

## POPULATION STRUCTURE OF THE MALARIA VECTOR *ANOPHELES DARLINGI* IN A MALARIA-ENDEMIC REGION OF EASTERN AMAZONIAN BRAZIL

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**Abstract.** *Anopheles darlingi* is the primary malaria vector in Latin America, and is especially important in Amazonian Brazil. Historically, control efforts have been focused on indoor house spraying using a variety of insecticides, but since the mid-1990s there has been a shift to patient treatment and focal insecticide fogging. *Anopheles darlingi* was believed to have been significantly reduced in a gold-mining community, Peixoto de Azevedo (in Mato Grosso State), in the early 1990s by insecticide use during a severe malaria epidemic. In contrast, although *An. darlingi* was eradicated from some districts of the city of Belem (the capital of Para State) in 1968 to reduce malaria, populations around the water protection area in the eastern district were treated only briefly. To investigate the population structure of *An. darlingi* including evidence for a population bottleneck in Peixoto, we analyzed eight microsatellite loci of 256 individuals from seven locations in Brazil: three in Amapa State, three in Para State, and one in Mato Grosso State. Allelic diversity and mean expected heterozygosity were high for all populations (mean number alleles/locus and  $H_E$  were 13.5 and 0.834, respectively) and did not differ significantly between locations. Significant heterozygote deficits were associated with linkage disequilibrium, most likely due to either the Wahlund effect or selection. We found no evidence for a population bottleneck in Peixoto, possibly because the reduction was not extreme enough to be detected. Overall estimates of long-term  $N_e$  varied from 92.4 individuals under the linkage disequilibrium model to  $\infty$  under the heterozygote excess model. Fixation indices and analysis of molecular variance demonstrated significant differentiation between locations north and south of the Amazon River, suggesting a degree of genetic isolation between them, attributed to isolation by distance.

### INTRODUCTION

The distribution of *Anopheles darlingi* within the Amazon Basin, where its primary importance in malaria transmission is well-documented,<sup>1–3</sup> appears to be continuous, with no obvious barriers that can isolate populations. A similar situation is found in *An. gambiae* in sub-Saharan Africa, except for the Rift Valley complex.<sup>4</sup> For both species, the isolation by distance (IBD) model,<sup>5</sup> which could promote genetic divergence<sup>6</sup> and ultimately speciation, seems a reasonable one (however, see Donnelly and others<sup>7</sup>). The IBD has also been proposed to explain genetic differentiation in the African malaria vector *An. funestus*.<sup>8</sup> However, a study comparing mitochondrial DNA (mtDNA) sequences and microsatellite loci of samples of another malaria vector, *An. albimanus*, from throughout Latin America found a weak IBD only in the microsatellite loci.<sup>9</sup> Support for this model for *An. darlingi* is mixed. The IBD was detected in samples from Venezuela, Brazil, and Bolivia using mtDNA restriction fragment length polymorphism (RFLP) data<sup>10</sup> and could be a factor influencing the 4–5% sequence divergence noted in the ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) in a sample from southeastern Brazil compared with four samples from Amazonian and northeastern Brazil.<sup>11</sup> Conversely, a recent analysis of 19 localities spanning much of the range of *An. darlingi* (southern Mexico to southern Brazil) using partial sequence from the mtDNA cytochrome oxidase subunit I (COI) gene, detected only a weak association between geographic and genetic distances, and did not fit a simple model of IBD.<sup>12</sup>

Intensive use of insecticides, most commonly on the inside walls of houses in the Amazon Basin to control *An. darlingi*,<sup>13</sup> may have resulted in a population bottleneck, and is believed to have caused behavioral changes from endophagy (indoor feeding)<sup>1,14</sup> to exophagy (outdoor feeding) at least in some Amazonian regions.<sup>2,15–18</sup> Of additional interest is the fact that *An. darlingi* was reported to have been eradicated from several districts of the Amazon city of Belem in 1968 after an intensive campaign, although it was detected there again in some districts in the mid 1990s.<sup>19</sup> The water protection area in the district of Entrocamento, Belem, to the east, which consists of forest surrounding two large lakes, was treated with insecticide during the 1990s only briefly and sparingly when malaria cases were detected in an illegal settlement that was soon dismantled.

Mato Grosso State has had a history of gold mining since the colonial period.<sup>20</sup> Typically, manual gold miners in Amazonian Brazil are vulnerable migrants who work in inaccessible, underdeveloped areas under poor conditions with little or no protection from vectors that may transmit parasitic diseases, particularly malaria.<sup>21</sup> Interestingly, such alluvial gold deposits are often located in habitats preferred by the primary malaria vector *An. darlingi*, and intensive malaria transmission is frequently associated with small-scale gold-mining.<sup>21</sup> Peixoto de Azevedo, one of our sample localities, was founded in 1986 because gold was discovered in the area,<sup>18</sup> and malaria prevalence as high as 20.5% was recorded.<sup>22</sup> Because insecticides were used extensively to control *An. darlingi* during a malaria epidemic in Peixoto de Azevedo that peaked in 1992,<sup>20</sup> we anticipated the possibility of detecting the signature of a bottleneck in this sample.

The focus of the present study was to use microsatellite markers<sup>23</sup> to assess population structure in *An. darlingi* and to look for the signature of a population bottleneck in an area of

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Brazil known to have had a history of insecticide use.<sup>13,24</sup> We used markers that are particularly sensitive to population differentiation.<sup>4</sup>

## METHODS

**Collections.** Localities (Figure 1) from Amapa State included Granja Alves (GA; 0°02'S, 51°05'W) and Lagoa dos Indios (LI; 0°02'S, 51°11'W), both on the outskirts of the capital city of Macapa, and Santana (STN; 0°01'S, 51°09'W), slightly southwest of Macapa. Localities in Para State included the district of Entrocamento in the capital city of Belem (BEL; 1°41'S, 48°40'W), Aracanga (ARA; 1°37'S, 48°36'W), approximately 12 km east of Belem, and Moju (MOJ; 1°52'S, 48°45'W), southwest of Belem. Peixoto de Azevedo (PEX; 10°23'S, 54°54'W) is in northern Mato Grosso State, just south of the border with Para State. In all sites, adult female *An. darlingi* were collected outdoors in 1997–1998 either resting in vegetation near houses or on the outer walls of houses, at cattle or water buffalo corrals, or by landing catches between approximately 6:30 PM and 8:30 PM, identified morphologically within 24 hours,<sup>25</sup> and stored in 95% ethanol until use. The landing catch protocol was reviewed and approved by the Institutional Review Boards at the University of Vermont and The New York State Department of Health and the Biosafety Committee of the Instituto Evandro Chagas in Belem, Brazil. The Walter Reed Army Institute for Research Human Use Review Committee reviewed and approved it as a minimal risk protocol. Six of seven of the collection sites are in the lowland tropical rain

forest ecoregion of the Amazon region,<sup>26</sup> the heart of the range of *An. darlingi*.<sup>2,3</sup> Peixoto is in the savannah ecoregion and along with BEL, MOJ, and ARA are in areas where *An. darlingi* is considered the main regional vector.<sup>26</sup> In contrast, in GA, LI, and STN, *An. darlingi* abundance is relatively low compared with *An. marajoara*, which is the local primary vector.<sup>27</sup> The number of mosquitoes analyzed ranged from 25 in PEX to 44 in BEL.

**Extraction of DNA and microsatellite genotyping.** Procedures and loci for the eight microsatellite markers used for this study are as previously described.<sup>23</sup> Repeat motifs included four GA dinucleotides, two AC dinucleotides, and two GT dinucleotides.<sup>23</sup> Microsatellite alleles were amplified by polymerase chain reaction with one fluorescent-labeled primer in a PTC 100 (MJR Research, Waltham, MA) thermal cycler using the following conditions: a 5-minute denaturation at 95°C; 30 cycles for 20 seconds at 95°C, 30 seconds at 60°C, and 20 seconds at 72°C; and a final extension for 5 minutes at 72°C.<sup>23</sup> Data were then analyzed using GENOTYPER software (Applied Biosystems, Foster City, CA). An ABI 373 automated sequencer (Applied Biosystems) with an internal size standard was used for allele size determination.

**Data analysis.** Unbiased estimates of expected and observed heterozygosities were calculated using genetic data analysis.<sup>28</sup> The inbreeding coefficient  $F_{IS}$  was obtained using GENEPOP.<sup>29</sup> Deviations from Hardy-Weinberg (HW) equilibrium were estimated using exact tests<sup>30</sup> and global tests in GENEPOP version 3.4 (updated version).<sup>31</sup> Tests for linkage disequilibrium between pairs of loci were performed in ARLEQUIN version 2.0.<sup>32</sup>

We estimated genetic differentiation by calculating  $F_{ST}$ ,<sup>29</sup> and  $R_{ST}$ ,<sup>33</sup> using Fstat version 2.9.3.2<sup>34</sup> and  $R_{ST}$ Calc version 2.2<sup>35</sup> with significance determined using permutation tests. Although  $F_{ST}$  estimates under the infinite alleles model (IAM) are considered more reliable when fewer than 20 microsatellite loci are used<sup>36</sup> compared with  $R_{ST}$  under the stepwise mutation model (SMM),<sup>33</sup> we used both estimates.<sup>37</sup> To examine the distribution of genetic variation among individuals, among sample locations, and between samples from Amapa and Para States, we performed an analysis of molecular variance in ARLEQUIN version 2 using  $F_{ST}$  (IAM values) and  $R_{ST}$  (SMM values).<sup>32</sup> We used the hierarchical model for genotypic data with groups of populations and no within-individual level. We grouped PEX first with the Para samples (because it is geographically closer to them) and compared this grouping with the placement of PEX with the Amapa samples (because the fixation indices demonstrated smaller distance values between PEX and these samples). We explored isolation by distance as a possible explanation for population differentiation by the regressions of  $F_{ST}/(1 - F_{ST})$  and  $R_{ST}/(1 - R_{ST})$  against the ln of straight line geographic distance between sample pairs.<sup>38</sup> To test the significance of the regression, we used a Mantel test<sup>39</sup> with 10,000 permutations as implemented in ARLEQUIN version 2.<sup>32</sup> We also performed separate Mantel tests for populations less than 500 km apart (i.e., all except PEX), as recommended.<sup>40</sup>

To investigate loci exhibiting heterozygote excess (HE), we used the heterozygosity tests in BOTTLENECK,<sup>41</sup> which compare two estimates of expected heterozygosity,  $H_e$ , based on allele frequencies and  $H_{eq}$  based on the number of alleles and sample size. At mutation-drift equilibrium (MDE), both estimates should be equal ( $H_e = H_{eq}$ ) in a population, whereas

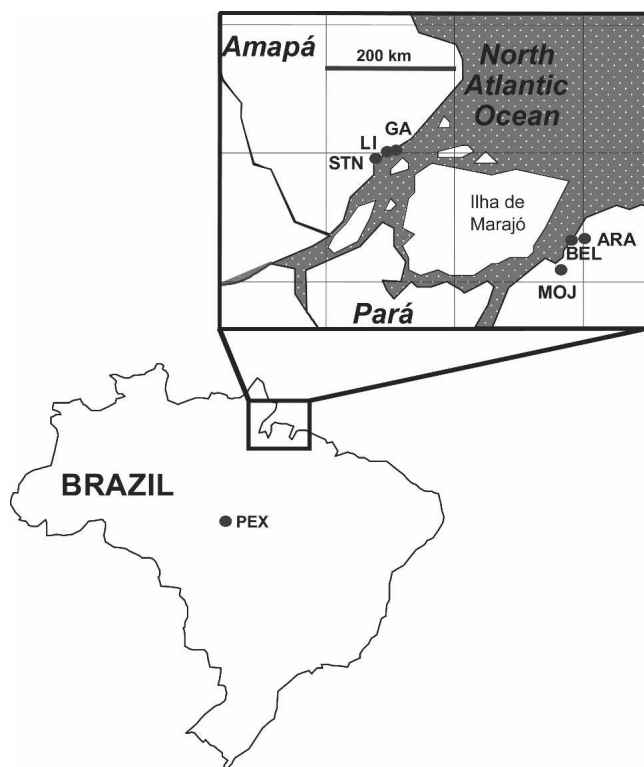


FIGURE 1. Sample localities of *Anopheles darlingi* in eastern Brazil. GA = Granja Alves; LI = Lagoa dos Indios; STN = Santana; ARA = Aracanga; BEL = Belem; MOJ = Moju.; PEX = Peixoto de Azevedo.

after a bottleneck  $H_e$  should be  $> H_{eq}$ , and the opposite,  $H_e < H_{eq}$ , may signal an expansion. Long-term effective population size ( $N_e$ ) was obtained using NeEstimator version 1.3.<sup>42</sup> Estimates were based on two mutation models, IAM and SMM, which are at the extreme ends of mutation models and should therefore provide good range estimates of long-term  $N_e$ .<sup>43</sup> In all analyses, when multiple tests were used, a sequential Bonferroni procedure was applied.<sup>44</sup>

## RESULTS

**Genetic diversity.** All eight microsatellites were polymorphic in the seven populations analyzed, and the highest numbers of alleles per locus were observed in the sample sites in Amapa State (5–26) compared with those in Para and Mato Grosso States (3–22) (Table 1). The mean expected heterozygosity ( $H_E$ ) value was high, 0.834, compared with the mean observed heterozygosity ( $H_O$ ) of 0.500. The lowest  $H_E$  value (0.215) was in MOJ in Para State, and the highest  $H_E$  value (0.966) was in PEX in Mato Grosso State.

**Hardy-Weinberg deviation and linkage disequilibrium.** Significant deviation from HW equilibrium was associated with heterozygote deficits in 26 of 56 possible tests after sequential Bonferroni correction (corrected  $\alpha = 0.0009$ ), and inbreeding

coefficients were positive in all cases of significant heterozygote deficits (Table 1). Heterozygote deficits are usually the result of null alleles, inbreeding, or a Wahlund effect (samples consisting of several pooled subpopulations).<sup>45,46</sup>

However, we also observed linkage disequilibrium across all populations for variable loci in 64 of 156 tests (Table 1). After Bonferroni correction, nine pairs of loci in PEX and one pair of loci in LI (corrected  $\alpha = 0.0002$ ) had significant linkage disequilibrium. In every case where significant linkage disequilibrium was observed, significant heterozygote deficits were also seen.

**Population differentiation.** The  $F_{ST}$  and  $R_{ST}$  values ranged from 0.0 to 0.1841 and from 0.0 to 0.297, respectively, and both values indicated that the same populations were significantly differentiated in 17 of 21 possible tests after the Bonferroni correction (Table 2). Significant differentiation was observed between the three localities in Amapa State (LI, GA, and STN) and the three localities in Para State (BEL, ARA, and MOJ, Figure 1). Within Amapa, there was little to no differentiation among the three localities. Significant differentiation was found between PEX and all populations surrounding the Amazon River where  $F_{ST}$  and  $R_{ST}$  ranged from 0.0880 to 0.1204 and from 0.0821 to 0.1404, respectively (Table 2).

TABLE 1

Sample size (N), number of alleles (A), expected ( $H_E$ ), and observed ( $H_O$ ) heterozygosities, as well as deviations from Hardy-Weinberg equilibrium ( $F_{IS}$ )\*

Locus		LI N = 41	STN N = 40	GA N = 40	ARA N = 37	BEL N = 39	MOJ N = 21	PEX N = 21	All populations
ADC01	A	20	19	22	8	8	15	26	26
	$H_E$	0.930	0.918	0.907	0.645	0.730	0.883	0.966	0.935
	$H_O$	0.615†	0.575†	0.6†	0.395†	0.614†	0.519†	0.696†	0.57†
	$F_{IS}$	0.351	0.377	0.342	0.391	0.161	0.417	0.250	
ADC02	A	11	14	10	4	4	4	10	14
	$H_E$	0.796	0.829	0.799	0.493	0.549	0.215	0.858	0.834
	$H_O$	0.537‡	0.475†	0.65‡	0.316†	0.442‡	0.154	0.375†	0.405†
	$F_{IS}$	0.329	0.430	0.189	0.362	0.198	0.288	0.586	
ADC107	A	7	6	8	4	4	9	13	13
	$H_E$	0.801	0.754	0.800	0.623	0.621	0.868	0.937	0.849
	$H_O$	0.667‡	0.6‡	0.675‡	0.184†	0.545‡	0.760	0.111†	0.529†
	$F_{IS}$	0.169	0.207	0.158	0.708	0.124	0.126	0.884	
ADC110	A	10	9	9	8	10	11	12	12
	$H_E$	0.836	0.824	0.863	0.834	0.800	0.865	0.897	0.866
	$H_O$	0.683†	0.850	0.7‡	0.763	0.727	0.692‡	0.917	0.755†
	$F_{IS}$	0.185	-0.032	0.191	0.086	0.091	0.203	-0.022	
ADC137	A	8	10	7	7	7	9	12	12
	$H_E$	0.736	0.774	0.707	0.801	0.765	0.871	0.842	0.838
	$H_O$	0.675‡	0.641	0.600	0.737‡	0.721	0.667‡	0.625‡	0.669†
	$F_{IS}$	0.084	0.174	0.153	0.081	0.059	0.238	0.262	
ADC138	A	7	6	9	6	5	8	12	12
	$H_E$	0.801	0.753	0.817	0.791	0.652	0.741	0.842	0.815
	$H_O$	0.244†	0.275†	0.25†	0.421†	0.19†	0.231†	0.2†	0.262†
	$F_{IS}$	0.698	0.638	0.697	0.471	0.711	0.693	0.766	
ADC28	A	4	4	3	4	4	4	5	5
	$H_E$	0.387	0.453	0.371	0.694	0.712	0.610	0.722	0.625
	$H_O$	0.275	0.333	0.366	0.368†	0.512‡	0.630	0.304†	0.394†
	$F_{IS}$	0.292	0.267	0.013	0.473	0.284	-0.033	0.584	
ADC29	A	11	12	11	11	15	12	14	15
	$H_E$	0.757	0.787	0.787	0.844	0.796	0.900	0.906	0.908
	$H_O$	0.439‡	0.525‡	0.525‡	0.378†	0.231†	0.429†	0.333†	0.414†
	$F_{IS}$	0.423	0.336	0.336	0.555	0.713	0.530	0.638	
All loci (mean)	A	9.8	10.0	9.9	6.5	7.1	9.0	13.0	13.5
	$H_E$	0.756	0.762	0.756	0.716	0.703	0.744	0.871	0.834
	$H_O$	0.517†	0.534†	0.546†	0.445†	0.498†	0.51†	0.398†	0.5†

\* LI = Lagoa dos Indios; STN = Santana; GA = Granja Alves; ARA = Aracanga; BEL = Belem; MOJ = Moju; PEX = Peixoto de Azevedo.

†  $P < 0.001$  by sequential Bonferroni procedure.

‡  $P < 0.05$  by sequential Bonferroni procedure.

TABLE 2

Pairwise estimates of genetic differentiation ( $F_{ST}$  below diagonal and  $R_{ST}$  above diagonal) between samples of *Anopheles darlingi* Brazil\*

	LI	STN	GA	ARA	BEL	MOJ	PEX
LI		0.0142	0.003	0.2529†	0.1807†	0.2522†	0.0821†
STN	-0.0039		0.0013	0.2970†	0.2094†	0.2937†	0.1351†
GA	-0.0044	-0.0008		0.2569†	0.1839†	0.2494†	0.1024†
ARA	0.1833‡	0.1710‡	0.1841‡		0.0265‡	0.1060‡	0.1596‡
BEL	0.1798‡	0.1674‡	0.1736‡	0.0114‡		0.1015‡	0.1334‡
MOJ	0.1579‡	0.1469‡	0.1564‡	0.0461‡	0.0549‡		0.1404‡
PEX	0.0955‡	0.0880‡	0.0938‡	0.1102‡	0.1204‡	0.0875‡	

\* For definitions of abbreviations, see Table 1.

†  $P = 0.05$  by Bonferroni correction (1,000 permutation).‡  $P < 0.05$ .

A significant positive correlation was found using both  $F_{ST}$  estimates under IAM and  $R_{ST}$  estimates under SMM with the Mantel test when populations  $< 500$  km apart were tested ( $R^2 = 0.9791$ ,  $P = 0.014$  for  $F_{ST}$  and  $R^2 = 0.9276$ ,  $P = 0.007$  for  $R_{ST}$ ; Table 3) and also when all populations were included in the analysis (Table 3).

Analysis of molecular variance showed that most variation (84%) was within sample locations, but there was significant variation (12–13%) between sample sites in Amapa and Para states, under both IAM and SMM models, irrespective of whether PEX was included with samples from Amapa or Para (Table 4).

**Effective population size and mutation drift equilibrium.** Estimates of  $N_e$  differed considerably depending on the model used. Under linkage disequilibrium, the overall  $N_e$  was 92.4, with 95% confidence intervals (CIs) of 87.8–97.4 (Table 5). Population  $N_e$  for PEX (54 individuals, 95% CI = 39.4–83.0) and for BEL ( $\infty$ , 95% CI = 242.3– $\infty$ ) were congruent with the known history of each population (intensive insecticide use in PEX versus little to no use in BEL). However, under heterozygote excess overall  $N_e$  values for both populations were  $\infty$ . Overlapping confidence intervals for populations using each model suggest that populations are subjected to similar amounts of genetic drift.<sup>47</sup>

In our populations of *An. darlingi*, results from the heterozygosity tests under SMM and IAM show contrasting results (Table 6). The stepwise mutation model indicates a heterozygote deficit at more loci in four of seven populations but none of these were significant at the 0.05 level. The infinite alleles model indicates that all populations have more loci demonstrating heterozygote excess. Two populations, GA and PEX, were significant for a heterozygote excess ( $P = 0.0091$  and  $0.0184$ , respectively). However, tests under IAM can wrongly detect heterozygote excess in populations that have not undergone a bottleneck.<sup>41</sup> Use of the strict SMM is recommended,<sup>41</sup> and under this model, no significant portion

of loci with heterozygote excess was detected in any sample (Table 6). We also examined microsatellite allele frequency distributions in each population, grouping alleles from all loci into allele frequency bins as indicated.<sup>48</sup> However, all seven graphs were virtually identical, showing the typical L-shape pattern consistent with populations at MDE, and a mode shift, as is predicted for a population having recently undergone a bottleneck, was not detected in any population.

## DISCUSSION

The mean number of alleles in *An. darlingi* (13.5) and mean  $H_E$  (0.834) are in the same range as values for *An. albimanus* (average = 10.9 alleles and average unbiased heterozygosity = 0.78), the only other primary neotropical malaria vector assessed with microsatellite markers.<sup>9</sup> All studies of *An. darlingi* regardless of marker type (polytene chromosomes, allozymes, mtDNA RFLPs and sequences, ITS2 sequences) have detected moderate levels of genetic variability,<sup>3</sup> and the present study is congruent with earlier work. We detected moderate levels of variability in both the populations where intensive insecticide treatment was carried out (PEX: mean  $H_E = 0.871$ ,  $A = 13.0$ ) and where it was not carried out (BEL: mean  $H_E = 0.703$ ,  $A = 7.1$ ) that were not significantly different from the five remaining sampling sites where levels of insecticide or frequency of application were known to be modest or irregular.

In two studies of allozymes in *An. darlingi*,<sup>49,50</sup> significant deviations from HW equilibrium were not detected, but more recently a comparison of two Amazonian populations (one in each of Amazonas and Amapa states) detected significant deviations in seven of eight loci examined.<sup>51</sup> Heterozygote deficits in relation to HW equilibrium are commonly detected at microsatellite loci in anophelines, e.g., *An. dirus*,<sup>52</sup> *An. gambiae*,<sup>4,53</sup> *An. funestus*,<sup>54</sup> and *An. albimanus*.<sup>9</sup> In our study, deficits were recorded for seven of eight loci and across all populations, with most significant values for *ADC01*, *ADC138*, *ADC29*, and *ADC02* (21 of 26 estimates that were significant after Bonferroni correction). A significant departure from HW equilibrium combined with linkage disequilibrium usually suggests inbreeding, population subdivision, more formally known as the Wahlund effect, or selection. Another possible explanation is that loci could be located on the same chromosome.

If one considers inbreeding, all loci should be affected equally by this phenomenon, with uniform heterozygote deficiencies. Our data demonstrate that deficits ( $H_O$  values in

TABLE 3

Correlation between geographic distances and genetic differentiation ( $F_{ST}$ ,  $R_{ST}$ ) among populations of *Anopheles darlingi* from Brazil

Grouping	Fixation index	Mantel test	
		$R^2$	$P$
All	$F_{ST}$	0.67606	0.0016
All	$R_{ST}$	0.541077	0.0189
$< 500$ km	$F_{ST}$	0.979163	0.0138
$< 500$ km	$R_{ST}$	0.927564	0.0073

TABLE 4  
Results of analyses of molecular variance (AMOVA) with eight microsatellite loci based on  $F_{ST}$  and  $R_{ST}$  values\*

Grouping of samples sites	Source of variation	df	$F_{ST}$		$R_{ST}$	
			Variance component	P	Variance component	P
PEX with Pará sites	Between groups	1	12.25%	< 0.001	36.63	0.028
	Among populations within groups	5	3.65%	< 0.001	4.47	< 0.001
	Within populations	505	84.09%	< 0.001	58.63	< 0.001
PEX with Amapá sites	Between groups	1	12.87%	< 0.001	37.03	< 0.001
	Among populations within groups	5	3.39%	< 0.001	4.75	< 0.001
	Within populations	505	83.73%	< 0.001	58.22	< 0.001

\* Significance levels were based on 1,023 permutations. PEX = Peixoto de Azevedo.

Table 1) are heterogeneous among loci. However, when related individuals mate in species such as mosquitoes with separate sexes, partial inbreeding can take place, creating a heterozygote deficit. In the life history of *An. darlingi* both sexes actually emerge the same day (an unusual phenomenon in mosquitoes<sup>55</sup>), but males cannot mate until their genitalia have rotated,<sup>56</sup> a mechanism that probably reduces the likelihood of inbreeding. *Anopheles darlingi* possess high fecundity and external fertilization, but females mate before questing for a blood meal,<sup>57</sup> and dispersal as far as 7.2 km has been measured by mark-release-recapture.<sup>58</sup> These life history factors taken together mean that inbreeding is a highly unlikely cause of deficits in the present study of *An. darlingi*.

The Wahlund effect is seen when different gene pools have inadvertently been sampled as though they were one.<sup>8</sup> Because we collected adults that are highly mobile, it is possible that a mixture of *An. darlingi* from different locations that differ in allele frequencies at a locus was sampled. The most likely sampling sites for this would be the three in Amapá State, which are 4–11 km apart, and BEL and ARA in Pará State, which are 12 km apart.

Another possibility is selection, when microsatellite loci (themselves considered to be neutral) are linked to loci that are under selection. Positional effects of microsatellite markers on  $F_{ST}$  values (inside or outside inversions) are well-known in *A. gambiae*,<sup>4,53</sup> but  $F_{ST}$  estimates were not affected when four microsatellite markers inside and four outside chromosomal inversions were compared in *An. funestus*.<sup>8</sup> Fourteen inversions in *An. darlingi* have been identified<sup>59–61</sup> (Conn JE, unpublished data), and mapping of *An. darlingi* microsatellite markers<sup>23</sup> onto the polytene chromosomes by *in situ* hybridization is currently under way (Conn JE, unpublished data). Only additional studies can determine whether any of the microsatellite loci in *An. darlingi* are linked to loci

under selection (i.e., inside inversions) and whether this has an influence on estimates of population differentiation.

Null alleles are also commonly cited as causing heterozygote deficits<sup>4,45,62</sup> but in the present study we detected considerable linkage disequilibrium across all populations for several loci in 64 of 156 tests. Linkage disequilibrium is not an expected outcome if the deficit is a result of null alleles because all individuals have the same probability of carrying a null allele.<sup>4</sup> Furthermore, in our study, the deficits were not clustered around one locus but were genome wide and across all populations.

The Mantel tests excluding PEX and for all populations were significant for  $F_{ST}$  and  $R_{ST}$  values (Table 3). Because the IAM-based  $F_{ST}$  estimates are considered to be sensitive to greater geographic distances where there is an overlap of the effect of mutation and the effect of isolation-by-distance,<sup>40</sup> the expected result was that the analysis including the sample site > 500 km distant (PEX) would not be significant. Our results were not consistent with this expectation, and suggest that the effect of geographic distances on genetic differentiation is very important, at least in this region of the range of *An. darlingi*.

Although the level of differentiation among Brazilian populations of *An. darlingi* located on either side of the Amazon River (Figure 1) is comparable to that detected using microsatellites in *An. albimanus* populations from Central and South America,<sup>9</sup> and also to *An. gambiae* populations in both west and east Africa,<sup>4,63</sup> the result was somewhat surprising, considering that all *An. darlingi* populations are from the center of its range.<sup>2</sup> It is possible that the substantial differences in  $N_e$  among populations under linkage disequilibrium (range = 54–∞) may have contributed to the genetic differentiation.<sup>7,64</sup> However, there are limits to detecting and interpreting heterogeneities in population structure in large-scale geographic studies,<sup>7</sup> such as those that found moderate gene flow and no differentiation among several populations of *An. darlingi* in South America.<sup>12,50</sup> Similarly, a study of *An. funestus* comparing east (Kenya) and west (Burkina Faso and Senegal) African samples, using mtDNA and rDNA ITS2 sequences did not detect significant population structure,<sup>65</sup> whereas a study based on microsatellite loci identified high genetic differentiation.<sup>66</sup> In *An. dirus* D a mitochondrial study detected no differentiation over a large area (> 1,200 km) in Thailand,<sup>52</sup> but a study using microsatellite markers found significant structure over approximately 400 km.<sup>67</sup> Our finding of population differentiation in *An. darlingi* in this region of Amazonian Brazil may be important for malaria control because it suggests that genetically modified mosquitoes re-

TABLE 5

Estimated  $N_e$  based on the linkage disequilibrium (LD) and heterozygote excess (HE) models\*

	LD	95% CI	HE	95% CI
ARA	780.4	120.5–∞	∞	NA
BE	∞	242.3–∞	∞	NA
GA	152.4	89.6–430.3	∞	NA
LI	133.6	81.7–321.5	∞	NA
MOJ	123.5	61.0–1945.5	∞	NA
PXT	54	39.4–83.0	∞	NA
STN	351.7	139–∞	∞	NA
All populations	92.4	87.8–97.4	∞	NA

\* CI = confidence interval; NA = not applicable. For definitions of other abbreviations, see Table 1.

TABLE 6

Number of loci exhibiting heterozygote excess ( $H_e$ ) and heterozygosity expected from the observed number of alleles ( $H_{eq}$ ) under both SMM and IAM models\*

		LI	STN	GA	ARA	BE	MOJ	PEX
SMM	$H_e < H_{eq}$	6	6	7	3	6	4	4
	$H_e > H_{eq}$	2	2	1	5	2	4	4
	$P(H_e > H_{eq})$	0.0583	0.057	0.0996	0.581	0.0516	0.4439	0.4312
IAM	$H_e < H_{eq}$	2	2	1	1	1	1	0
	$H_e > H_{eq}$	6	6	7	7	7	7	8
	$P(H_e > H_{eq})$	0.3034	0.298	0.0091	0.0907	0.0895	0.1043	0.0184

\* SMM = stepwise mutation model; IAM = infinite alleles model. For definitions of other abbreviations, see Table 1.

leased in this area would experience a substantial barrier to gene flow. We think it unlikely that the Amazon River *per se* is a barrier, since this eastern part of the Amazon Delta has many small islands as well as the large Ilha de Marajó (Figure 1), with suitable *An. darlingi* habitat.<sup>68</sup> A more likely explanation for the differentiation is a combination of IBD and the heterogeneity of the  $N_e$ . We also note that the properties of markers used in previous studies of *An. darlingi* (i.e., less polymorphic, lower mutation rate<sup>69,70</sup>) were probably not sensitive enough to detect this level of differentiation, and most of them were assessing samples at much greater geographic scales.

The overall  $N_e$  of 92.4 individuals under linkage equilibrium is similar to that estimated for the malaria vector *An. albimanus* ( $N_e = 96$ ), but under heterozygote excess all values of *An. darlingi* are  $\infty$ . If we consider that these two models provide range estimates,<sup>43</sup> *An. darlingi* has a very broad range with the upper bound greater than that found in several estimates for *An. gambiae*.<sup>43,47,71</sup> Estimates of  $N_e$  are based on expected heterozygosity assuming equilibrium,<sup>43</sup> but the populations of *An. darlingi* under study deviated significantly from HW equilibrium and also displayed linkage disequilibrium. Such a violation may have contributed to the higher than expected values detected under heterozygote excess.

If we focus on the lower bound provided by the IAM estimates, the small  $N_e$  of *An. darlingi* could be the result of an overall bottleneck due to insecticide use or seasonal fluctuations as proposed for *An. albimanus*.<sup>9</sup> Seasonal fluctuations in *An. darlingi* are quite pronounced<sup>2,72</sup> and there is ample evidence for insecticide use over many years.<sup>13</sup> The large  $N_e$  and allele distribution indicating MDE of *An. darlingi* in Belem could reflect the very low use of insecticide in this eastern district of Entrocamento, the source of the municipal water supply for the city of Belem. We think it likely that the small  $N_e$  of 54 individuals in PEX, a site of high malaria prevalence in the early 1990s<sup>22</sup> where insecticides were used repeatedly to reduce *An. darlingi*,<sup>24</sup> could be attributed to a bottleneck that took place within the  $4N_e$  generation time necessary for detection.<sup>41</sup> In theory, populations that have undergone a recent bottleneck, such as PEX in the early 1990s, should have lost rare alleles even if each maintained substantial heterozygosity. We found some evidence for a bottleneck in PEX (a significant heterozygote excess under IAM, Table 6) but this was not supported under the SMM model. Unexpectedly, we also detected a significant heterozygote excess in GA, Amapa State, where there is no record of substantial insecticide treatment until 2001,<sup>73</sup> which is after our samples had been collected. Both the number of polymorphic microsatellite loci we sampled ( $n = 8$ ) and our mean sample size ( $n = 36.6$  indi-

viduals) are within the range where bottlenecks will most likely be detected (8–10 loci and 30 individuals per locus<sup>48</sup>). However, the power of the bottleneck test is such that approximately 20% of the time an actual bottleneck will remain undetected.<sup>41,48</sup> Perhaps, as was suggested for *Aphidius ervi*,<sup>74</sup> the reduction in population size of *An. darlingi* in PEX was not extreme enough to be detected by BOTTLENECK. The  $N_e$ s of the three sampling sites in Amapa (GA, LI, and STN), which ranged from 133.6 to 351.7 under IAM, were also of interest because in these sites *An. marajoara* is presently the primary malaria vector and the abundance of *An. darlingi* is relatively low,<sup>27</sup> although it is common in a nearby riverine habitat (Sao Raimundo do Pirativa) where it is considered to be the principal vector.<sup>72</sup>

Many analyses of population structure assume MDE, but this is violated (at least using the mtDNA marker) in studies of several malaria vectors, including *An. dirus*,<sup>52</sup> *An. gambiae* and *An. arabiensis*,<sup>75</sup> *An. marajoara*<sup>76</sup> and *An. darlingi*.<sup>12</sup> Subsequent studies of some of the same species<sup>7,67</sup> confirmed the violation of MDE using multi-locus tests. The demographics of these species are thus unstable, usually in the form of bottlenecks and (or) expansions, and make accurate estimates of gene flow from  $F_{ST}$  estimates particularly difficult.<sup>4</sup> Our assessment of *An. darlingi* in the present study suggests that in this eastern area of Amazonian Brazil these populations are in MDE, whereas among South American samples a Pleistocene expansion was detected by mtDNA sequences.<sup>12</sup>

The study of population structure in *An. darlingi* also has important implications, particularly in relation to its role in the transmission of *Plasmodium falciparum* in the Amazon.<sup>77</sup> A study of genetic structure in *P. falciparum* in five locations in Amazonian Brazil including Serra do Navio in Amapá State, which is near the three study sites GA, LI, and STN in the present study of *An. darlingi* and another, Tailândia, which is near MOJ in Para State in the present study, found that despite the increase in the number of cases of infection with *P. falciparum* in Brazil from the 1970s to the 1990s,<sup>13</sup> there was no evidence of a recent epidemic expansion of any particular clone.<sup>78</sup> This study also detected substantial divergence and low levels of gene flow between populations of *P. falciparum*, which did not conform to IBD. Studies of *P. falciparum* and *An. darlingi*, its primary Amazonian host, based on sample sites in Amapa and Para States detected similar aspects of population structure: significant linkage disequilibrium and genetic divergence, although the effect of IBD was not the same. Although recognizing that differences in *Plasmodium*<sup>79</sup> and *Anopheles*<sup>56</sup> mating systems will influence each organism's population structure, we advocate studies of population structure in both *P. falciparum* and *An. darlingi*

from the same localities because it seems likely they would provide insights that could result in more effective control measures that are specific to particular malaria-endemic regions. This is particularly important in Amazonian Brazil where *P. falciparum* resistance to several commonly used drugs, e.g., chloroquine,<sup>80</sup> sulfadoxine-pyrimethamine,<sup>81</sup> and mefloquine<sup>82</sup> has been detected, and where a recent study determined that there has been significant under-reporting of cases of infection with *P. falciparum*, a trend that appears to be worldwide and is especially notable outside Africa.<sup>83</sup>

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