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MOLECULAR POPULATION GENETICS OF THE PRIMARY NEOTROPICAL MALARIA VECTOR *ANOPHELES DARLINGI* USING mtDNA

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ABSTRACT. Samples of the neotropical malaria vector *Anopheles darlingi* from Bolivia, Brazil, and Venezuela were analyzed to test for differences in mitochondrial haplotype frequencies. With the use of molecular variance components and F-statistics, significant genetic variability of *An. darlingi* was found apportioned primarily among populations within regions or within populations, with regions defined either as biomes (n = 5) or ecoregions (n = 2). The Mantel analysis resulted in a significant correlation [Prob (r) = 0.009] between genetic and geographic distances, evidence that these populations are genetically isolated by distance. Such isolation could reflect differences in phenotypes for factors affecting vector capacity.

KEY WORDS Neotropical, malaria vector, *Anopheles darlingi*, mtDNA, population structure

INTRODUCTION

Throughout its range in tropical Central and South America, *Anopheles darlingi* Root transmits human malaria parasites and is of great health and economic significance. It is also considered the most important malaria vector in the Amazon Basin, where its recent resurgence is caused by factors that include invasion of its primary breeding sites along warm lowland rivers by gold miners, construction workers, and immigrants living under crowded and precarious conditions (Deane 1988, Alecrim 1992). This development, coupled with its high anthropophily (Deane et al. 1946), is alarming. For example, *An. darlingi* has recently expanded its geographic range via human-assisted colonization to include Iquitos, Peru, where it caused a recent outbreak of malaria (Fernandez et al. 1997).

Even though single species taxonomic status appears to have been clearly demonstrated for *An. darlingi* from a combination of random amplified polymorphic DNA (RAPD), isozyme, Internal Transcribed Spacer 2 (ITS2) sequences, and morphological data for samples collected from Belize to southern Brazil (Manguin et al. 1999), substantial heterogeneity exists in behavior, such as peak biting time (reviewed in Rosa-Freitas et al. 1992), and size as measured by wing lengths (Lounibos et al. 1995, Charlwood 1996). Geographic differences in blood-seeking periodicities may have resulted from adaptation to local environmental conditions, whereas wing length variability could be attributed to heterogeneous larval habitats (Lounibos et al. 1995). Furthermore, evidence has been found for genetic variation at the population level from larval polytene chromosome (Kreutzer et al. 1972, Tadei et al. 1982), allozyme (Steiner et al. 1982, Rosa-Freitas et al. 1992), and mtDNA restriction fragment length polymorphism (RFLP) data (Freitas-Sibajev et al. 1995).

The documented heterogeneity in *An. darlingi* may be explained, in part, by population structure and patterns of gene flow, which we propose to examine with mitochondrial DNA (mtDNA) RFLP data. The mtDNA genome in insects evolves at a rate that is distinctive from the nuclear genome (reviewed in Simon et al. 1994). This distinction, combined with the mtDNA genome's maternal inheritance and extremely low level of recombination, has led to its use in population and species level analyses in many organisms including anopheline mosquitoes from the Neotropics (Conn et al. 1998, de Merida et al. in press) and Africa (Besansky et al. 1997).

MATERIALS AND METHODS

Sample collection

Adult *An. darlingi* were collected by aspiration from human subjects outdoors around nightfall (approximately 1800–2000 h) in Bolivia (GU = Guayaramerín, Beni department, 7.XII.91, n = 5; SR = San Ramón, Riberalta, Beni department, 8&10.VIII.90, n = 15), and Venezuela (AY = Puerto Ayachucho, Amazonas state, 19.VII.92, n = 22). Collections in Brazil (CP = Capanema, Pará state, 20.VIII.91, n = 42; UR = Urucuri, Pará state, 21.VIII.91, n = 10) were taken from cattle stables. Mosquitoes were identified with the morphological key of Deane et al. (1946) then either placed in

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Table 1. Presence (1) or absence (0) of mtDNA restriction sites in haplotypes of *Anopheles darlingi*. Numbers below each restriction enzyme refer to the base position in the mtDNA sequence.

	*Bgl*II				*Cla*I					*Eco*RV				*Nsi*I			*Pst*I				*Pvu*II				*Sst*I			
Haplotype	6800	9200	10879	13058	3550	4450	7771	9600	12860	750	2950	10000	11300	1700	10456	13250	2919	4400	5400	7300	2300	5000	11000	11300	1683	2283	5139	7440
22 CDABAEA	1	0	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1
23 BEEAAAA	1	1	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1
24 ADACACA	1	1	0	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1	1
25 CDABAEB	1	0	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	1
26 BDACAAA	1	1	1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	1	1	0	1	1	0	1	1	1	1
27 BDAABBA	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
28 ADFCACA	1	1	0	1	1	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	1	0	0	0	1	1	1	1
29 BEAAABA	1	1	1	1	1	1	1	0	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
30 CDACAEB	1	0	1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	1	1	1	1	0	0	1	1	0	1
31 BEECAAA	1	1	1	1	1	1	1	0	1	1	0	1	0	1	0	0	1	1	1	1	0	1	1	0	1	1	1	1
32 ADACAEA	1	1	0	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1	1
10 AAAAAAA	1	1	0	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1
33 AEAAAAA	1	1	0	1	1	1	1	0	1	0	1	0	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1
34 ADAADAA	1	1	0	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	0	1	0	1	1	0	1	1	1	1
35 BDEBAAA	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1
36 ADABAEA	1	1	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1
37 BEEBAAA	1	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	1	1	1	0	1	1	0	1	1	1	1

Fig. 1. Locations of *Anopheles darlingi* sampled populations. Sites in the present study are AY, Puerto Ayacucho; CP, Capanema; GU, Guayaramerín; SR, San Ramón; UR, Urucuri, and are represented by solid squares. Open squares represent sites of collections analyzed by Freitas-Sibajev et al. (1995) and used in the present study for the distance, divergence, and hierarchical analyses: CM, Costa Marques; CU, Cuiabá; DO, Dourado; MN, Manaus. CP has both an open and a solid square near it because samples from this site were used in both studies.

liquid nitrogen or isopropanol, or else females were bloodfed and carried alive to Florida to obtain eggs. After oviposition and eclosion, larvae from the bloodfed females were reared at 12L:12D photoperiod and 27°C at the Florida Medical Entomology Laboratory in Vero Beach, FL. Prior to DNA extraction, specimens were stored at −70°C. Voucher specimens of adults from selected sites are deposited at the Florida Medical Entomology Laboratory, the National Museum of Natural History, Washington, DC, and the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

DNA extraction

The procedure for DNA extraction from individual mosquitoes and hybridization to the complete mtDNA genome of *Anopheles quadrimaculatus* Say species A followed that in Conn et al. (1997). Conserved sites were mapped by comparison with earlier RFLP work with *An. darlingi* (Freitas-Sibajev et al. 1995) and with the complete mtDNA sequences of *An. quadrimaculatus* (Mitchell et al. 1993) and *Anopheles gambiae* Giles (Beard et al. 1993). Composite mtDNA haplotypes for each individual mosquito were identified by both number and enzyme profile (Table 1).

Table 2. Distribution and frequency of mtDNA haplotypes in South American populations of *Anopheles darlingi*.[1]

	Population					
	Bolivia		Brazil		Venezuela	
Haplotype	GU	SR	CP	UR	AY	Total
22 CDABAEA	1					1
23 BEEAAAA	2	4				6
24 ADACACA	1	1				2
25 CDABAEB	1					1
26 BDACAAA		2				2
27 BDAABBA		1				1
28 ADFCACA		3				3
29 BEAAABA		1				1
30 CDACAEB		1				1
31 BEECAAA		1				1
32 ADACAEA		1				1
10 AAAAAAA			41	9		50
33 AEAAAAA				1		1
34 ADAADAA			1			1
35 BDEBAAA					1	1
36 ADABAEA					20	20
37 BEEBAAA					1	1
Total	5	15	42	10	22	94

[1] GU, Guayaramerín; SR, San Ramón; CP, Coupanema; UR; Uracuri; AY, Puerto Ayacucho.

Haplotypes were numbered commencing at 22 so they would not be confounded with those for *An. darlingi* in Freitas-Sibajev et al. (1995). However, we maintained haplotype 10 from Freitas-Sibajev et al. (1995) for the haplotype from Capanema in eastern Brazil (Table 1) because the haplotypes in the two studies were identical.

Statistical analyses

Nucleotide (Π) and haplotype (h) diversities within populations and nucleotide divergence among populations were calculated with the computer program REAP (McElroy et al. 1992). F-statistics (ϕ) and molecular variance components (σ^2) were calculated according to Cockerham (1969, 1973) for each of the following in the combined data set of *An. darlingi*: among regions, among populations/regions, and within populations. Their significance was determined by running 1,000 permutations with analysis of molecular variance (Excoffier et al. 1992). We defined population as sample site (n = 9; see Fig. 1). For region, sample sites were grouped according to neotropical biome (Eisenberg 1989), which resulted in 5 groupings: 1) Campos limpos–Amazonian savannas, AY; 2) Madeiran, CP, UR, Costa Marques (CM); 3) Amazonian, SR, GU, Manaus (MN); 4) Brazilian rain forest, Dourado (DO); and 5) Campos cerrados, Cuiabá (CU). We did a 2nd analysis where region was defined as ecoregion, specifically for neotrop-

Table 3. Haplotype and nucleotide diversities (SD in parentheses) of *Anopheles darlingi* from 5 collection sites in Bolivia, Brazil, and Venezuela.

Site[1]	Haplotype diversity(*h*)	Nucleotide diversity (II)
GU	0.900 (0.161)	0.0300
SR	0.905 (0.054)	0.0254
CP	0.048 (0.045)	0.0002
UR	0.200 (0.154)	0.0008
AY	0.178 (0.106)	0.0047
Mean	0.446 (0.035)	0.0122 (0.00004)

[1] GU, Guayaramerín; SR, San Ramón; CP, Capanema; UR; Urucuri; AY, Puerto Ayacucho.

ical malaria vectors (Rubio-Palis and Zimmerman 1997). This analysis resulted in only 2 groupings: 1) interior lowland forest, CM, CP, DO, GU, MN, UR; and 2) savanna, AY, CU, SR.

GENEPOP (Raymond and Rousset 1995) was used to examine isolation by distance via a Mantel analysis (Mantel 1967) of a pairwise matrix of geographic distances and genetic distances (estimated by F_{ST}). This procedure provided a test for the null hypothesis of the independence of the geographic and genetic distances between each of the 9 populations. Significance was determined by a permutation test of n = 1,000, creating a distribution that was used to evaluate the significance of the Mantel analysis. Pairwise F_{ST} values were transformed to $F_{ST}/(1 - F_{ST})$, and a regression was performed on the pairwise geographic distances among 9 populations. The regression was performed a 2nd time with the geographic distance transformed with the natural logarithm. Transformations and regression analyses were done with JMP (Version 3.1, SAS Institute, Inc., 1989–94).

RESULTS

Seven restriction enzymes produced a total of 17 haplotypes for the 94 individuals analyzed from the 5 collection sites (Table 1) in the present study. Haplotypes were shared between San Ramón and Guayaramerín in Bolivia (approximately 48 km apart), as well as between Capanema and Urucuri in Brazil (separated by approximately 19 km). No haplotypes were shared between localities more than 200 km apart (Table 2).

Haplotype and nucleotide diversity within populations varied considerably, with GU and SR displaying high diversities and CP low diversities (Table 3). When all 9 collection sites (including data from Freitas-Sibajev et al. [1995]) were analyzed, low nucleotide divergence was found between collection sites that were close geographically (e.g., GU–SR, 0.000499; CP–UR, 0.000009; Table 4); all other sites varied from 0.002102 (CM–GU) to 0.020178 (MN–CU; Table 4).

No significant differences were found among biomes (Eisenberg 1989), but populations within biomes and intrapopulation variances were significant (Table 5). The values for the ϕ-statistics at the population level (ϕ_{SC} = 0.447 and ϕ_{ST} = 0.640) suggest structuring in *An. darlingi* among populations within biomes and within populations. Similarly, when the ecoregions of Rubio-Palis and Zimmerman (1997) were used, variances within ecoregions (ϕ_{SC} = 0.596) and within populations (ϕ_{ST} = 0.634) were significant. The correlations between nontransformed geographic distances and genetic distances, as well as those between transformed geographic distances and genetic distances, were significant as computed by the Mantel analysis (Prob [r] = 0.009 and Prob [r] = 0.006; Fig. 2A, 2B, respectively).

DISCUSSION

The levels of haplotype and nucleotide diversities for *An. darlingi* are in the same range as those of some populations of other neotropical anophelines, e.g., *Anopheles rangeli* Gabaldon, Cova Garcia, and Lopez and *Anopheles trinkae* Faran from Bolivia and Ecuador (Conn et al. 1997) and *Anopheles nuneztovari* from Brazil (Conn et al. 1998). The overall nucleotide divergence for *An. darlingi*

Table 4. Nucleotide divergence among 9 collection sites (this study plus data from Freitas-Sibajev et al. 1995[1]) of *Anopheles darlingi*.[2]

	GU	SR	CP	UR	AY	DO	CM	CU	MN
GU	—								
SR	0.000499	—							
CP	0.006800	0.008352	—						
UR	0.006496	0.008041	0.000009	—					
AY	0.004131	0.011996	0.011196	0.011184	—				
DO	0.002269	0.004597	0.005438	0.005370	0.012083	—			
CM	0.002102	0.004334	0.003192	0.003046	0.012374	0.000772	—		
CU	0.005795	0.007357	0.008296	0.007532	0.019165	0.004280	0.002296	—	
MN	0.003850	0.014673	0.019716	0.019774	0.011118	0.011702	0.014169	0.020178	—

[1] Because both studies included individuals from CP, they were combined for this analysis.

[2] GU, Guayaramerín; SR, San Ramón; CP, Capanema; UR; Urucuri; AY, Puerto Ayacucho; DO, Dourado; CM, Costa Marques; CU, Cuiabá; MN, Manaus.

Table 5. Hierarchical analysis of molecular variance in *Anopheles darlingi*.

Variance component		Observed partition		P[1]	ϕ-statistics
		Variance	% total		
Among biomes[2]	σ_a^2	0.741	34.92	0.031	ϕ_{CT} = 0.349
Among populations/biomes	σ_b^2	0.617	29.09	<0.001	ϕ_{SC} = 0.447
Within populations	σ_c^2	0.763	35.99	<0.001	ϕ_{ST} = 0.640
Among ecoregions[3]	σ_a^2	0.199	9.52	0.239	ϕ_{CT} = 0.095
Among populations/ecoregions	σ_b^2	1.124	53.88	<0.001	ϕ_{SC} = 0.596
Within populations	σ_c^2	0.763	36.60	<0.001	ϕ_{ST} = 0.634

[1] Probability of having a more extreme variance component and ϕ-statistic than the observed values by chance alone. Probabilities were calculated by a random permutation procedure (Excoffier et al. 1992). ϕ_{CT} and σ_a^2 are tested under random permutations of whole populations across biomes or ecoregions; ϕ_{SC} and σ_b^2 are tested under random permutations of individuals across populations but within the same biome or ecoregion; ϕ_{ST} and σ_c^2 are tested under random permutations of individuals across populations without regard to original populations or biomes (ecoregions).

[2] As in Eisenberg (1989).

[3] As in Rubio-Palis and Zimmerman (1997).

is fairly low (0.6%, this study; 0.9%, Freitas-Sibajev et al. [1995]), more characteristic of a panmictic species with little population structure on a continental scale, such as the nearctic species *An. quadrimaculatus* A (Perera et al. 1995). Other anophelines that have been sampled over a similar range in the Neotropics have higher values (1.5% for both *An. nuneztovari* and *An. rangeli* [Conn et al. 1997]), but one of these, *An. nuneztovari,* displays significant population structure and appears to be in the process of speciation (Conn et al. 1998), whereas the other, *An. rangeli,* was recently shown to be polyphyletic in an analysis of 655 bp of the mtDNA COII gene for several individuals from Ecuador and Bolivia (Danoff-Burg and Conn, unpublished data).

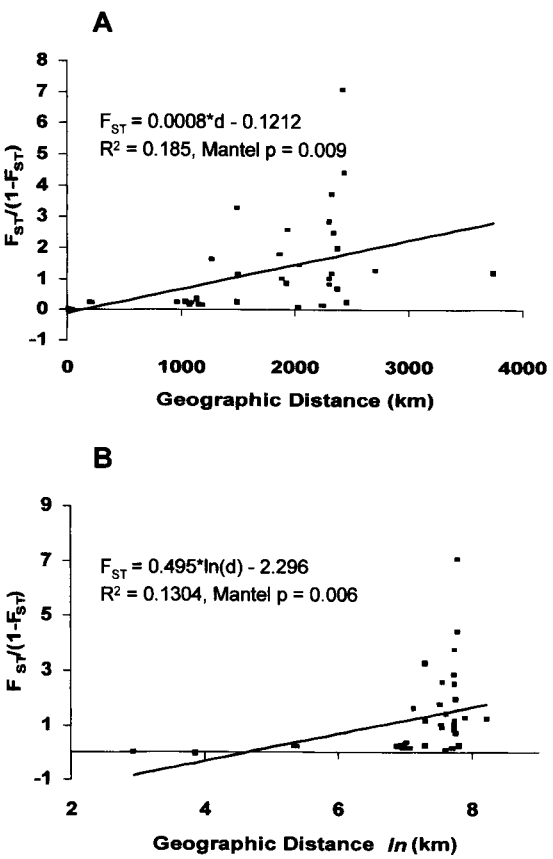

Fig. 2. Regression analysis of pairwise $F_{ST}/(1 - F_{ST})$ compared with (A) untransformed and (B) natural logarithms of geographic distances among all 9 sites.

The hierarchical analysis demonstrates that most genetic structure is found among populations within regions or within populations. Such a pattern can imply immigration from previously isolated populations (Avise et al. 1992), large or heterogeneous long-term effective population size (N_e, [Slatkin and Maddison 1989]), undersampling, or a distinctive history for mosquito haplotypes such that some once geographically widespread lineages become locally extinct while others persist, resulting in highly divergent haplotypes that are present in the same population (Conn et al. 1998). We detected no evidence in this data set for immigration from previously isolated populations in *An. darlingi,* although testing this may require samples from more than the 9 collection localities surveyed. We note that substantial variation in N_e was found between two Kenyan populations of the major African malaria vector *An. gambiae* (Lehmann et al. 1998), and, because we have no data on N_e for *An. darlingi,* we cannot rule out large or heterogeneous N_e as a possible explanation for our results. Although undersampling may be a factor, other studies that used larger sample sizes in neotropical anophelines (Conn et al. 1998, de Merida et al. in press) show a similar pattern, i.e., in hierarchical analysis, most structure is found among populations within regions or within populations. This suggests a broader explanation, which could be multiple colonization events with loss of some lineages as mentioned above.

We have also demonstrated significant isolation

by distance, which is likely to be restricting gene flow in *An. darlingi* on a local level, even though we cannot distinguish whether this is contemporary or historical (Bossart and Pashley Prowell 1998). Populations that are isolated may possess distinctive phenotypes for factors affecting vector capacity, such as feeding behavior, longevity, and diel activity, and such information is important to effectively design vector control strategies at a local level.

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