Am. J. Trop. Med. Hyg., 60(3), 1999, pp. 364–376

POPULATION STRUCTURE OF THE PRIMARY MALARIA VECTOR IN SOUTH AMERICA, *ANOPHELES DARLINGI*, USING ISOZYME, RANDOM AMPLIFIED POLYMORPHIC DNA, INTERNAL TRANSCRIBED SPACER 2, AND MORPHOLOGIC MARKERS

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Abstract. A genetic and morphologic survey of *Anopheles darlingi* populations collected from seven countries in Central and South America was performed to clarify the taxonomic status of this major malaria vector species in the Americas. Population genetics was based on three techniques including isozyme, random amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR), and internal transcribed spacer 2 (ITS2) markers. The results of the isozyme analysis indicated moderate differences in the allele frequencies of three putative loci (glutamate oxalaoacetate transaminase-1, isocitrate dehydrogenase-1, and phosphoglucomutase) of the 31 analyzed. No fixed electromorphic differences separated the populations of *An. darlingi*, which showed little genetic divergence (Nei distances = 0.976–0.995). Fragments produced by RAPD-PCR demonstrated evidence of geographic partitioning and showed that all populations were separated by small genetic distances as measured with the 1 − S distance matrix. The ITS2 sequences for all samples were identical except for four individuals from Belize that differed by a three-base deletion (CCC). The morphologic study demonstrated that the Euclidean distances ranged from 0.02 to 0.14, with the highest value observed between populations from Belize and Bolivia. Based on these analyses, all the *An. darlingi* populations examined demonstrated a genetic similarity that is consistent with the existence of a single species and suggest that gene flow is occurring throughout the species' geographic range.

Anopheles (*Nyssorhynchus*) *darlingi* Root 1926 is the most efficient malaria vector in the New World.[1] In South America, its distribution stretches from Colombia to northeastern Argentina. In Mexico, it is found as far north as Tabasco and Chiapas States, and in Central America, it occurs in Belize, Guatemala, Honduras, and occasionally in El Salvador.[2] However, this species has never been officially reported from Nicaragua, Costa Rica, or Panama, resulting in an apparent discontinuity in its distribution between Central and South America.[3]

Transmission of human malaria by *An. darlingi* occurs mainly in warm and humid forests along rivers in lowland areas (less than 550 meters above sea level), sometimes in coastal and piedmont ecoregions with high rainfall and tropical forests.[4] *Anopheles darlingi* is a very serious malaria vector throughout its geographic range because of its association with humans and its high rates of infection with the malaria parasites *Plasmodium falciparum, P. vivax,* and *P. malariae*.[5] It is the most anthropophilic and endophagic anopheline in the Americas, factors that obviously account for much of its potency as a major malaria vector.[5–8] As a result of complex sociologic, economic, and biologic factors, malaria has resurged in Brazil.[9] *Anopheles darlingi* is the primary vector of human malaria in the Amazon Basin,[7] and is the major contributor to the present resurgence of disease in South and Central America.

Anopheles darlingi was first described by Root[10] in 1926 from Rio de Janeiro in Brazil. In 1937, from samples collected near São Paulo (Brazil), Galvão and others[11] described a variety of *An. darlingi* named *paulistensis* based on adult and egg characteristics. The latter characters seem to be at least quite variable in Brazil[12,13] and Venezuela.[14] Studies on the biology of *An. darlingi* also showed considerable variation in Brazil,[8,15–19] Suriname,[20,21] French Guiana,[22] Guyana,[23] Colombia,[24] and Venezuela.[25] In addition, variation has been described in its morphology,[26,27] chromosomes,[28,29] and isozymes.[18,30–32] Some of these reports have suggested that *An. darlingi* may be a species complex.[19,30]

In 1988, Linthicum[3] asserted that populations of *An. darlingi* collected throughout its geographic range showed little interpopulation variation among the diagnostic morphologic characteristics. Therefore, *An. darlingi* was defined as a monotypic subgroup and placed in the Argyritarsis section. However, a recent phylogenetic analysis (Danoff-Burg JA, Conn JE, unpublished data) using morphologic and molecular data suggested that the Argyritarsis section is paraphyletic with respect to the Albimanus section and *An. darlingi* has been placed in a basal grade in the *Nyssorhynchus* subgenus (Figure 1).

In spite of these reports, few comparative studies of *An. darlingi* populations have been undertaken. The present study was based on populations of *An. darlingi* from throughout much of its geographic range in Central America and South America (Figure 2). In Brazil, *An. darlingi* was collected in different states (Table 1), including the type-locality of the variant *paulistensis* (from São Paulo State).

The approach we took to evaluate the taxonomic status of *An. darlingi* was based on morphometric and genetic analyses using isozyme, random amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR), and internal transcribed spacer 2 (ITS2) markers. These genetic techniques have proven to be excellent for distinguishing species of insects[33–41] that otherwise are very difficult to separate on the basis of morphologic features. Our main objectives included 1) analysis of the differentiation and variability of *An. darlingi*, 2) comparison of *An. darlingi* populations with

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1999		**00-00-1999 to 00-00-1999**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Population Structure of the Primary Malaria Vector in South America, Anopheles Darlingi, Using Isozyme, Random Amplified Polymorphic DNA, Internal Transcribed Spacer 2, and Morphologic Markers	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Walter Reed Army Institute of Research,Department of Entomology,Washington,DC,20307**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
see report

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**13**	

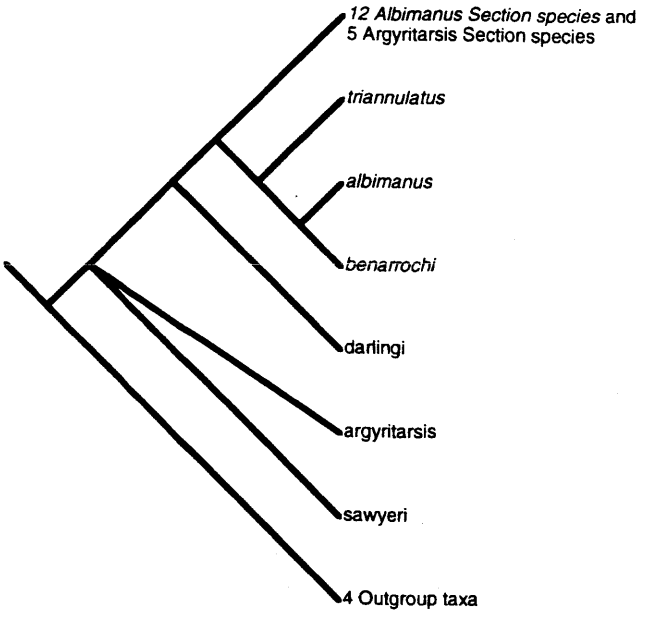

FIGURE 1. Basal portion of *Nyssorhynchus* subgenus phylogeny based on data from complete sequences of NADH dehydrogenase subunit 2, NADH dehydrogenase subunit 6, cytochrome oxidase subunit I, and internal transcribed spacer 2, as well as egg, larval, pupal, and adult morphologic characteristics.[32] Species in italics are those that were postulated to be in the Albimanus section, whereas those in roman font were said to be in the Argyritarsis section.[3,26,70] Relationships among the terminal species in the subgenus were simplified to demonstrate the basal placement of *Anopheles darlingi*.

FIGURE 2. Collecting areas of *Anopheles darlingi* in seven countries throughout the geographic distribution of this species. Each symbol (squares and circles) corresponds to a collection of multiple samples. Dark squares are samples used in genetic analyses, white squares in morphologic studies, and dark circles in both genetic and morphologic studies. ALB = Fortuna de Albarical (Amazonas, Venezuela); ARA = Araraquara (São Paulo, Brazil); ARI = Aripao (Bolivar, Venezuela); BAH = Itabela (Bahia, Brazil); BAR = Pereira Barreto (São Paulo, Brazil); BEL and BHZ = Belize (Central America); BEM = Belem (Pará, Brazil); BOL = Beni (Bolivie); CAP = Capanema (Pará, Brazil); CAS = Caicara (Bolívar, Venezuela); CMB = Costa Marques (Rondonia, Brazil); COL = Beté (Chocó, Colombia); CUI = San Antonio de Leverger (Mato Grosso, Brazil); DOU = Rio Jacare Pepira (São Paulo, Brazil); ELJ = El Juval (Trujillo, Venezuela); IQ = Iquitos (Peru); ITU = Ituxi River (Amazonas, Brazil); MAN = Manaus (Amazonas, Brazil); MAR = Maripasoula (French Guiana); MG = Mato Grosso (Brazil); OCA = Ocamo (Amazonas, Venezuela); PEX = Peixoto de Azevedo (Mato Grosso, Brazil); PIN = El Pinal (Bolivar, Venezuela); PR = Velha Timboteua (Pará, Brazil); SJM = San Juan de Manapiare (Amazonas, Venezuela); TRP = Trou-Poisson (French Guiana); URU = Urucuri (Pará, Brazil); YUC = La Yuca (Bolivar, Venezuela).

the variant *paulistensis* described in the literature, and 3) comparison of the morphologic and genetic profiles of *An. darlingi* populations with other *Anopheles* species used as outgroups.

MATERIALS AND METHODS

Mosquito populations. From 1990 to 1996, we collected samples of *An. darlingi* from seven countries including Belize in Central America, and Bolivia, Brazil, Colombia, French Guiana, Peru, and Venezuela in South America (Figure 2 and Table 1). The countries were chosen to provide a spatial representation of the geographic distribution of this species and to examine samples from the type-locality of the variant *paulistensis*. Populations (Per 1–5) from Peru (Iquitos, 3°46′S, 73°15′W) were used only for the RAPD-PCR. In the morphologic study, populations of *An. darlingi* from northern Colombia (Beté, Departamento del Chocó, 5°45′N, 77°W) were also analyzed.

Samples used were primarily single individuals from isofemale progeny broods, wild-caught adults, and occasionally larvae or pupae reared individually to the adult stage. For the latter, fourth instar larval and/or pupal exuviae were preserved and each specimen was recorded, identified to species, and kept frozen at −70°C.

Other *Anopheles* species were included in the isozyme, RAPD-PCR, and morphologic analyses as outgroup taxa. We used *An.* (*Nys.*) *argyritarsis* Robineau-Desvoidy and *An.* (*An.*) *pseudopunctipennis* Theobald for the isozyme analysis, *An.* (*Nys.*) *nuneztovari* Gabaldon for the RAPD-PCR, and four species in the *Nyssorhynchus* subgenus: *An. albitarsis* Lynch Arribalzaga, *An. argyritarsis, An. braziliensis* (Chagas'), and *An. marajoara* (Galvão and Damasceno) for the morphologic analysis. When relationships among 21 other *Nyssorhynchus* taxa were reconstructed using only ITS2, *An. darlingi* was monophyletic and included in a basal unresolved polytomy. Therefore, no outgroups were used in the present study.

Isozyme electrophoresis. Isozymes were separated by horizontal starch gel electrophoresis. Experimental procedures are detailed in the publication by Manguin and others.[42] A total of 35 enzyme systems was screened on two different buffers: the morpholine buffer system[43] (Morph, pH 6.1), and the Tris-citrate buffer system[44] (TCss, pH 6.7). Of the 35 enzyme systems tested, 23 showed good allelic resolution, including 31 putative loci (Table 2). For each locus, the most frequent electromorph was designated the 100 allele and all other alleles were measured relative to it. Electromorph genotype frequencies were used as input for BIOSYS-1.[45] Analysis of each population included computation of allele frequencies, heterozygosity per locus, additional

TABLE 1

Geographic information on the sample sites of *Anopheles darlingi*

Country	State	Locality and collection no.	Longitude/latitude	Collection code for each technique,[1,2,3,4,5]*
Belize	Cayo	Sibun River	17°07–18′N/88°34–40′W	BHZ[1,3] (542, 556–9, 565, 571–3, 578–82, 615), Bel[2] 1–10, BEL[4]
	Cayo	5 Blues Lake, Novelo	17°08-09′N/88°36–38′W	BHZ[1] (548–9, 567, 611), BHZ[1] (563, 612)
	Cayo	Churchyard	17°18′N/88°34′W	BHZ[1] (584–5, 604)
Bolivia	Beni	Guayaramerin	10°51′S/65°21′W	BOL: GU,[1,4,5] BOL[2] (1–4)
	Beni	Riberalta, San Ramon	11°02′S/66°04′W	BOL: SR[1,5], BOL[2] (5–8, 10–17, 19–22)
	Beni	Riberalta, Antofagasta	12°56′S/64°30′W	BOL: Ant,[1,5] BOL[2] (9,18)
Brazil	Amazonas	Manaus	03°08′S/60°01′W	Man[2] (1–5), MAN[5]
	Amazonas	Ituxi River: Floresta, Labrea, Estirao	7°59′-8°01′S/65°14–16′W	ITU[1,3]: BR (5, 10), BR (2), BR (7)
	Amazonas	Ituxi River: Ilha do Jucia	7°47S/65°07′W	ITU[1]: BR (13)
	Bahia	Itabela	16°34′S/39°24′W	BAH: Bah[2] (1–5)
	Mato Grosso	Peixoto de Azevedo	10°23′S/54°54′W	PEX: BR[1,5] (544, 546, 020, 701), Pex[2] (1–28)
	Mato Grosso	Mato Grosso	15°00′S/59°55′W	MG[4]
	Mato Grosso	Santo Antonio de Leverger	15°51′S/56°03′W	Cui[2]
	Para	Capanema, Velha Timboteua, Urucuri	01°00–12′S/47°11–18′W	Cap[2] (1–8), PR,[4,5] CAP[5], URU[5]
	Para	Belem, Embrapa	01°25′S/48°77′W	Bem[2] (1, 2)
	Rondonia	Costa Marques	12°26′S/64°13′W	CMB[4]
	São Paulo	Pereira Barreto	20°46′S/51°09′W	BAR: BR[1,3] (21, 23, 25, 26, 27), Igu[2] (1, 2)
	São Paulo	Araraquara	22°05′S/48°26′W	ARA[1]: BR (28, 29)
	São Paulo	Rio Jacare Pepira (near Dourado)	22°07′S/48°18′W	Dou[2] (1–20)
French Guiana	Itacoubo	Trou-Poisson	5°26′N/53°05′W	TRP: FG[1,3,4] (1,3 14); FRG[2] (1–5)
	Maripasoula	Maripasoula	3°38′N/54°03′W	MAR[1,4]
Venezuela	Amazonas	Fortuna de Albarical, Puerto Ayacucho	05°58–59′N/67°25′–76°39′W	ALB,[1,4,5] Ven[2] (2, 4–12)
	Amazonas	San Juan de Manapiare	5°22′N/66°05′W	SJM[4]
	Amazonas	Ocamo	2°50′N/65°14′W	OCA[4]
	Bolivar	Aripao	7°20′N/65°10′W	ARI[4]
	Bolivar	Corobal, La Yuca	7°48–51′N/65°40–42′W	YUC[1,4]: COR, VZ (9, 10)
	Bolivar	Caicara	7°49′N/66°12′W	CAS[4]
	Bolivar	El Pinal	6°30′N/61°40′W	PIN[4]
	Trujillo	El Juval	9°33′N/70°39′W	ELJ,[1,5] Ven[2] (1,3)

* [1] = isozymes; [2] = random amplified polymorphic DNA–polymerase chain reaction; [3] = internal transcribed spacer 2 (ITS2); [4] = morphology; [5] = ITS2 (Danoff-Burg JA, Conn JE, unpublished data). For definitions of abbreviations, see Figure 2.

TABLE 2
Electrophoretically detected enzyme systems of *Anopheles darlingi*

Enzyme system	E.C. number*	Symbol	No. of loci†	Buffer‡
1. Adenylate kinase	2.7.4.3	AK	1	Morph
2. Aldehyde oxidase	1.2.3.1	AO	1	Morph
3. Arginine kinase	2.7.3.3	ARGK	1	TCss
4. Esterase	3.1.1.1	EST	2	Morph
5. Fumarase	4.2.1.2	FUM	1	TCss
6. Glycerol dehydrogenase	1.1.1.72	GCD	1	Morph
7. Glutamate oxaloacetate transaminase	2.6.1.1	GOT	2	Morph
8. α-Glycerophosphate dehydrogenase	1.1.1.8	GPDH	1	Morph
9. Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	G3PDH	1	Morph
10. Glutathione reductase	1.6.4.2	GR	2	TCss
11. β-Hydroxyacid dehydrogenase	1.1.1.30	HAD	1	Morph
12. Hexokinase	2.7.1.1	HK	2	Morph
13. Isocitrate dehydrogenase	1.1.1.42	IDH	2	TCss
14. Leucine amino peptidase	3.4.11.1	LAP	2	TCss
15. Malate dehydrogenase	1.1.1.37	MDH	2	Morph
16. Malic enzyme	1.1.1.40	ME	1	Morph
17. Mannose-6-phosphate isomerase	5.3.1.8	MPI	1	Morph
18. 6-Phosphogluconate dehydrogenase	1.1.1.44	6PGD	1	Morph
19. Phosphoglucose isomerase	5.3.1.9	PGI	1	TCss
20. Phosphoglucomutase	5.4.2.2	PGM	1	Morph
21. Pyruvate kinase	2.7.1.40	PK	2	Morph
22. Triose phosphate isomerase	5.3.1.1	TPI	1	TCss
23. Xanthine dehydrogenase	1.2.1.37	XDH	1	TCss
Total			31	

* Enzyme commission number.
† Number of scorable bands per phenotype.
‡ Refers to electrophoresis buffer: Morph = morpholine; TCss = Tris-citrate.

measures of genetic variability, and a test for conformance to Hardy-Weinberg equilibrium at single loci by chi-square analysis. Differentiation among the populations was measured by F-statistics. Nei's[46] unbiased genetic distances and identities and Rogers[47] genetic distances were clustered by the unweighted pair group method using arithmetic average (UPGMA) to produce the phenogram.

Random amplified polymorphic DNA–polymerase chain reaction. Experimental procedures can be found in the publications by Wilkerson and others.[34,35,48] Sixty decamer primers (Operon sets A, B, C; Operon Technologies, Alameda, CA) were screened using three individuals from a single family of *An. darlingi* from Peixoto de Azevedo (Brazil). From these, a subset of eight primers that produced consistent bands that could be scored in each of the three specimens was chosen to analyze the entire sample of 124 individuals from 13 populations (Table 1). Five of the eight gave consistent results for all specimens in the study. In contrast, 13 individuals of *An. nuneztovari* from two populations were analyzed using the same five primers. *Anopheles nuneztovari* is placed in a sister clade to the one including *An. darlingi*. The resultant data set was formatted as described by Black[49] and a 1 − S distance matrix was generated using the similarity option in the RAPDPLOT (FORTRAN programs for the analysis of RAPD-PCR markers in populations; Black IV, WC, Colorado State University, Fort Collins, CO) program. The formula is derived from the Nei and Li[50] similarity index: $S = 2N_{AB}/(N_A + N_B)$ where N_{AB} are the fragments that two individuals share in common and N_A and N_B are the number of fragments in individuals A and B, respectively. The matrix was analyzed in PHYLIP 3.5C (Phylogeny inference package; Felsenstein J, University of Washington, Seattle, WA) using the NEIGHBOR program with the UPGMA option and a phenogram was produced with DRAWGRAM, also in PHYLIP 3.5C RAPDBOOT[51] was used to generate 100 pseudoreplicate distance matrices, which were collapsed to form 100 trees with UPGMA. The bootstrap consensus tree was derived from the 100 UPGMA trees with the CONSENSUS program in PHYLIP 3.5C.

Sequencing of ITS2. Total DNA was extracted from specimens using the extraction technique of Collins and others.[52] The PCR temperature profile consisted of 37 cycles of 1 min at 95°C, 1 min at 45°C, and 1.5 min at 72°C. Primers specific for the ITS2 region[53] annealed to the conserved regions of the 5.8S and 28S ribosomal subunits that flank this region. All 13 specimens were manually sequenced and representatives from each of the 12 sites were resequenced using an ABI 373 automated sequencer using the ABI PRISM Dye terminator cycle sequencing kit (Perkin Elmer, Norwalk, CT). Contig assembly and related sequence analysis was performed using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI). The GenBank accession number of the ITS2 for *An. darlingi* populations from South America is U92337, and the number for populations from Belize is AF051274 (Danoff-Burg JA, Conn JE, unpublished data).

Morphology. Selected wing, leg, and head characteristics were measured and analyzed on 305 adult females of *An. darlingi* from 17 populations (Figure 2 and Table 1) using a dissecting microscope. The head characteristic was the length of the proboscis and those of the wings and legs are illustrated in Figure 3A and B, respectively. The morphologic terms and abbreviations used in this figure follow those of Harbach and Knight,[54,55] Wilkerson and Peyton,[56] and Peyton (unpublished data). The Program Statistica[57] was used for statistical analysis among populations with $N \geq 10$. To measure the closeness of *An. darlingi* populations based on

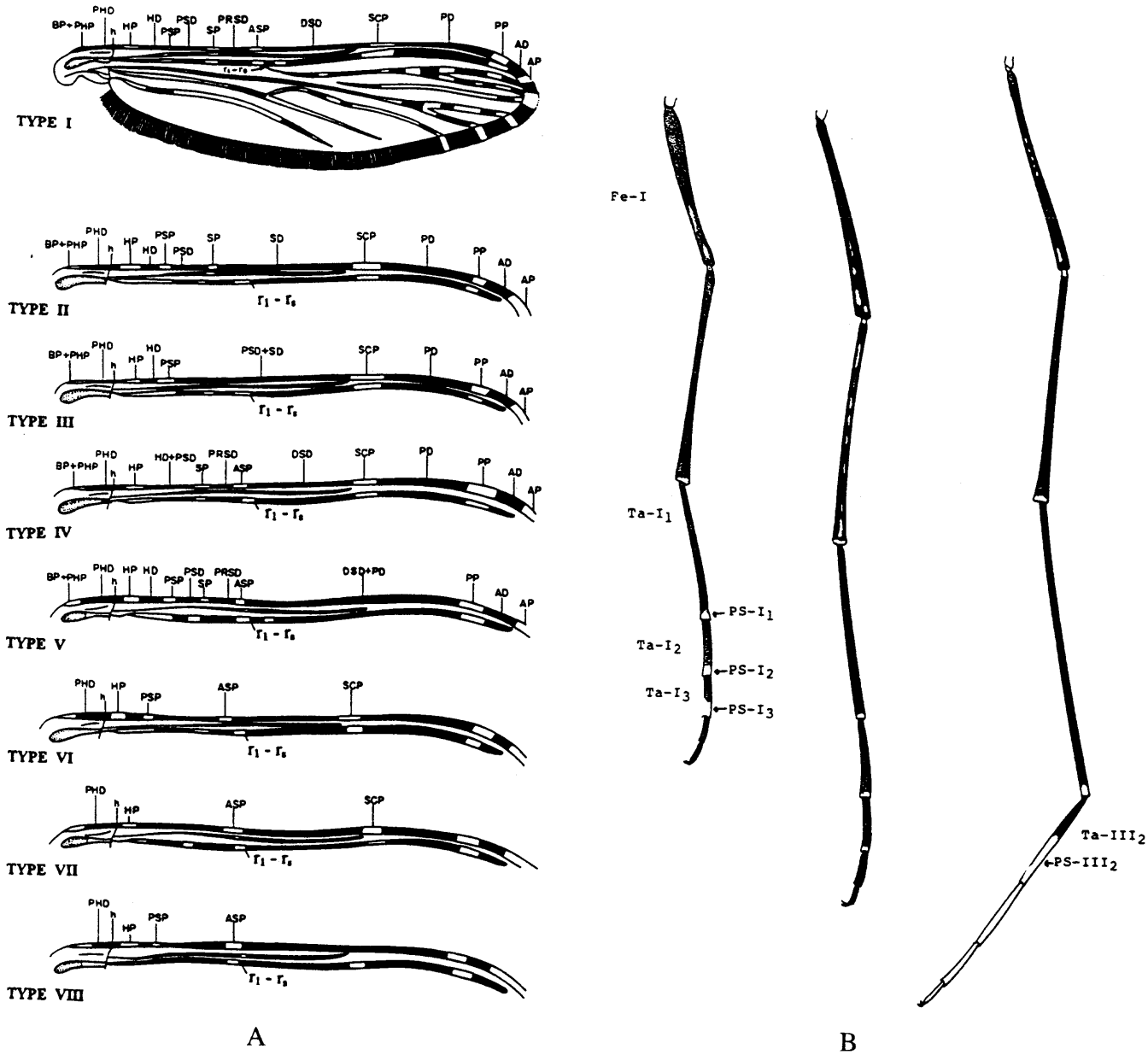

FIGURE 3. Morphologic wing and leg characteristics of *Anopheles darlingi*. **A,** wing types based on patterns of dark and pale spots on the costa. BP + PHP = basal pale + prehumeral pale; PHD = prehumeral dark; h = humeral crossvein; HP = humeral pale; HD = humeral dark; PSP = presector pale; PSD = presector dark; SP = sector pale; SD = sector dark; PRSD = proximal sector dark; ASP = accessory sector pale; DSD = distal sector dark; SCP = subcostal pale; PD= preapical dark; PP = preapical pale; AD = apical dark; AP = apical pale (wing spot); $r_1 - r_s$ = radius one and cell minus radial sector. **B,** legs showing the characteristics measured. Fe-I = forefemur; Ta-I_1 = foretarsomere 1; pale spot (PS-I_1) on Ta-I_1; Ta-I_2 = foretarsomere 2; pale spot (PS-I_2) on Ta-I_2; Ta-I_3 = foretarsome 3; pale spot (PS-I_3) on Ta-I_3; Ta-III_2 = hindtarsomere 2; pale spot (PS-III_2) on Ta-III_2.

linear continuous characteristics such as morphologic ones, the Euclidean distances among populations were estimated by cluster analysis.[58]

RESULTS

Isozyme analysis. An isozyme comparison of 31 loci (Table 2) among the populations of *An. darlingi* indicated that mean heterozygosity ranged from 0.063 to 0.122 (Table 3) with a mean ± SE of 0.1 ± 0.023 across all populations.

The F-statistic (F_{ST}), a measure of the amount of differentiation among subpopulations, showed a low mean value of 0.102 and the mean index of fixation of individuals relative to the total of subpopulations (F_{IS}) had a moderate mean value of 0.039. Three F_{ST} values with moderate differentiation, were found for glutamate oxaloacetate transaminase-1 (*Got-1*) (0.170), isocitrate dehydrogenase-1 (*Idh-1*) (0.170), and phosphoglucomutase (*Pgm*) (0.204).

There were five different alleles for *Got-1*. Populations of *An. darlingi* from São Paulo (Brazil) had a 62% frequency for allele 100 and 37% for allele 85, whereas all the other populations had a frequency more than 80% for allele 100

TABLE 3

Measures of genetic variatoin of *Anopheles darlingi* (1–7), *An. pseudopunctipennis* (8), and *An. argyritarsis* (9)*

Populations	No. of samples	Mean sample size/locus	Mean no. of alleles/locus	% Polymorphic loci†	Mean heterozygosity	
					Direct-count	HdyWbg expected‡
1. Belize	4	109.4	2.0	16.1	0.063	0.065
		(1.2)	(0.2)		(0.020)	(0.020)
2. Amazonas, Brazil	4	99.6	2.5	45.2	0.101	0.104
		(1.2)	(0.2)		(0.018)	(0.019)
3. São Paulo, Brazil	3	93.4	2.6	48.4	0.122	0.130
		(1.2)	(0.2)		(0.023)	(0.025)
4. Mato Grosso, Brazil	1	18.0	1.9	48.4	0.122	0.128
		(0.0)	(0.2)		(0.029)	(0.029)
5. Venezuela	3	104.7	2.7	35.5	0.098	0.102
		(0.9)	(0.2)		(0.024)	(0.024)
6. French-Guiana	2	93.5	2.4	19.4	0.079	0.084
		(0.6)	(0.2)		(0.021)	(0.023)
7. Bolivia	3	23.2	2.0	45.2	0.117	0.125
		(0.5)	(0.2)		(0.025)	(0.026)
8. *An. pseudopunctipennis*	1	5.8	1.3	29.0	0.081	0.088
		(0.1)	(0.1)		(0.028)	(0.029)
9. *An. argyritarsis*	1	6.0	1.2	19.4	0.048	0.051
		(0.0)	(0.1)		(0.025)	(0.021)

* Values in parentheses are standard errors.
† A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.99.
‡ Unbiased estimate. HdyWbg = Hardy-Weinberg equilibrium.

(Table 4). In the case of *Idh-1,* which has four alleles, all populations displayed a frequency greater than 72% for allele 100, except the ones from Belize, which had a 40% frequency for allele 100 and 59% for allele 86. The third locus is *Pgm,* which had five alleles. The Venezuelan populations had a frequency of 70% for allele 80 and 28% for allele 100, whereas all the other populations had a frequency greater than 69% for allele 100.

Regardless of the allele frequency differences, Nei's index of genetic identity (above the diagonal) indicated a high degree of similarity among all populations, with values greater than 0.975 (Table 5). The phenogram illustrated that *An. darlingi* populations from Brazil and Bolivia had high genetic identities (> 0.99). Then, by decreasing order of similarity, populations from French Guiana, Belize, and finally Venezuela followed with a genetic identity of 0.981 (Figure 4A).

The comparison of *An. darlingi* populations with two other anopheline species showed that with *An. (Nys.) argyritarsis,* the genetic distance was 0.331, and with *An. (An.) pseudopunctipennis,* the genetic identity was 0.149 (Figure 4B). This comparison emphasizes the genetic homogeneity found within the *An. darlingi* populations studied. The population of the variant *paulistensis* presented no significant differences compared with the other *An. darlingi* populations.

Random amplified polymorphic DNA–polymerase chain reaction. The five primers used in this study produced 35 bands that could be scored in *An. darlingi* and 17 in *An. nuneztovari,* including four shared bands. These bands ranged in size from 0.350 to 2.323 kilobasepairs (Table 6). All individuals of *An. darlingi* clustered together, as did all *An. nuneztovari,* each with 100% bootstrap support (Figure 5). Within both species, there was some evidence of geographic partitioning of most populations. Samples of *An. darlingi* were partitioned into four main clusters (Figure 5). Cluster I comprises most (23 of 28) of the Peixoto de Azevedo individuals (central Brazil); Cluster II includes samples from Bolivia, many parts of Brazil, French Guiana, and Venezuela; Cluster III contains many (16 of 20) of the Rio Jacare Pepira individuals (southern Brazil); and Cluster IV contains most (9 of 10) of the Belize specimens. Genetic distances separating all clusters of *An. darlingi* were small in comparison with those separating the two clusters of *An. nuneztovari,* which included samples from central Brazil (Peixoto de Azevedo) and northeastern Brazil (Belem).

Sequencing of ITS2. Based on the characterization of the ITS2 region of *An. darlingi,* no variation was observed among the 406 basepairs of the ITS2 region of the nine individuals that were sequenced in the present study from South America, including Brazil (Ituxi River and Pereira Barreto) and French Guiana (Trou-Poisson and Maripasoula). However, the four individuals from Belize all had an identical small deletion of CCC (Figure 6). The three basepair differences between populations from Belize and South America represents a value of 0.74% intraspecific variation in the ITS2. When relationships among the taxa were reconstructed using only ITS2, the *An. darlingi* populations from South America and Belize were a monophyletic taxon that was not allied with any other species and included in a basal unresolved polytomy.

Morphology. The morphologic study showed wide variation in the pattern of pale and dark spots on wing costa, making it possible to differentiate eight classes of wing patterns (Figure 3A). Costal spots are characteristic for the subgenus *Nyssorhynchus,*[56] in which the basal dark spot (BD) is absent and the basal pale (BP) and prehumeral pale (PHP) are fused (BP plus PHP). In Type I, the accessory sector pale (ASP) is present and divides the sector dark (SD) into proximal sector dark (PRSD) and distal sector dark (DSD);[59] in Type II, the absence of the ASP resulted in the fusion of the PRSD and DSD, which become the SD; in Type III, the SP and ASP are absent; in Type IV, the presector pale (PSP) is absent; in Type V, the subcostal pale (SCP) is absent; thus, the DSD and PD are fused; in Type VI, the SP is absent; in

TABLE 4
Relative allele frequencies for three loci of *Anopheles darlingi**

Locus†	Allele‡	Belize	Brazil			Venezuela	French Guiana	Bolivia
			Amazonas	São Paulo	Mato Grosso			
Got-1	n	114	103	99	18	107	95	24
	117	—	—	—	—	0.009	—	—
	112	—	—	0.01	0.056	0.023	0.021	—
	100	**0.982**	**0.981**	**0.616**	**0.944**	**0.808**	**0.968**	**0.958**
	85	0.018	0.015	**0.374**	—	0.056	—	0.042
	70	—	0.005	—	—	0.103	0.011	—
	(H)	0.035	0.039	0.343	0.111	0.346	0.063	0.083
Idh-1	n	102	102	99	18	107	95	24
	115	0.02	0.029	0.056	0.111	0.084	0.137	0.042
	100	0.392	**0.936**	**0.889**	**0.722**	**0.752**	**0.805**	**0.896**
	86	**0.588**	0.034	0.051	0.167	0.164	0.058	0.063
	72	—	—	0.005	—	—	—	—
	(H)	0.52	0.127	0.222	0.556	0.439	0.326	0.208
Pgm	n	111	105	101	18	108	97	24
	120	—	—	0.005	0.028	—	—	0.021
	115	—	0.095	0.089	0.083	0.009	0.026	0.167
	100	**0.797**	**0.719**	**0.851**	**0.694**	0.282	**0.964**	**0.729**
	80	0.203	0.181	0.05	0.194	**0.704**	0.01	0.083
	64	—	0.005	0.005	—	0.005	—	—
	(H)	0.315	0.343	0.277	0.5	0.426	0.072	0.458

* Dashes represent 0; **bold numbers** represent the allele frequencies discussed in the Results.
† *Got-1* = glutamate oxaloacetate transaminase; *Idh-1* = isocitrate dehydrogenase; *Pgm* = phosphoglucomutase.
‡ n = number of specimens; h = heterozygosity (direct-count) per locus.

Type VII, the PSP and SP are absent; and in type VIII, the SP and SCP are absent.

The observed frequency distribution of wing types in the different populations analyzed varied significantly and are population dependent ($\chi^2 = 161.34$, $P = 0.0016$). In general, Type I was the most frequent type of wing followed by Type VI. In the Belize population, only Type I and II were present, while Type II was absent from the populations of French Guiana, Brazil, and Bolivia. The Venezuela populations of Caicara and Albarical showed up to six different types of wings.

Analysis of variance (ANOVA) of the proportions of the length of each costal wing spot divided by the length of the wing for the 13 populations showed that the proportion prehumeral dark/humeral pale (PHD/HP) (Figure 3A) is a valid diagnostic characteristic for *An. darlingi* ($F = 78.1361$, $P = 0.000001$) and can be used as a marker to differentiate this species from other morphologically similar ones such as *An. marajoara, An. braziliensis, An. argyritarsis,* and *An. albitarsis.*

The highest Euclidean distance (ED) was observed between the Belize and Bolivia populations (0.14). The Bolivian population is separated from the others by a distance of 0.08. The Brazil populations of Costa Marques and Pereira Barreto are similar to each other (ED = 0.04) and to the Venezuela populations. The population from Colombia located west of the Andes Mountains is very close (ED = 0.02) to the population of San Juan de Manapiare, located in the Venezuelan Amazon, east of the Andes (Figure 2).

Analysis of the morphology of the proboscis and legs resulted in valid diagnostic characteristics that could separate *An. darlingi* populations such as proboscis (P) and forefemur (P/Fe-I), pale spot on foretarsomere ($PS\text{-}I_1/Ta\text{-}I_1$, $PS\text{-}I_2/Ta\text{-}I_2$, $PS\text{-}I_3/Ta\text{-}I_3$) and on hindtarsomere ($PS\text{-}III_2/Ta\text{-}III_2$) (Figure 3B). Galvão and others[11] pointed out that the variant *paulistensis* had more dark scales on the hindtarsomere 2 than the *An. darlingi* described by Root. Nevertheless, for this characteristic, we found that the Pereira Barreto population (*paulistensis* type locality) was similar to the Bolivian population. The ANOVA of these proportions for *An. darlingi, An. marajoara, An. braziliensis, An. argyritarsis,* and *An. albitarsis* was significantly different ($P < 0.0001$), and the best

TABLE 5
Matrix of genetic distance of *Anopheles darlingi* (1–7), *An. pseudopunctipennis* (8) and *An. argyritarsis* (9)*

Population	1	2	3	4	5	6	7	8	9
1. Belize	****	0.987	0.978	0.988	0.983	0.985	0.980	0.114	0.307
2. Amazonas, Brazil	0.014	****	0.992	0.995	0.987	0.992	0.993	0.134	0.334
3. São Paulo, Brazil	0.022	0.008	****	0.990	0.976	0.984	0.992	0.131	0.341
4. Mato Grosso, Brazil	0.012	0.005	0.010	****	0.986	0.988	0.991	0.125	0.337
5. Venezuela	0.017	0.013	0.024	0.014	****	0.979	0.979	0.124	0.322
6. French Guiana	0.015	0.008	0.016	0.012	0.022	****	0.985	0.126	0.313
7. Bolivia	0.020	0.007	0.008	0.009	0.021	0.015	****	0.118	0.367
8. *An. aygyritarsis*	1.181	1.098	1.076	1.087	1.134	1.161	1.003	****	0.324
9. *An. pseudopunctipennis*	2.172	2.007	2.032	2.081	2.089	2.069	2.140	1.128	****

* Values above the diagonal are Nei's unbiased[34] genetic identities; values below the diagonal are Nei's unbiased[33] genetic distances.

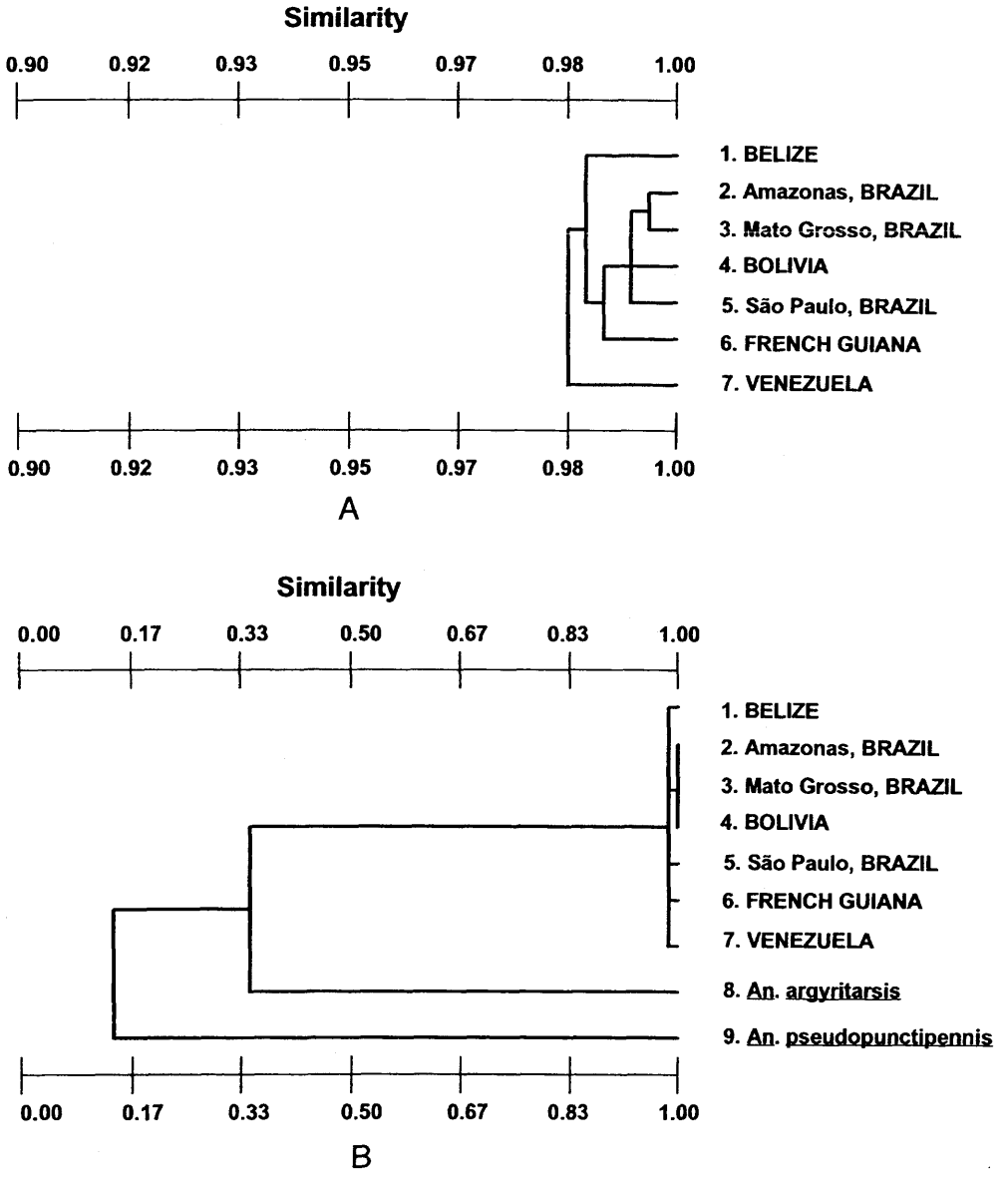

FIGURE 4. Unweighted phenogram from Nei's[46] unbiased genetic distance matrix. **A,** for all *Anopheles darlingi* populations (cophenetic correlation = 0.815). **B,** for all *An. darlingi* populations, *An. pseudopunctipennis,* and *An. argyritarsis* (cophenetic correlation = 0.983).

TABLE 6

Approximate sizes of random amplified polymorphic DNA (RAPD) fragments used in comparison of *Anopheles darlingi* populations and comparison of *An. darlingi* with *An. nuneztovari*

Primer	Size of RAPD fragments (kilobasepairs)*
B12	1.569, 1.501†, 1.226, 1.140, 1.135, 0.940, 0.911, 0.892†, 0.888, 0.861†, 0.705† 0.675, 0.552, 0.439‡, 0.423†
A09	1.888, 1.593, 1.323‡*, 1.071, 0.872, 0.817†, 0.781, 0.449, 0.432‡, 0.350
B04	1.150‡, 0.760, 0.707, 0.468†, 0.429, 0.422†, 0.409, 0.358
A07	2.322†, 1.721†, 1.489, 1.297, 1.152, 1.045†, 0.976†, 0.790, 0.735, 0.636, 0.589, 0.509
C18	1.935‡, 1.252, 0.718

* Occurrence of fragments: no annotation = *An. darlingi.*
† *An. nuneztovari.*
‡ Both species.

diagnostic characteristic to separate all four species is the proportion PS-III_2/Ta-III_2. This characteristic was previously used by Linthicum[3] to separate *An. albitarsis* and *An. marajoara* only.

DISCUSSION

Based on the isozyme analysis, the mean ± SE heterozygosity of 0.100 ± 0.023 for *An. darlingi* (index that reflects the genetic variability) was similar to the average value found in other Diptera (0.115 ± 0.009).[60] This species showed a higher genetic variability than the *An. pseudopunctipennis* populations (0.059 ± 0.020) studied by Manguin and others.[42] However, lower genetic variability was found for the Belize populations (0.063) compared with South American populations (0.079–0.122, with a mean ± SE of 0.107 ± 0.017). Belize and other countries of Central

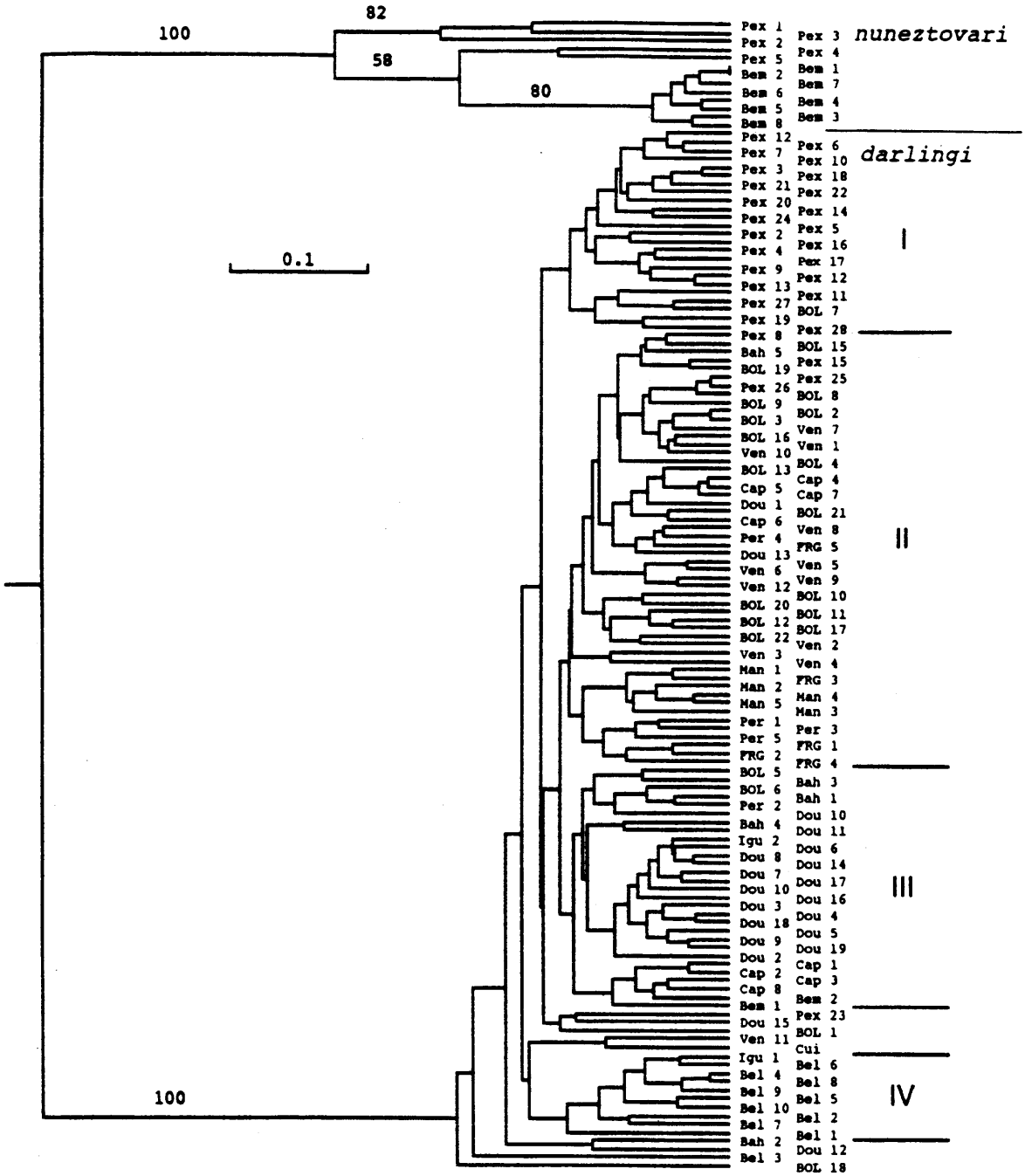

FIGURE 5. Phenogram showing relationships among 124 individuals of *Anopheles darlingi* and 13 *An. nuneztovari*. Branch lengths are proportional to 1 − S, and the scale bar is equal to 0.1. On selected branches, the bootstrap value of 100 repetitions is shown. The roman numerals indicate the four primary clusters.

America are part of a rather narrow and restricted land mass that is scored by many short, parallel river systems. As a consequence, populations of *An. darlingi* from Belize, and more globally from Central America, have a restricted distribution compared with the large land mass and extensive river systems occurring in South America. It may be that the loss of population heterozygosity in Belize reflects the normally expected loss of polymorphic alleles in populations that are confined to restricted and isolated environments. In other words, the gene flow of *An. darlingi* populations in Central America occurs in a more reduced area than in South America, causing a bottleneck effect that resulted in less polymorphic populations. Our hypothesis is that a northern extension of populations from South America established *An. darlingi* populations in Central America. It may also be that the modern discontinuous geographic distribution of this species occurred secondarily to the original northward extension of *An. darlingi* populations.

Overall genetic identity within *An. darlingi* populations was high (0.976–0.995), and these identities are markedly

```
A. darlingi SA CAGTGCGAGG TACACATTTT TGAGTGCCCA CATTCACCGC AGAACCAACT  50
A. darlingi BE .......... .......... .......... .......... ..........  50

A. darlingi SA AGCGAGGTCG TGCCGCCGCC GGTCAGCCGG CCGGTTAGCT GGCTACTGAT 100
A. darlingi BE .......... .......... .......... .......... .......... 100

A. darlingi SA GATTTGATTG ACGCGCCGCG TCCCCAACCG GACGCGCCCG TGTGTGGTCA 150
A. darlingi BE .......... .......... .---...... .......... .......... 150

A. darlingi SA AGCATTGAAG GACTGTGGCG TGGTGGGTGC ACCGTGTGTC GTTGCTTAAT 200
A. darlingi BE .......... .......... .......... .......... .......... 200

A. darlingi SA ACGCGACCCT CTCTCCGGTT TCACATCTGG AGCGGGCTAT CCAGTCACAA 250
A. darlingi BE .......... .......... .......... .......... .......... 250

A. darlingi SA TCCCCAGCGA AATGTGCCGA TACACGGGTA GCCCCGATGT GGAGATCCAA 300
A. darlingi BE .......... .......... .......... .......... .......... 300

A. darlingi SA GCGAGGACCT CCCTCAAAAC CATTGTGATG AAACCCCACC ACACAAGAGA 350
A. darlingi BE .......... .......... .......... .......... .......... 350

A. darlingi SA GAAGAGAGAG AGCGACCAAA AGCAACGTTC GCACGCGCTG TCAGCTCATC 400
A. darlingi BE .......... .......... .......... .......... .......... 400

A. darlingi SA GAGCGCGCAC ACGGATCTAG  GAACTAGGAT CTCAAGTGGG CCTCAATATG 450
A. darlingi BE .......... .......... .......... .......... .......... 450

A. darlingi SA TGTGACTACC CCTA                                        500
A. darlingi BE .......... ....                                        500
```

FIGURE 6. Sequence of 34 nucleotides of the 5.8S region, the entire 406 nucleotides of the internal transcribed spacer 2 (ITS2) region, and 24 nucleotides of the 28S region for *Anopheles darlingi.* The **arrowheads** represent the first and last sequences of the ITS2 region. SA = South America; BE = Belize, which differs only by a small three-base deletion (CCC) at position 179-181. The sequence has been deposited in GenBank (accession number U92337).

higher than the value of 0.85, suggested by Avise[61] as the lower limit for conspecific populations. For instance, in other anopheline species, values ranged from 0.84 to 0.88 between both species, *An. gambiae s.s.* and *An arabiensis,*[62–64] and from 0.92 to 1.0 among *An. pseudopunctipennis* populations.[42] In the study of Steiner and others,[30] an *An. darlingi* population from southern Brazil had values ranging between 0.592 and 0.772, suggesting the existence of more than one species. In comparison with another study,[18] genetic identity of southern and northern Brazil populations has been found to be much higher, with a value of 0.952 within the range of intrapopulational variation. Similarly, the range of our genetic identity values for all the Brazil populations was both narrower and higher (0.976–0.992) than the former one.

Although some moderate differentiation was found in three loci (*Got-1, Idh-1,* and *Pgm*), the mean F_{ST} values showed negligible differentiation among *An. darlingi* and no fixed differences were found. In addition, the moderate mean F_{IS}, with a value of 0.039, suggests that random mating among the populations of *An. darlingi* is occurring.[65] Comparison of the isozyme analysis between *An. darlingi* and *An. pseudopunctipennis*[42] showed a higher homogeneity among populations of the former species, with F_{ST} values of 0.102 and 0.375, and Nei genetic identities ranging between 0.976 and 0.995, and 0.924 and 1.0, respectively. In the case of *An. darlingi,* there were no clear groupings of populations as found for *An. pseudopunctipennis.*

Our RAPD-PCR results support the conclusion that the widespread sampled populations of *An. darlingi* are conspecific. In addition to high similarity values among the populations, comparisons with *An. nuneztovari* demonstrated the level of differentiation expected between species. Of the 35 markers used in this analysis, seven are monomorphic, i.e., occur in > 95% of the individuals tested, and two of these occur in all individuals. Wilkerson and others[34,35] found similar numbers of correlated diagnostic markers in each of the four species of the Albitarsis complex (3–7) using the same 60 RAPD primers as in the present study.

Our analysis produced four large and three small clusters, indicative of geographic partitioning. Since *An. darlingi* clusters usually include individuals from widely separated populations, e.g., cluster III includes individuals from central and northern Brazil and Bolivia, the case can also be made that the level of mixis implied by the clustering would likely be sufficient to ensure panmixis (random mating).

Interestingly, the two clusters in the RAPD-PCR data for *An. nuneztovari* (Belém, northeastern Brazil and Peixoto, central Brazil) are similar to the distinctive mitochondrial DNA lineages of 12 *An. nuneztovari* populations in Belém and central Amazonian Brazil (Manaus and Boa Vista),[66] underscoring some congruence, in this case, between two different molecular markers.

Intraspecific variation in the ITS2 region of 21 members of the *Nyssorhynchus* subgenus varies from 0% to 2.8%. Therefore, the value of 0.74% for *An. darlingi* represents a low value for intraspecific variation of the subgenus. Most of this variation is in the form of insertion-deletions involving a few base pairs (as is the case for *An. darlingi* from Belize in the present study) or single base point mutations. The identical ITS sequences across all of South America suggest a single panmictic breeding population.

We conclude that samples from Mexico, as well as other Central American populations and those from Colombia, would have to be sequenced to determine whether the small deletion in the Belize population represents isolation by distance, immigration, or extinction events. Isolation by distance could result from Belize populations being near the northern end of a north-south cline or could be due to immigration, or extinction events could be related to the reported discontinuity of *An. darlingi* populations in Central

America. Both distance explanations seem to be relatively weak since there is no evidence of clinal variation (for example, the ITS2 sequence is identical between El Juval in western Venezuela and São Paulo). If the Belize populations had been independently evolving for a long period of time due to an historical vicariant (of different origin) event, variance in the ITS2 from Belize compared with populations in South America should be greater. Therefore, we suggest that the small deletion in the Belize populations of *An. darlingi* is most likely the result of a recent introduction event caused either by human action or some other extrinsic force. Future studies could test this hypothesis by determining which South American populations have the greatest similarity with the Belize populations and whether other Central American and Mexican samples are more similar to populations from Belize or from South America.

The study of head, legs, and wings of 17 populations of *An. darlingi* showed wide variation in morphologic characteristics. On the wing costa, eight different patterns were described based on the presence or absence of one or more spots. Geographic variations in the pattern of the wing costa have been reported for other *Nyssorhynchus* such as *An. albitarsis*[67] and *An. nuneztovari.*[68] The Euclidean distances separating groupings of diagnostic wing characters suggest that gene flow occurs among populations across all geographic locations of populations included in these analyses. A previous study[69] on Brazil populations showed that the variations were intraspecific "because no patterns were population specific".

Based on isozyme, RAPD-PCR, ITS2 sequence, and morphologic analyses, all *An. darlingi* populations collected from most parts of the species' range in Central and South America demonstrated a genetic similarity that is consistent with the existence of a single species. While some genetic and morphologic variations were found among the populations surveyed, no complete barriers to gene flow were identified. The relative homogeneity of *An. darlingi* populations might indicate that the isolated population of Central America could have originated from South America. As presently defined, the limited range of *An. darlingi* in Central America suggests a recent introduction of the species. This limited distribution also raises the possibility that the species could, over time, expand into or reinvade more Central American countries and more states in southern Mexico. The former hypothesis could be tested using microsatellites, RAPD-PCR, or ITS2 sequences for the characterization of *An. darlingi* populations throughout southern Mexico, Central and South America to determine more precisely the phylogeny of the species.

Acknowledgments: We thank the following individuals for assistance with the field collections: in Belize: S. Hakre and L. Reyes (Epidemiology Research Lab, Belize City); in Bolivia: J. Cespedes, J. Cuba, C. Toffen, and A. Melgar (Unidad Sanitaria, Riberalta); in Brazil: W. A. Alecrim (Instituto de Medecina Tropical, Manaus), W. Tadei (Instituto Nacional de Pesquisas da Amazonia, Manaus), R. Lourenço-de-Oliveira and D. Couto Lima (Fundacao Oswaldo Cruz, Rio de Janeiro), O. P. Forattini and M. A. Mureb Sallum (Universidade de São Paulo), L. C. Meneguetti, F. de Azevedo Corréa, J. E. Bracco, and E. Bergo (Superintendência de Controle de Endemias, São Paulo), A. Cruz Marques, J. Fonseca Sandoval, F. dos Santos, R. Da Luz Lacerda, A. Wanderley, and G. Calderón (Fundacao Nacional da Saude), M. Marins Povoa (Instituto Evandro Chagas, Belém); in Colombia: W. Rojas and S. Zuloaga (Cooperacion para Investigaciones Biologicas, Medellin), H. Polo (Servicio de Eradicacion de Malaria, Chocó); in French Guiana: J.-M. Reynes (Pasteur Institute, Cayenne); and in Venezuela: J. Scorza, E. Rojas, and E. Brown (Universidade de los Andes, Trujillo); J. Mora, J. Espana, J. Aguilar, and C. Menaré (Dirección de Malariología y Saneamiento Ambiental, Maracay), and H. Guzmán and J. Manuel Arcia (Escuela de Malariology y Saneamiento Ambiente, Maracay). We also thank R. E. Harbach who helped in collecting specimens in São Paulo State (Brazil). We are grateful to E. L. Peyton, who provided some voucher specimens of the Smithsonian Institution and for most valuable discussions. Special thanks are due to W. C. Black IV for helping with the RAPD-PCR analysis and to A. Gutierrez for the statistical analysis of the morphologic data.

Financial support: This research was sponsored by Uniformed Services University of the Health Sciences (USUHS) grant R087EL to Sylvie Manguin, in part by National Aeronautics and Space Administration (NASA) grant W-16306 for the isozyme study, by NIH grant AI-31034 to L. P. Lounibos (Florida Medical Entomology Laboratory, Vero Beach, FL) and NIH grant AI-40116 to Jan E. Conn for the ITS2 sequencing, by Consejo Nacional de Investigaciones Cientificas y Tecnologicas (MPS-RPIV-130032-9), and the Pan American Health Organization for the morphologic study.

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