

## A NEWLY RECOGNIZED SPECIES IN THE *ANOPHELES* (*NYSSORHYNCHUS*) *ALBITARSIS* COMPLEX (DIPTERA: CULICIDAE) FROM PUERTO CARREÑO, COLOMBIA

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**Abstract.** We report a previously unrecognized mosquito species from eastern Colombia belonging to the *Anopheles* (*Nyssorhynchus*) *albitarsis* complex. We provisionally name this taxon *An. albitarsis* species “F.” Until now, the only members of the *Albitarsis* Complex recorded from north of the Amazon River have been *An. marajoara* and a putative phylogenetic species, *An. albitarsis* “E.” As with the other largely monomorphic species in the complex, we were able to detect its presence using ribosomal DNA internal transcribed spacer 2 (rDNA ITS2) and partial *white* gene sequences. Unlike *An. marajoara*, but in common with other species in the complex, *An. albitarsis* F lacks the *white* gene fourth intron. This species is sympatric with *An. marajoara* in a malaria-endemic area in Puerto Carreño, Vichada Department, Colombia. It could be an important current and/or historical vector of human malaria parasites at this locality and, depending on its actual distribution, elsewhere in Colombia and Venezuela.

### INTRODUCTION

Forty-three *Anopheles* species are reported from Colombia, with most of the malaria vectors belonging to subgenus *Nyssorhynchus*. Vectors include *An. darlingi* Root, *An. nuneztovari* Gabaldon, *An. albimanus* Wiedemann, *An. rangeli* Gabaldon, *An. oswaldoi* (Peryassu), and *An. marajoara* Galvão and Damasceno.<sup>1–3</sup> The last was recently implicated as the primary vector of malaria in northeastern Brazil,<sup>4</sup> and it is a possible urban auxiliary vector in Villavicencio, Colombia.<sup>2,5,6</sup> In Colombia, it was found naturally infected with *Plasmodium falciparum*,<sup>5</sup> experimentally infected with *P. vivax*,<sup>7</sup> and has evident resistance to Dichloro-Diphenyl-Trichloroethane (DDT).<sup>8</sup> In eastern Colombia, *An. marajoara* was shown to be the most abundant and widely distributed anopheline in urban areas, with a preference for man-made breeding habitats and apparently highly adapted to human environments.<sup>6</sup> *An. marajoara* is a member of the *Albitarsis* Complex,<sup>9</sup> which, based on molecular evidence,<sup>10–15</sup> also includes: *An. albitarsis* Lynch-Arribalzaga, *An. albitarsis* B, *An. deaneorum* Rosa-Freitas, and *An. albitarsis* E.

More than 85% of Colombia's land area is suitable for permanent malaria transmission,<sup>1</sup> with the areas of highest risk being the Pacific Coast, the Cauca River Valley, and the Urabá, Orinoquía, and Amazonia Regions.<sup>16</sup> Puerto Carreño, in the Orinoquía region bordering Venezuela, is the capital of the Vichada department, and is a study site in a national program of malaria surveillance. Malaria incidence around Puerto Carreño is significant: in 2005, there were 4.08 cases/1,000 inhabitants for *P. falciparum*, 8.98 for *P. vivax*, and 1.81 mixed infections. Local rural and peri-urban malaria transmission is aided by year-round migration of infected and susceptible humans and enhanced by intensive land exploitation with resultant creation of abundant *Anopheles* larval habitats. The area is characterized as savanna and has a mean temperature range of 27–30°C, with yearly monomodal rainfall.<sup>17</sup> Because Puerto Carreño has peri-urban malaria and the primary proven vector, *An. darlingi*, is usually restricted to rural areas, it is likely that other vectors, such as *An. marajoara*, are

maintaining malaria endemicity in this region. It was therefore interesting to find molecular evidence of a genetically distinct species in Puerto Carreño sympatric with and morphologically similar to *An. marajoara*. We refer to this species here as *An. albitarsis* “F.” Larvae of one or both of these species are recorded in habitats exposed to sunlight located near houses (Instituto Nacional de Salud, personal communication). As such, it could be playing an important role as a primary or auxiliary vector in some areas. The occurrence of a cryptic species sympatric with the known malaria vectors *An. marajoara* and *An. darlingi* adds additional complexity to the study of the dynamics of malaria transmission in Colombia.

### MATERIALS AND METHODS

**Source of specimens, identifications, and sequences.** Collections that yielded *An. albitarsis* F were made using a human landing catch method in an urban environment in Puerto Carreño, Vichada Department, Colombia, on 23 July, 26 August, and 8 September 2004 by technical personnel of the Entomological Unit of Vichada's Health Department. Specimens were identified as *An. marajoara* using morphologic taxonomic keys<sup>18,19</sup> and were maintained in 95% ethanol at 4°C before DNA extraction and sequencing. The letter designations used for the unnamed species is based on random amplified polymorphic DNA (RAPD) markers,<sup>10,11</sup> where “A,” “B,” “C,” and “D” to refer to *An. albitarsis* s.s., *An. albitarsis* B, *An. marajoara*, and *An. deaneorum*, respectively. *An. albitarsis* E was next named using this convention,<sup>12</sup> and we follow with *An. albitarsis* F. For a phylogenetic estimation of *An. albitarsis* F in relation to other members of the *Albitarsis* Complex, we used 10 previously published *white* gene sequences<sup>15</sup> (i.e., an ingroup of *An. albitarsis* s.s., *An. albitarsis* B, *An. deaneorum*, and *An. marajoara*, and an outgroup species, *An. albimanus* Wiedemann). To the ingroup, we added single individuals of *An. marajoara* (putative *An. albitarsis* E<sup>12</sup>) from Boa Vista, Roraima State, Brazil (BR36-2), Trinidad B.W.I. (TR3-4), and Puerto Carreño, Colombia (CV9-6), and two *An. albitarsis* F (CV9-7, CV14-1) from Puerto Carreño. To provide additional contrast to the other species of the *Albitarsis* Complex and to find species-specific

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sequence for a polymerase chain reaction (PCR)-based identification tool, we used a previously reported ITS2 sequence<sup>13</sup> and also sequenced ITS2 from Colombian *An. marajoara* and *An. albitarsis* F. Because there is known intragenomic variation in ITS2<sup>20</sup> among species of the complex, we only used sequences that were the result of direct sequencing (i.e., consensus sequence).<sup>13</sup>

#### DNA extraction, amplification, sequencing, and alignment.

For the above new sequence, a wing and a leg from each mosquito were used for DNA extraction, and the remainder of the specimen was deposited at the Instituto Nacional de Salud, Bogotá, Colombia. DNA vouchers were deposited at the Smithsonian Institution, National Museum of Natural History. DNA was extracted by a phenol-chloroform method.<sup>10</sup> *White* gene and ITS2 sequences were produced following published protocols.<sup>14,15</sup> Alignment was unambiguous and done manually.

**ITS2-based PCR diagnostic.** We compared the ITS2 sequence of *An. albitarsis* with the sequences of the four ITS2-distinguishable species in the Albitarsis Complex.<sup>14</sup> Observed sequence differences were used to design a species-specific primer, albF (5'-TGGCTTTGAGGAAGGTGGTATATC-3'). We tested this primer on known samples of the other four species in the complex to verify that no amplification was occurring that could lead to misidentifications. As previously described,<sup>14</sup> in addition to species-specific primers, we also included conserved ITS2 forward and reverse primers in all reactions. This serves as an internal control for the presence of template DNA (i.e., a band will still be produced with non-members of the Albitarsis Complex).

**Genetic distance and phylogenetic analyses.** *White* gene sequences were analyzed by maximum parsimony as implemented in PAUP\* version 4.0b10.<sup>21</sup> Each gap was treated as a single character regardless of the length of the gap under the assumption that a given gap is a result of one mutational event.<sup>22</sup> In the Albitarsis Complex, the fourth *white* intron is present only in *An. marajoara*.<sup>15</sup> Neither intron presence/absence nor intron sequence was included in our analysis. Analysis was conducted using the heuristic search option with tree-bisection-reconnection (TBR) branch-swapping algorithm. Bootstrapping was done with 1,000 pseudoreplicates with 10 random-taxon-addition replicates per pseudoreplicate. The ITS2 was visually aligned and genetic distances calculated using uncorrected "P," also in PAUP\*. Our objective at this time was only to contrast *An. albitarsis* F with the other species and not to test known phylogenetic hypotheses. Therefore, because there was strong branch support based on *white* gene and correlated results from ITS2, we did not attempt to further refine the analyses.

## RESULTS

Based on the *white* gene and rDNA ITS2 sequences, we report the discovery of an additional species in the Neotropical *An. albitarsis* complex. We compared previously published sequence and analyses to conclude that this newly recognized taxon, *An. albitarsis* F, was at least as different from the four species initially recognized using RAPDs,<sup>10,11</sup> rDNA-ITS2,<sup>13,20</sup> partial sequences of *COI*, *ITS2*, *ND4* and *28S*,<sup>13</sup> and *white* gene,<sup>15</sup> as the four other species (*An. albitarsis*, *An. albitarsis* B, *An. marajoara*, and *An. deaneorum*) are from each other. Based on sequence data from the entire

mitochondrial *cytochrome B oxidase I* gene, another putative species, *An. albitarsis* E, was reported.<sup>12</sup> However, with our *white* gene dataset, we found that *An. marajoara* and *An. albitarsis* E clustered together. In addition, as previously reported,<sup>14,20</sup> the ITS2 sequences of these species showed no species-level differences. Further weight is given to the conclusion that *An. albitarsis* F is reproductively isolated, because it is found in sympatry with its close relative, *An. marajoara*.

**White gene.** As was the case in the other members of the Albitarsis Complex, PCR amplification of the *white* gene resulted in a single size fragment for *An. albitarsis* F, confirming our assumption that there is a single copy of this gene. As described below, we found abundant sequence differences (3.7–4.8% difference from the other four species) to distinguish *An. albitarsis* F from the others. In addition, as previously published,<sup>15</sup> the fourth intron of the *white* gene was absent in all species in the complex except *An. marajoara*. We found that this intron is also absent in *An. albitarsis* F.

Of the 692 total exon bases sequenced, there were 67 polymorphic and parsimony informative sites as follows: 8 at position 1, 5 at position 2, and 54 at position 3. Parsimony analysis produced the same topology as previously published<sup>15</sup> with the additional taxon, *An. albitarsis* F, falling in place with moderate support (88% bootstrap) as a sister taxon to the clade (*An. albitarsis* s.s., *An. deaneorum*; Figure 1). There was 100% support for the monophyly of the Albitarsis Complex in relation to the outgroup species, *An. albimanus*. Although the

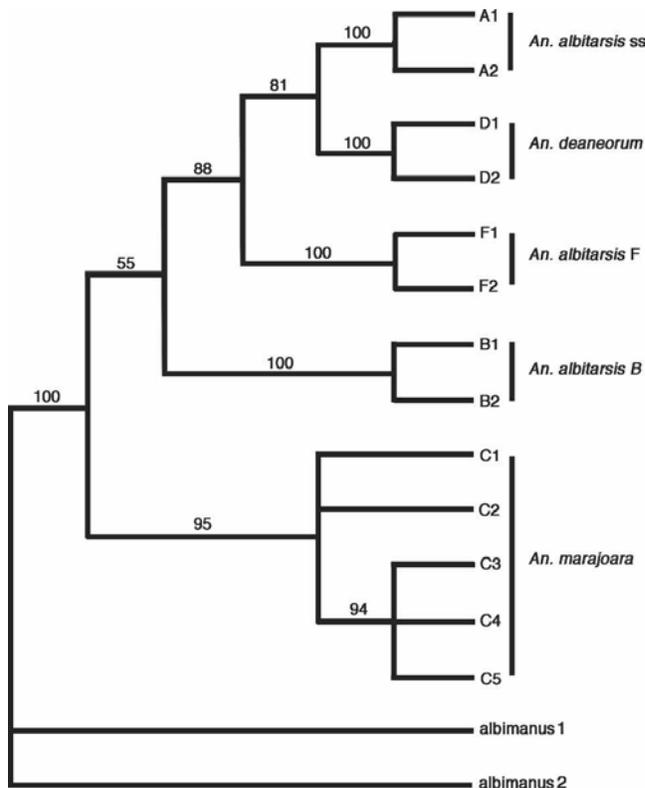


FIGURE 1. Maximum parsimony tree generated from *white* gene sequence. Two sequences of *An. albimanus* were used as outgroup (albimanus1, GenBank accession no. U73839, albimanus2, L76302). Ingroup: previously published sequences<sup>15</sup>: A1-2, *An. albitarsis*; B1-2, *An. albitarsis* B; C1-2, *An. marajoara*; D1-2, *An. deaneorum*; GenBank accession nos. are AY956295-302. Specimens C3, C4, C5 (putative *An. marajoara* species E) and F1, F2 are first reported here.

basal relationships within the ingroup were not particularly well supported (55–88%), there was high support (95–100%) for all branches leading to the species clusters (Figure 1). Because *An. albitarsis* F is as differentiated as the other nominal species, we conclude that it is a distinct species. All samples of *An. marajoara*, including putative *An. albitarsis* E (C3, C4, C5 in Figure 1), clustered together with high support, but there is a suggestion of geographic differentiation, because the specimens suggested to be *An. albitarsis* E<sup>12</sup> clustered together with 94% support.

**Ribosomal DNA (ITS2).** We obtained clear differences between consensus sequences (i.e., direct sequence, not data from clones<sup>13</sup>) of the four previously recognized species and *An. albitarsis* F. Our small sample ( $N = 3$ ) of *An. albitarsis* F had identical sequences. Total ITS2 length for all five species is similar, ranging between 344 and 365 bp, with that of *An. albitarsis* F being 365 bp. A pairwise comparison (uncorrected “*P*”) of the five taxa showed a range of genetic distances among all the species based on ITS2 from 0.57% to 1.97%. The distance between *albitarsis* F and the other four species ranged from 1.41% to 1.97%, which is greater than the distances among the four previously known species (0.57% to 1.14%). A notable feature of the ITS2 of this complex is the existence of two variable microsatellite regions. These were previously found to be intraspecifically and intragenomically variable.<sup>20</sup> The two regions are also present in *An. albitarsis* F, but the extent of variability in this species, if any, will not be known until cloning is carried out.

**ITS2-based PCR diagnostic primers.** The species diagnostic primer reported here, in combination with other published primers,<sup>14</sup> unambiguously identify five species in the Albitarsis Complex. Because the previously published primers<sup>14</sup> were based on constant but slight sequence differences, it was not possible to multiplex reactions for the four species treated. The ITS2 sequence of *An. albitarsis* F, however, is more divergent than the others, and the primer designed for it can be used with the *An. marajoara* primer to allow identification of these two species in a single reaction (Figure 2). The *An. albitarsis* F primer did not amplify fragments in any of the other species (i.e., because the conserved forward and reverse primers were used in all reactions, only an ITS2 amplicon appeared [Figure 2] when the species-specific target sequence was absent). *An. marajoara* and *An. albitarsis* F,

therefore, can be separated by a combination of the universal primers ITS2F, ITS2R, and species-specific albF, albC using a variation of conditions described by Li and Wilkerson<sup>14</sup> (annealing temperature of 62.5°C and primer concentrations of 12 pmol/50 µL and 4 pmol/50 µL for albC and albF, respectively).

## DISCUSSION

Various methods have been used to resolve species identities in the Albitarsis Complex, including chromosomes, allozymes, mtDNA restriction fragment length polymorphism (RFLPs), RAPD-PCR, morphology, and behavior.<sup>10,11,23–26</sup> The species are distributed in Central and South America: *An. albitarsis* (southern Brazil, northern Argentina, Paraguay), *An. deaneorum* (northern Argentina to western Brazil), *An. marajoara* (Brazil, Venezuela, Colombia, southern Central America), and *An. albitarsis* B (south, central, and eastern Brazil). The complete taxonomic picture for *An. marajoara* is not yet clear, because, based on *COI* sequence<sup>12</sup> and on *ND5* sequence (K. Shaw and J. Conn, personal communication), what is called *An. marajoara* in Colombia is actually likely to be the recently characterized putative *An. albitarsis* E.<sup>12</sup> It remains a question whether *An. albitarsis* E is distinct from *An. marajoara*, because the finding was not confirmed by us using *white* gene and ITS2 sequences, or by others.<sup>10,11,14,20</sup> The existence of *An. albitarsis* E therefore remains uncorroborated by selected nuclear markers, although using microsatellite markers, three distinctive populations in northern South America were detected (J. Conn and C. Li, personal communication). For purposes of this discussion, we refer to all specimens that can be diagnosed with ITS2 and *white* gene, including putative *An. albitarsis* E, as *An. marajoara*. No such ambiguity exists with the designation of *An. albitarsis* F, however, because both ITS2 and *white* gene sequences are distinct.

Correct identifications will permit informed studies on biting behavior and malaria susceptibility. However, there are currently no morphologic characters to distinguish any species of the Albitarsis Complex except for the larva of *An. deaneorum* (multi-branched head seta 2-C<sup>27</sup>). Further study is needed to detect possible morphologic characters to distinguish *An. albitarsis* F from the others species. For now, the ITS2-based PCR identification method presented here can easily be used to distinguish these species. In addition, the presence of *white* gene intron four in *An. marajoara* and its absence in *An. albitarsis* F can serve to separate the two wherever they are sympatric, as long as no other species in the complex are present (e.g., in northern Amazonian Brazil and southern Colombia and Venezuela).

To understand relative malaria susceptibility of the species in the Albitarsis Complex, it would be useful to see if there is a correlated phylogenetic component. Currently, two or three species are known vectors, *An. marajoara*, including *An. albitarsis* E, and *An. deaneorum*. Unfortunately, the phylogenetic relationships among species of the Albitarsis Complex remain unresolved, because there are conflicting tree topologies. One study used partial sequences of mitochondrial *COI* and *ND4* and rDNA ITS2 and D2,<sup>13</sup> and showed the relationships (*An. albitarsis*, *An. albitarsis* B) and (*An. marajoara*, *An. deaneorum*). Another used the complete *COI* sequence,<sup>12</sup> which resulted in a tree similar to above except that *An. deaneorum* came out in an ambiguous position in relation

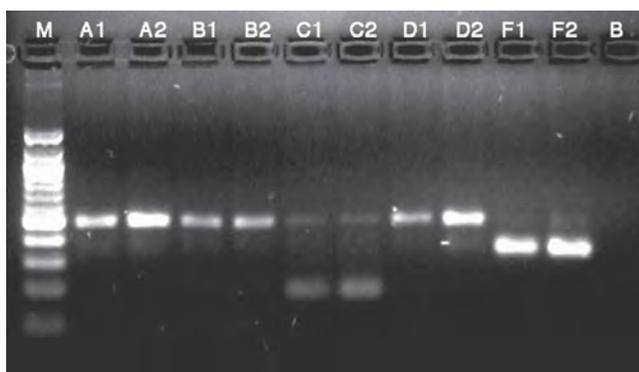


FIGURE 2. Result of amplification of rDNA ITS2 of five species in the Albitarsis Complex with a combination of four primers (ITS2F, ITS2R, albC, albF): A1-2, *An. albitarsis*; B1-2, *An. albitarsis* B; C1-2, *An. marajoara*; D1-2, *An. deaneorum*; F1-2, *An. albitarsis* F; M, 100 bp DNA ladder (Biolabs); B, negative control.

to two clades that contained *An. marajoara* and *An. albitarsis* E. A preliminary conclusion that there could be a phylogenetic component is supported by both studies. However, *An. albitarsis* F was unknown at the time of the above analyses. A third analysis, apparently not supporting a phylogenetic component to malaria susceptibility, is based on partial *white* gene sequence<sup>15</sup> and shows quite a different topology (similar to Figure 1 but without species F), albeit with relatively low bootstrap support for the basal branches, with the exception of the one leading to *An. marajoara*. The *white* gene tree is compelling because a parsimonious solution would be to assume a single intron loss event, which would place *An. marajoara* basal to {*An. albitarsis* B (*An. albitarsis* F[*An. albitarsis*, *An. deaneorum*])} (but see Krzywinski and Besansky<sup>28</sup>). This topology, with *An. marajoara* basal, was recovered by Merritt and others<sup>15</sup> even though intron sequence was not scored for their analysis. Note that there is apparent geographical structuring of the *An. marajoara* clade, as one might expect with the existence of an *An. albitarsis* E, with MAR3, TR3-4, MAR4 from Boa Vista, Trinidad, and Colombia, respectively, clustering together. A recent report<sup>29</sup> addresses the issue of conflicting datasets in the resolution of species boundaries and phylogenetic relationships in the *An. gambiae* complex. Evidence was found that supports introgression and reproductive isolation, as well as different tree topologies, depending on which sequence was sampled. It was concluded that phylogenetic analysis of closely related species can result in recovery of highly supported wrong answers depending on the gene(s) used. Clearly, more evidence is needed to resolve the relationships of species belonging to the Albitarsis Complex and their respective roles in malaria transmission.

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