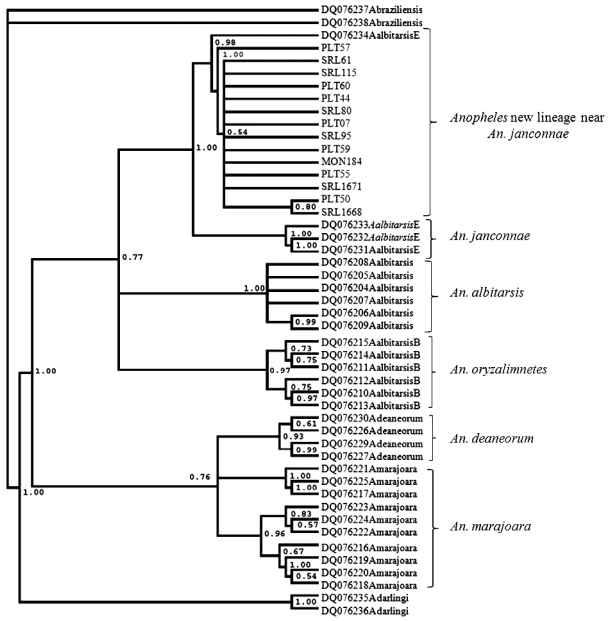

Fig. 3: Bayesian topology of the cytochrome oxidase I sequences. Numbers above branches indicate posterior probabilities. DQ076234: *Anopheles albitarsis* E (?) specimen from Portuguesa, Venezuela; DQ076231-DQ076233: *An. albitarsis* E (*Anopheles janconnae*) from the type locality in Roraima, Brazil. Outgroup comprises *Anopheles darlingi* and *Anopheles braziliensis*.

DISCUSSION

In this study, after applying the scheme to identify specimens of the Albitarsis complex from Caribbean localities to species based on ITS2-PCR (Li & Wilkerson 2005), it was not possible to determine whether they were *An. marajoara*, *An. janconnae* or a new lineage closely related to *An. janconnae*. However, phylogenetic analyses of mitochondrial COI gene sequences of *An. albitarsis* s.l. specimens from the Caribbean localities in Colombia (Fig. 3 and Supplementary data) strongly supported monophyly with specimens previously identified as *An. albitarsis* E from Venezuela (Lehr et al. 2005), but not with *An. janconnae* from the type locality in RR.

The DNA sequences from ribosomal and nuclear genes placed sequences of a new lineage closely related to *An. janconnae* and *An. janconnae* in close relationship to *An. marajoara*. Results of the NJ analyses of the ITS2 is unresolved with most sequences clustered as a polytomy and the *white* gene topology showed the new lineage closely related to *An. janconnae* within a strongly supported clade (99% bootstrap support) formed by *An. marajoara*. The NJ analyses showed that genetic differentiation for species of the Albitarsis complex based on the ITS2 and *white* gene sequences lacked sufficient resolution to support a new lineage closely related to *An. janconnae* or the *An. janconnae* clade, probably due to the following: (i) homoplasy, (ii) lineage divergence within the Albitarsis complex is still undetectable using these molecular markers, in particular among *An. marajoara*, the new lineage closely related to *An. janconnae* and *An. janconnae* and/or (iii) the possibility that the mtDNA lineages are the result of allopatric fragmentation rather than speciation. This result suggests that the ITS2 and single copy nuclear *white* gene markers have limitations in discriminating between the incipient or recently separated species/lineages proposed for the Albitarsis complex and that the mtDNA genome is a more sensitive indicator of that divergence (Zink & Barrowclough 2008).

As with other species complexes, females of the Albitarsis complex are difficult to distinguish using the available morphological keys. They may exhibit high intraspecific morphological variability (Rubio-Palis et al. 2003) and also interspecific similarity; hence, multivariate analysis of measurements of morphological features (Motoki et al. 2009) and molecular analyses based on mtDNA sequences have been shown to be effective approaches for distinguishing among morphologically similar species in the complex (Lehr et al. 2005).

A recent population genetics study using microsatellite markers detected population structure for *An. marajoara* in Colombia (Brochero 2006, Brochero et al. 2010). These authors found that populations at different locations in Colombia showed departures from Hardy-Weinberg equilibrium associated with heterozygote deficits because of the Wahlund effect. However, a different study (Posso et al. 2006), using random amplification of polymorphic DNA-PCR, reported the occurrence of gene flow among *An. marajoara* populations from eastern and western Colombia that was higher among the eastern populations. Results of the present study, based on the phylogenetic analyses of COI sequences, suggest the presence of more than one species of the Albitarsis complex in Colombia. Therefore, results of the previous studies may have been biased, as only Brochero et al. (2010) included molecular analyses to support or confirm species identification. The differentiation detected in *An. marajoara* populations from Colombia may have two possible explanations. First, the genetic variation observed among *An. marajoara* populations from Colombia may be influenced by allopatric distribution (potential isolation by distance) in addition to possible reproductive isolation. Second, different lineages/species of *An. albitarsis* s.l., particularly a new lineage closely related to *An. janconnae*, may be present in these areas of Colombia.

In this study, MP, ML and Bayesian analyses of COI sequences of *An. albitarsis* s.l. from Colombian Caribbean specimens were grouped into a strongly supported clade together with a single sequence reported as *An. albitarsis* E from Venezuela, as well as grouped separately from the monophyletic lineage of *An. janconnae* (Fig. 3 and Supplementary data). However, a low to moderate level of genetic variability was detected among the specimens forming the *An. janconnae* clade and samples from Colombia, as might be expected for mosquitoes from different populations. In summary, the topology of all optimal trees under MP, ML, and Bayesian methods, in addition to the strong nodal support and the values of genetic distance calculated for *An. albitarsis* s.l. specimens from the Caribbean localities, showed that a new lineage closely related to *An. janconnae* is distributed

in this region. The occurrence of a new lineage closely related to *An. janconnae* in sympatry with other known malaria vectors in the Caribbean region (Gutiérrez et al. 2008, 2009) adds additional complexity to the understanding of malaria transmission dynamics in Colombia, as *An. janconnae* is known as a locally important malaria vector in Brazil. Further studies will be essential to verify if this putative species plays an important role as a secondary or local vector in the Caribbean region of Colombia. Also, these data suggest that more intensive morphological and molecular characterization of the species of the Albitarsis complex not only from Colombia but throughout their distribution in the Neotropical biogeographic region should be undertaken to complete the descriptions of the unknown life stages for all members of this complex (for instance, fourth-instar larvae and pupae of *An. janconnae* and species F remain undescribed), including the putative new lineage suggested here.

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