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Short Report: *Anopheles darlingi* (Diptera: Culicidae) in Panama

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Abstract. We report *Anopheles darlingi* in Darien Province in eastern Panama. Polymerase chain reaction–restriction fragment length polymorphism profiles of the single copy nuclear *white* gene and sequence comparisons confirmed the presence of 66 specimens of the northern lineage of *An. darlingi*. The parsimony network depicted 5 *CO1* haplotypes in 40 specimens of *An. darlingi*, which connected through 7–8 mutational steps with sequences from Central and South America. Furthermore, the presence of haplotypes in Biroquera, Darien Province identical to those previously published from northern Colombia suggests that Panamanian samples originated in Colombia. Results of neutrality tests (R_2 and Fu's F_S) were not significant and the mismatch distribution was multimodal and did not fit the model of sudden population growth. These findings may indicate a long and stable presence of *An. darlingi* in eastern Panama.

Panama (9°00′N, 80°00′W) reported 5,095 malaria cases during 2004, which represented a six-fold increase in the incidence since 2001. The highest prevalence of malaria occurs in rural areas where more than 60% of the population is indigenous, living in extreme poverty and more vulnerable to malaria infection.[1] Four moderate-high risk areas for malaria transmission in Panama are Bocas Del Toro, Ngöbe Buglé Comarca, Kuna Yala Comarca, and Darien. Some of these locations are currently undergoing extensive changes in landscape because of an increase in tourism.

Among the reasons proposed for the recent increase in malaria cases in Panama is a change in vector species composition because of colonization and possible adaptation of some anophelines to new settings that is prompted by changes in landscape features. This scenario has taken place in other geographic contexts, i.e., *Anopheles darlingi*, the primary malaria vector in South America, has undergone extensive colonization in Iquitos, Peru, the western Amazon region in Brazil,[2,3] and the city of Belém.[4] In parts of the northeastern region of the Amazon, *An. darlingi*, has been replaced mostly by *An. marajoara*.[5] Moreover, *An. darlingi* has increased in abundance during the past 10 years in the Peruvian Amazon most likely because of deforestation. Consequently, *Plasmodium falciparum* has reinvaded areas in which malaria was previously eradicated.[2,6] In Panama, studies on changes in *Anopheles* species composition have not been conducted despite substantial anthropogenic perturbation during the past three decades. Loaiza and others[7] recently examined the distribution of *Anopheles* mosquitoes in Panama. They identified 14 species and suggested that *An. albimanus*, *An. aquasalis*, and *An. punctimacula* are each potential malaria vectors. However, *An. darlingi* was not collected during this survey.

Anopheles darlingi is broadly distributed from southern Mexico to northeastern Argentina, and in some parts of Central America, but has never been officially reported in Nicaragua, Costa Rica, or Panama, resulting in an apparent discontinuity in its distribution.[8] One hypothesis to explain finding *An. darlingi* in Darien is a relatively recent invasion from Colombia into eastern Panama that may have been enhanced by local changes in the landscape because of deforestation and other anthropogenic changes.[9,10] However, *An. darlingi* may have been present in eastern Panama for a longer time, but it has never collected or may have been misidentified.

In the present study, we report *An. darlingi* in Darien, the region in eastern Panama with the highest prevalence of drug-resistant *P. falciparum*. In addition, we identified which lineage of *An. darlingi* is present in Panama and investigated the genetic relatedness between our samples and previous published sequences of the mitochondrial cytochrome oxidase subunit 1 (*CO1*) gene.

We collected adult mosquitoes using the human landing catch technique and Centers for Disease Control and Prevention (CDC) miniature light traps (LTs) for four uninterrupted hours (6:00 PM–10:00 PM). Anti-malarial drugs were taken by collectors on the basis of recommendations by Instituto Conmemorativo Gorgas de Estudios de la Salud (ICGES) in Panama, and prior consent was obtained. The ICGES safety board reviewed and approved the mosquito collecting protocol. Adult females were killed in the field with chloroform, placed individually in 1.5-mL tubes, and stored in plastic bags with desiccant. Larval stages of *Anopheles* species were caught using a standard dipping technique during three consecutive days per locality.[11] Third- and fourth-larval stages were retained for adult emergence and species confirmation. Mosquitoes were sorted by species using the morphologic key of Wilkerson and Strickman.[12] These mosquitoes were subsequently hand carried to the United States, where molecular identification and sequencing was carried out by the Wadsworth Center Molecular Genetics Core and the Griffin Laboratory, New York State Department of Health (Albany, NY).

Molecular identification of *An. darlingi* was carried out using two approaches. First, we amplified by polymerase chain reaction (PCR) and sequenced a portion of the mitochondrial *CO1* gene and confirmed the sequences as that of *An. darlingi CO1* using the BLAST algorithm nucleotide search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Second, we used PCR–restriction fragment length polymorphism (PCR-RFLP) of the single copy nuclear *white* gene to distinguish between northern and southern *An. darlingi*, which are thought to be in the early stages of lineage divergence.[13] The PCR-RFLP profiles were confirmed by sequencing 800 basepairs of the *white* gene, and nucleotide sequences were compared with known sequences of *CO1* and *white* gene lineages.[13] Procedures and primers used for DNA isolation, PCR amplification and sequencing of the *CO1* and *white* genes have been reported.[10,13]

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Genealogic relationships among *An. darlingi CO1* haplotypes were investigated using a statistical parsimony network implemented in the program TCS version 1.12.[14] For this analysis, we used other previously published *CO1* sequences from several countries encompassing the geographic distribution range of *An. darlingi* (GenBank accession nos. DQ298209–DQ298244).[10] Neutrality tests of Fu's F_S[15] and R_2[16] were used to test for population size changes assuming neutrality[17] and calculated in Dna*SP*, version 4.50.02.[18] In addition, the mismatch distribution,[19] and the haplotype and nucleotide diversities were computed in Arlequin version 3.11.[20]

A total of 1,056 female mosquitoes belonging to 10 *Anopheles* species were collected in Yaviza, Jaque, and Biroquera (Table 1 and Figure 1). *Anopheles punctimacula* was the most common species (51.5%), confirming previous findings in Darien where it seems to be largely predominant.[7] In contrast, *An. albimanus* was less prevalent overall (26.7%), but more common than *An. punctimacula* in Jaque. *Anopheles darlingi* was more prevalent than *An. albimanus* in Biroquera, but proportionally less abundant than *An. punctimacula* and *An. apicimacula* (Table 1). *Anopheles darlingi* was only collected in Jaque and Biroquera by human landing catch, and not recorded in either LTs or in larval collections. Furthermore, *An. albimanus* was the most common species in larval collections and *An. punctimacula*, *An. apicimacula*, and *An. oswaldoi* s.l. were the only species collected in LTs. We also collected eight specimens of *An. nuneztovari* s.l. in Biroquera where previous sampling has not identified this species (Table 1).[7] The *CO1* gene sequences from Jaque and Biroquera showed 100% identity with *An. darlingi CO1* gene sequences (GenBank accession nos. AF417698 and AF270932) reported by Sallum and others.[21] The PCR-RFLP profiles of the single copy nuclear *white* gene and sequence comparison confirmed the presence of 63 mosquitoes of the northern *An. darlingi* lineage in Biroquera and 3 in Jaque.

Five *CO1* haplotypes were detected in 40 *An. darlingi* mosquitoes, three from Jaque and 37 seven from Biroquera (GenBank accession nos. FJ550354–FJ550358). All haplotypes were separated in the parsimony network by 7–8 mutational steps from sequences of Central and South America. Moreover, six sequences from Biroquera were identical to haplotype M (D2 in Figure 2), which had been previously found in Nuqui, Colombia,[10] thus suggesting a Colombian origin for the Panamanian samples (Figure 1). The haplotype diversity (Hd) and nucleotide diversity (Nd) for the *CO1* gene were moderate to low (Hd = 0.58, SD = 0.07 and Nd = 0.0006, SD = 0.0003, respectively), which could suggest a past bottleneck and subsequent expansion with a rapid buildup of mutations.[22] Nevertheless, results of neutrality tests ($F_S = 2.23 > 0.05$[17] and $R_2 = 0.014 > 0.05$[18]) were not significant and the mismatch distribution was multimodal and did not fit the model of sudden population expansion.

A history of malaria cases and the almost exclusive occurrence of *P. falciparum* in eastern Panama, where *An. albimanus* is not as prevalent as other *Anopheles* species, suggests that its incidence there may be related to the presence of a more efficient and possibly unidentified vector in this region.[7] In 2007, an outbreak of *P. vivax* and *P. falciparum* malaria (97 cases) occurred in Puerto Piña, which is located approximately 5 km from Jaque. Although no *Anopheles* species survey was conducted at the time, we hypothesize that *An. darlingi* could have been the main vector during this episode. Panama has undergone significant changes in land use, urbanization, and human migration since 1960,[23] and these changes may have altered the habitats for *Anopheles* larvae. However, we believe these changes have not yet affected areas such as Jaque and Biroquera, which are still geographically isolated, and where *An. darlingi* breeds in partially shaded and clean bodies of water, often associated with rivers and floating vegetation.

Seventy percent of Darien is still covered by forests, which provide ample breeding sites for *An. darlingi* and other *Anopheles* species.[24] Our results, in which we collected 45% of the total *Anopheles* species reported from Panama in Darien, support this conclusion.[7] Moreover, the balance in the proportion of low-, intermediate-, and high-frequency *CO1* gene haplotypes in Panama, nonsignificant neutrality test results, and a multimodal mismatch distribution support a stable past effective population size for *An. darlingi* in eastern Panama and argue against a bottleneck caused by a possible recent invasion. Nevertheless, a larger sample size, more localities, other molecular markers, and a more sophisticated simulation analysis that does not depend on summary statistics to construct the mismatch distribution will be required to fully evaluate the presence of *An. darlingi* in eastern Panama.

In the present study, *An. darlingi* was not collected in Yaviza, which is located approximately 300 km from Panama City.

Table 1
Anopheles species collected from Yaviza, Jaque, and Biroquera, Darien Province, Panama during August 11–19, 2008*

Anopheles species by subgenera	No. of mosquitoes (%) by species	No. of mosquitos by collecting method			No. of adult mosquitoes by locality		
		HLC	LT	LC	Yaviza	Jaque	Biroquera
Nyssorhynchus							
albimanus	281 (26.7)	216	0	65	7	204	5
darlingi	66 (6.3)	66	0	0	0	3	63
oswaldoi s.l.	7 (> 1)	6	1	0	5	0	2
triannulatus s.l.	9 (> 1)	6	0	3	6	0	0
nuneztovari s.l.	8 (> 1)	8	0	0	0	0	8
Anopheles							
punctimacula	541 (51.5)	528	7	6	350	91	94
malefactor	7 (> 1)	7	0	0	1	1	5
apicimacula	112 (10.6)	111	1	0	25	0	87
neomaculipalpus	21 (2)	3	0	17	1	2	0
pseudopunctipennis s.l.	6 (> 1)	6	0	0	6	0	0
Total	1,056	956	9	91	401	302	256

* HLC = human landing catch; LT = light trap; LC = larval collection.

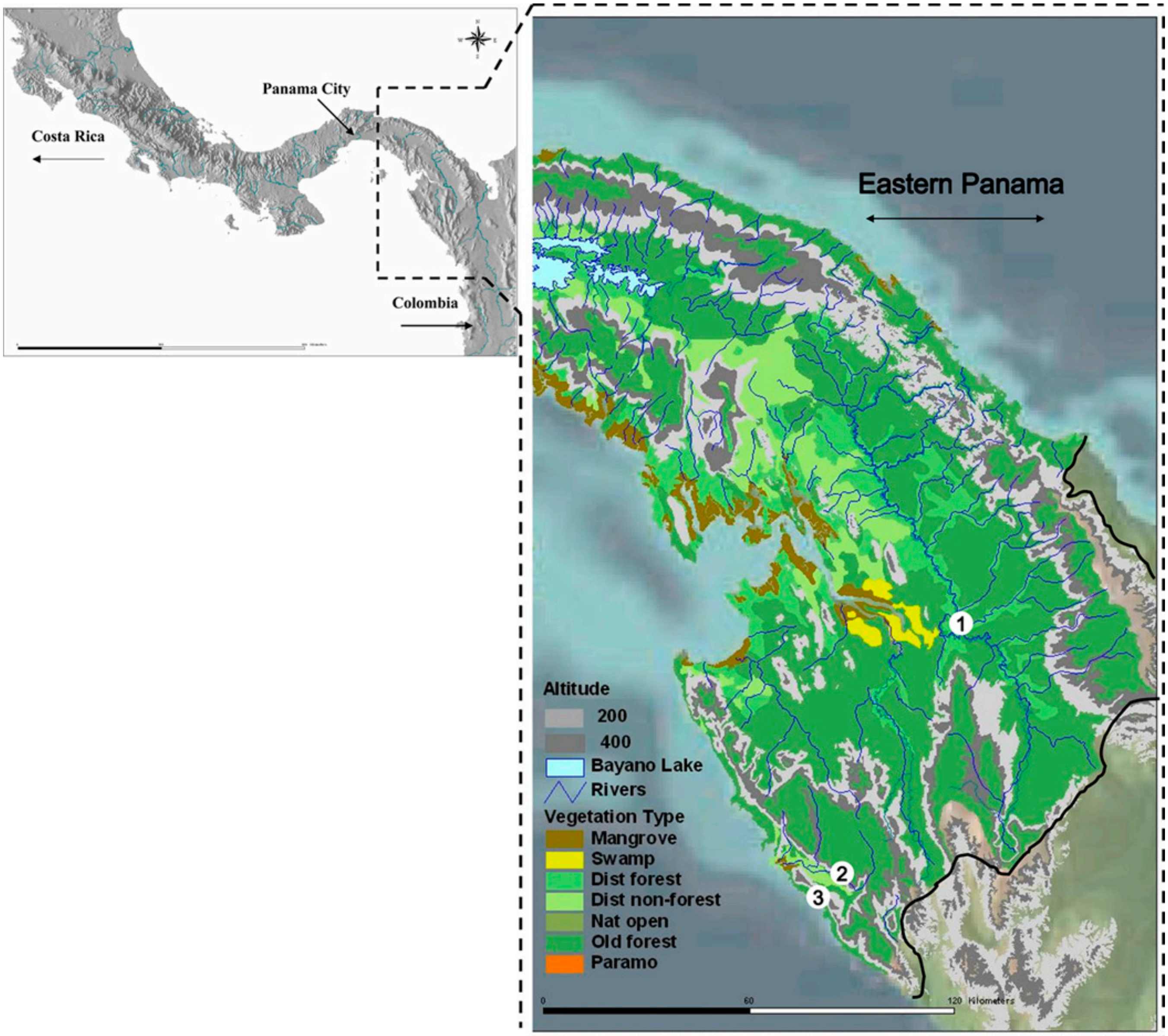

FIGURE 1. Geographic position of Yaviza (**1**), Biroquera (**2**), and Jaque (**3**) in Panama. Altitude is in meters. Dist non-forest, Dist forest, and Nat open represent areas with high, medium and low deforestation, respectively. Old forest represents non-disrupted primary forest. Paramo is a neotropical ecosystem at 3,100–5,000 meters above sea level. This figure appears in color at www.ajtmh.org.

However, with future environmental perturbation in Darien, *An. darlingi* could expand towards central and western Panama and contribute to a range expansion of *P. falciparum* into new areas, aggravating the malaria situation. Additional sampling throughout Darien and Kuna Yala Comarca may identify *An. darlingi* in other localities where *P. falciparum* has been previously encountered. At the local level, the influx of either refugees escaping from armed conflict in Colombia or travelers visiting eastern Panama, plus the presence of *An. darlingi*, are likely to intensify malaria outbreaks by *P. falciparum* in Darien. In the short term, it will be necessary to avoid unplanned deforestation through more sustainable methods of development for agriculture and human settlements in Darien. This in combination with traditional malaria control measures will reduce the likelihood of a possible expansion of *An. darlingi* into western Panama.

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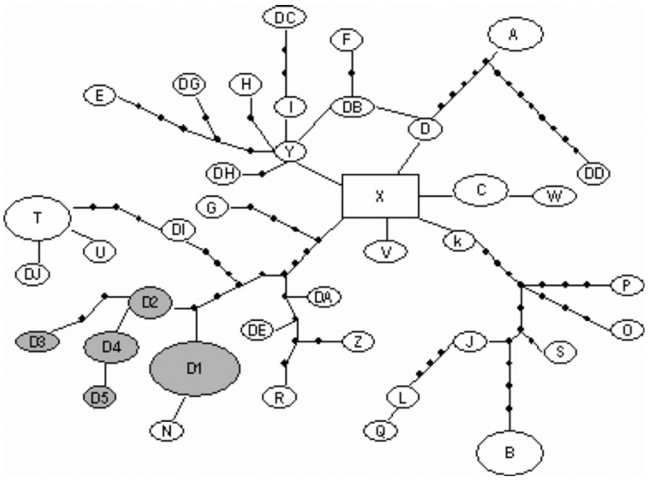

FIGURE 2. Minimum-spanning network re-drawn from Mirabello and Conn (2006). Shaded haplotypes (D1-D5) are recorded from Jaque and Biroquera in the Darien, eastern Panama. Haplotype D2 is identical to M[10] from Nuqui, Colombia.

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