

LIMITED DIVERSITY OF *ANOPHELES DARLINGI* IN THE PERUVIAN AMAZON REGION OF IQUITOS

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Abstract. *Anopheles darlingi* is the most important malaria vector in the Amazon basin of South America, and is capable of transmitting both *Plasmodium falciparum* and *P. vivax*. To understand the genetic structure of this vector in the Amazonian region of Peru, a simple polymerase chain reaction (PCR)-based test to identify this species of mosquito was used. A random amplified polymorphic DNA-PCR was used to study genetic variation at the micro-geographic level in nine geographically separate populations of *An. darlingi* collected in areas with different degrees of deforestation surrounding the city of Iquitos. Within-population genetic diversity in nine populations, as quantified by the expected heterozygosity (H_E), ranged from 0.27 to 0.32. Average genetic distance (F_{ST}) among these populations was 0.017. These results show that the nine studied populations are highly homogeneous, suggesting that strategies can be developed to combat this malaria vector as a single epidemiologic unit.

INTRODUCTION

Peru has the second highest number of malaria cases in South America. Most of these cases are reported in the Peruvian Amazon, where malaria is a re-emerging disease.¹ This high prevalence is attributed to the presence of *Anopheles darlingi*, a highly competitive and anthropophilic vector that colonized the Amazon Region at the start of the 1990s.² *Anopheles darlingi* is the most significant vector of malaria in South America and is capable of transmitting both *Plasmodium falciparum* and *P. vivax*.^{3,4}

In tropical zones, anthropogenic activities involving deforestation, such as the construction of highways, agriculture, and fish farming, produce ecologic changes and can alter the community of disease vectors.^{5,6} For instance, deforestation has caused partial or total substitution of *Anopheles* species in the Himalayas⁷ and Colombia.⁸ In the Peruvian Amazon, the population of *An. darlingi* has increased over the last 10 years and is now the major vector of malaria.¹ Before 1991, *An. darlingi* was not found in the areas around Iquitos, the major city of the Peruvian jungle.⁹

The recent spread of *An. darlingi* in this zone of the Amazon is a public health problem. As such, we need cost-effective methodologies to not only identify this species but to understand the patterns of genetic diversity for this and other vectors. Identification of *An. darlingi* can be performed either by using specific primers (as was done in this study) or the use of internal transcribed spacers as described by Marrelli and others.¹⁰

Genetic diversity of *Anopheles* has been studied using random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR),^{11–15} internal transcribed spacer 2 (ITS2) markers,^{13,16} microsatellite loci,¹⁷ mitochondrial DNA,^{17,18} single nucleotide polymorphism genotyping,¹⁹ and isoenzy-

matic analysis.^{13,20} RAPD-PCR is based on the amplification of random DNA using short sequence primers (approximately 10 bases) at low annealing temperatures. It has been used to analyze the genetic variation of *An. darlingi*¹³ and other species of mosquitoes²¹ and as a tool to construct genetic maps.²²

In this study, we present a new PCR-based assay that permits a simple and low cost identification of *An. darlingi*. Additionally, we used RAPD-PCR to analyze the genetic variation of natural populations of *An. darlingi* collected from areas with different levels of deforestation in Iquitos (Loreto, Peru), in the Amazonian region of Peru.

MATERIALS AND METHODS

Collection and manipulation of *Anopheles*. Female *Anopheles* adults were collected near the city of Iquitos (3°45'18"S and 73°14'40"W). Iquitos is the principal city of the Peruvian Amazon and is located on the western bank of the Amazon River in the northeast region of Peru at an altitude of 110–125 meters above sea-level in the department of Loreto, an administrative unit that borders with Ecuador, Colombia, and Brazil. The area in the study is tropical and humid with an annual average temperature that ranges between 24°C and 28°C, an annual relative humidity that varies between 80% and 90%, and an annual average precipitation of 1,500 mm.⁵ We defined the level of deforestation of the areas sampled according to a previously used classification: rural villages (Vil); shrub areas (Shrub), with young vegetation returning approximately five years after deforestation; secondary forest (SecFor), deforested approximately 15 years before the study; and primary forest (PriFor), referring to closed-canopy, tall forest.²

The areas sampled include five rural villages: Zungarococha, Santa Clara, Varillal, San Gerardo, and El Dorado; two shrub areas: San Gerardo and San Jose; one secondary forest: Monte Calvario; and one primary forest: Tiberias Pintuyacu I. The villages and shrub areas are located along the Iquitos-Nauta highway, which was completed in 2005, and the forests

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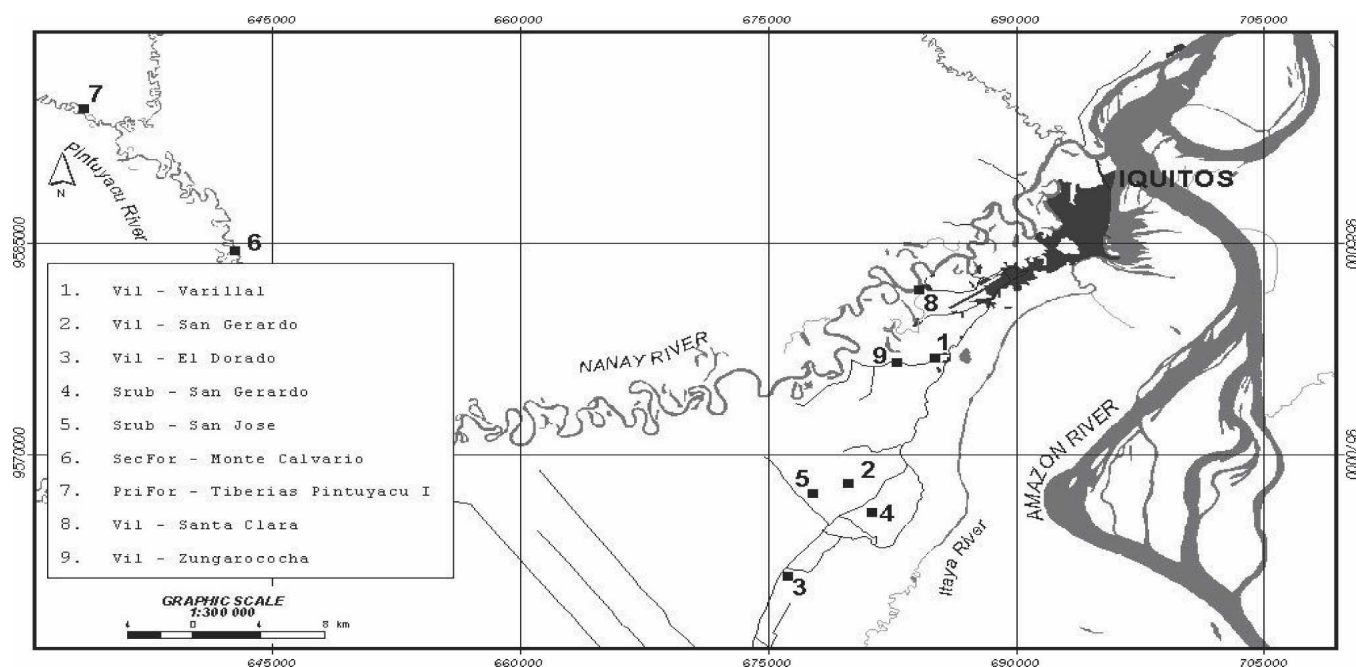


FIGURE 1. Map of the study "population" areas' relation to the city of Iquitos, showing the approximate locations of *Anopheles darlingi* collections with different levels of deforestation.

are located to the west of Iquitos (Figure 1). Samples were obtained by human bite collections (previously described) from December 1998 to June 1999 and from September 2000 to August 2001.² The study was reviewed and approved by the ethics committee of the Johns Hopkins Bloomberg School of Public Health and Asociación Benéfica Prisma.

Identification of *Anopheles darlingi*. The adult females of *An. darlingi* were identified with the diagnostic characteristics described by Linthicum and others,²³ individually preserved in 96% alcohol, and stored at -20°C . We developed a PCR-based test to specifically identify *An. darlingi* individuals. First, we searched Genbank for the *An. darlingi* sequences showing lowest similarity with other homologous sequences from other *Anopheles* species. We selected a sequence in the internal spacers of subunits 5.8S and 28S of the genes coding ribosomal RNA (Genbank accession no. AF462389) and designed the primers 5'-CCC GTG TGT GGT CAA GCA TT-3' and 5'-TTG AGG CCC ACT TGA GAT CC-3'. We then tested the specificity of these primers by performing a PCR on genomic DNA extracted following the same protocol from *An. darlingi* and the following related species: *Aedes aegypti*, *Culex quinquefasciatus*, *An. oswaldoi*, *An. rangeli*, *An. benarrochi*, *An. nuneztovari*, *An. punctimacula*, *An. albimanus*, *An. triannulatus*, *An. mediopunctatus*, *An. mattogrossensis*, and *An. forattini*. The conditions for the PCRs were the following: 2 mM MgCl_2 , 0.2 mM dNTPs, 0.5 μM of each primer, 0.5 units of *Taq* polymerase (Invitrogen, Carlsbad, CA), and 2 ng of genomic DNA in a final volume of 12 μL per tube. The reaction mixture was incubated for one cycle at 94°C for five minutes; 35 cycles at 94°C for one minute, 57°C for one minute, and 72°C for one minute, and at 72°C for five minutes. Electrophoresis was conducted on a 2% agarose gel at 75 V for two hours.

Extraction of genomic DNA. DNA was isolated according to the method of Snounou and others,²⁴ and the quantifica-

tion of the DNA was carried out by comparison with bacteriophage λ genomic DNA (Invitrogen) of known concentration.²⁵

RAPD-PCR. RAPD analyses were carried out on 270 DNA samples from 9 zones (30 per population) according to the protocol described by Wilkerson and others¹⁴ with some modifications, such as the use of *Taq* (Biolase, Santa Clara, CA); four oligonucleotides: A09, A05, A08 and B04;¹² and oligonucleotide 1283, which provided high rates of differentiation in studies on genetic variation of *Helicobacter pylori* (Table 1).²⁶

The specific reaction conditions were 2.5 mM MgCl_2 , 0.3 mM dNTPs, 0.8 μM of each oligonucleotide (Table 1), 1 unit of *Taq* polymerase (Biolase), and 0.5–1.0 ng of genomic DNA in a final volume of 25 μL per tube. The reaction mixtures were incubated for one cycle at 94°C for five minutes; 44 cycles at (94°C for one minute, 36°C for one minute, and 72°C for two minutes, and 72°C for five minutes. Electrophoresis was conducted on a 2% agarose gel at 60 V for four hours with a 100-basepair molecular mass marker (Invitrogen).

Genetic and statistical analysis. DNA ProScan version 2.39 (DNA Proscan, Inc., Nashville, TN) was used for analysis of polymorphic patterns from the populations of *An. darlingi*.

TABLE 1
RAPD-PCR oligonucleotides, their sequences, number of bands, and margins of analysis*

Oligonucleotides	Sequence (5' to 3')	No. of polymorphic bands	Margins of analysis (basepairs)
OPA09	GGGTAACGCC	15	400–1570
OPA08	GTGACGTAGG	12	436–1100
OPA05	AGGGGTCTTG	14	387–1433
OPB04	GGACTGGAGT	11	400–1277
1283	GCGATCCCCA	13	434–1249

* RAPD-PCR = random amplified polymorphic DNA–polymerase chain reaction.

This program calculates the length of the DNA fragments based on the migration coefficient (R_F) normalized to the molecular weight of the 100-basepair molecular mass marker (Invitrogen). The molecular weights of the bands from each polymorphic pattern are stored in a database (and compared with 5% error to new polymorphic patterns). A polymorphic pattern was considered new for *An. darlingi* when we found a minimum of three different bands for each primer. For the final analysis, a binary matrix was constructed based on the presence or absence of the bands amplified, which considered bands with frequencies within the margins of $> 5\%$ and $< 96\%$. Unless differently specified, polymorphic alleles from the RAPD-PCR were analyzed assuming that RAPD-PCR products segregate as dominant traits in a Mendelian fashion, the DNA sequences with equal molecular weight are homologs, the different loci segregate independently, and populations are in Hardy-Weinberg equilibrium.

Within-population genetic diversity was quantified by the method of expected heterozygosity (H_E).²⁷ The 95% credibility intervals of H_E were calculated using the program Hickory version 1.3.²⁷ Pairwise F_{ST} (the between-population component of genetic variance) were estimated using two estimators: Wright's F-coefficient²⁸ and the θ statistic.²⁹ Assuming an island model of population structure, we used F_{ST} to estimate the number of migrants per generation (N_M) using the formula $F_{ST} = 1/(1 + 4N_M)$.³⁰ Genetic distances (F_{ST}) and the N_M estimators were calculated using the RAPDFST computer program.²⁹ Genetic-distance matrices were graphically summarized by non-metric multidimensional scaling (MDS),³¹ through use of the software NewMDSX (Sigma Essex Research and Consultancy, Argyll, United Kingdom). MDS uses an iterative process to transform a similarity/dissimilarity matrix into distances represented in a Euclidean n-dimensional space. We assessed the correlation among matrices of genetic and geographic distances by the Mantel test,^{32,33} as implemented in Arlequin version 2.0 (Schneider S, Roessli D, Excoffier L, Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland). To control for the effects of deforestation, we created a matrix of ecologic distances. For this purpose, we coded populations as 0 if they were primary forest, 1 if secondary forest, 2 if shrub area, and 3 if rural village. For each pair of populations, we defined the ecologic distance as the absolute value of the subtraction of their codes. We then calculated a partial correlation between genetic and geographic distance matrices, controlling for the ecologic distances, using the Mantel test as suggested by Smouse and others.³⁴

Analysis of molecular variance (AMOVA) was used to quantify the proportion of the total genetic variance within population, between populations, and among groups of populations (i.e. rural villages, shrub areas, secondary and primary forest), using a framework similar to the analysis of variance adapted for genetic data.³⁵ The AMOVA calculations were performed from the band frequencies using the program Arlequin version 2.0. Significance of the AMOVA results was assessed by a randomization test (10,000 repetitions). To verify that AMOVA calculations were not affected by deviations from Hardy-Weinberg equilibrium, we also used the Bayesian approach developed by Holsinger and others to calculate θ_B , an estimator of F_{ST} among the nine populations.³⁶ This probabilistic approach uses the full data set and incorporates the uncertainty regarding Hardy-Weinberg equilib-

rium, while the previously mentioned analyses assume Hardy-Weinberg equilibrium.

RESULTS

Population and identification of *An. darlingi*. Approximately 1,000 mosquitoes were collected and identified morphologically as *An. darlingi* from the nine study areas: five rural villages, Zungarocoha, Santa Clara, San Gerardo, Varrillal, and El Dorado; two shrub areas, San Jose and San Gerardo; one secondary forest, Monte Calvario; and one primary forest, Tiberias Pintuyacu I.² Thirty mosquitoes were randomly selected from each of the populations, from which DNA was extracted and the species verified by PCR. The result of the PCR with different species of mosquito is shown in Figure 2. The expected result for *An. darlingi* was 300 basepairs. There was no amplification in the other species of mosquito tested.

Descriptive analysis of the RAPD-PCR loci. We identified 65 polymorphic bands with molecular weights in the range of 387–1,570 basepairs in the 270 samples of *An. darlingi* (Figure 3). Seventy-five percent of the total samples were analyzed twice; none of the repeats differed by three or more bands. The number of polymorphic bands for each type of oligonucleotide is shown in Table 1.

Within-population genetic diversity. Within-population genetic diversity was low (Table 2). This genetic diversity was quantified by the average expected heterozygosity across the 65 loci (for each locus, $H_E = 1 - p^2$, where p is the frequency of the presence of the band). The average values across the 65 RAPD loci varied from 0.27 to 0.32. In general, there was no association among the environment of mosquito populations and level of within-population variability.

Between-population genetic diversity. The correlation between the Wright's F_{ST} (Table 3) and the θ matrices of genetic distances was very high ($R^2 = 0.95$, $P < 0.0001$, by Mantel test). Therefore, hereafter we used the Wright's F_{ST} estimator to compare genetic diversity between populations. The average genetic distance among the nine populations was 0.017. In



FIGURE 2. Specific polymerase chain reaction products for *Anopheles darlingi* (300 basepairs [bp]). Lane 1, *Culex quinquefasciatus*; Lane 2, *An. albimanus*; Lane 3, *An. mattogrossensis*; Lane 4, *An. mediopunctatus*; Lane 5, *Aedes aegypti*; Lane 6, *An. rangeli*; Lanes 7, 8, 14, 15, 18, and 19, *An. darlingi*; Lane 9, *An. oswaldoi*; Lane 10, 100-bp DNA ladder; Lane 11, *An. nuneztovari*; Lane 12, *An. benarrochi*; Lane 13, *An. triannulatus*; Lane 16, *An. forattini*; Lane 17, *An. punctimacula*; Lane 20, negative control.

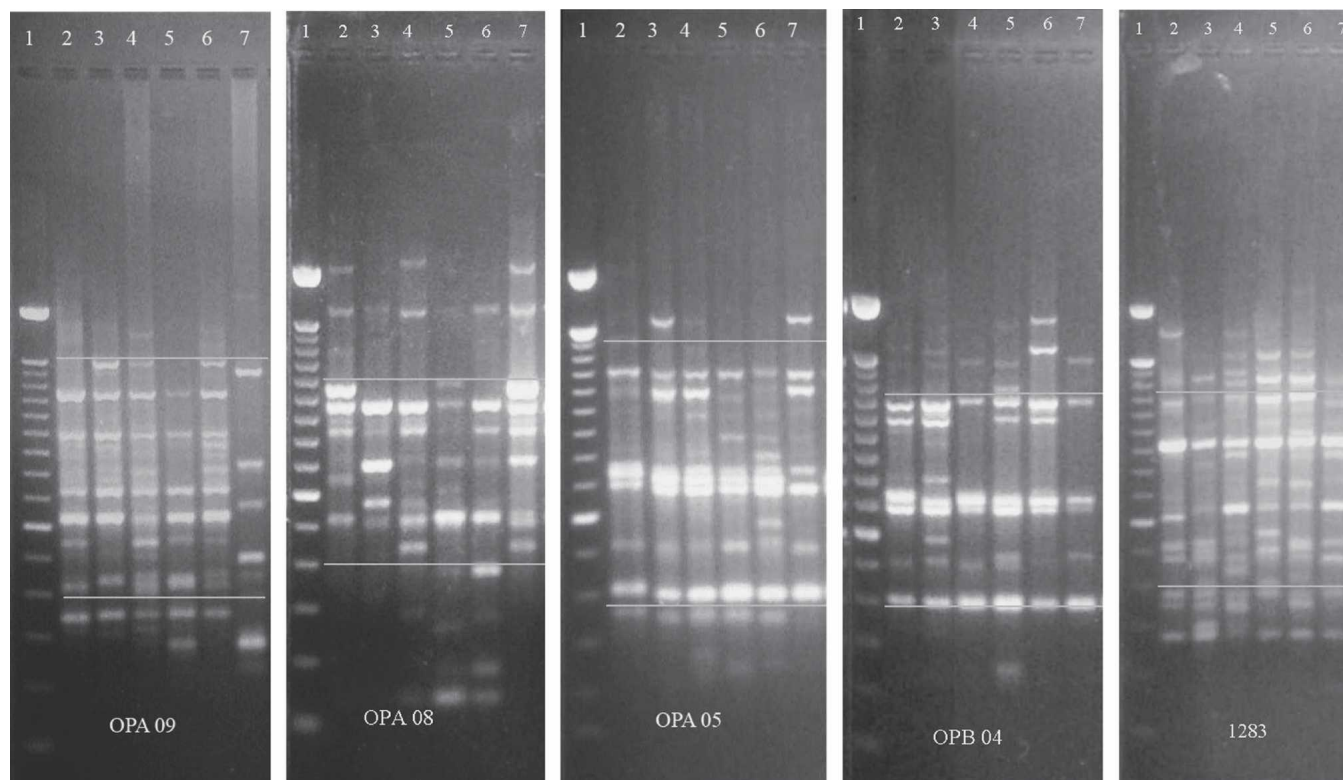


FIGURE 3. Results of random amplified polymorphic DNA amplification patterns of *An. darlingi* samples (lanes 2–7) with the oligonucleotides specified in Table 1. Lane 1, 100 basepair DNA ladder, horizontal lines indicate the limits of analysis (negative control not shown).

general, genetic distances were very low. For each population, Table 3 also shows, across the diagonal, the average genetic distance with the other eight populations.

Assuming an island model of population structure, we estimated N_M (i.e., the number of migrants per generation) from the F_{ST} values (Table 3). All N_M values are greater than 8, which suggests that gene flow, rather than genetic drift, is the predominant evolutionary force shaping the genetic structure of these mosquito populations.³⁷ Alternatively, all populations studied derived from a unique ancestral population that has expanded very recently, without time to differentiate.

The MDS of the F_{ST} genetic distance matrix is shown in Figure 4. Consistent with the genetic distance matrix, the populations of Vil-Varillal and Vil-Santa Clara, which show the largest average genetic distances with the other populations (0.19 in both cases), are the more peripheral in the graphic. El Dorado and San Jose, which show the smallest average genetic distances (0.13 and 0.15, respectively), are central in the graphic. In general, the distribution of the populations in the MDS does not match their geographic distances. The scatterplot of genetic distances (F_{ST}) against geographic distances (Figure 5) confirms this result. In this figure, two sets of observations are evident. On the left of the scatterplot, a set of points represents comparisons among geographically close populations (i.e., those rural villages and shrub areas located along the Iquitos-Nauta highway near Iquitos) and between SecFor-Monte Calvario and PriFor-Tiberias Pintuyacu I. On the right of the scatterplot, we observe comparisons among each of the populations along the highway and the distant forest populations, and it is clear that the genetic distances for the former, geographically close populations are

not smaller than for the latter, geographically distant populations. The Mantel test does not show any correlation between the F_{ST} and geographic distances ($R^2 = -0.06$, $P = 0.62$), even when the effect of ecologic distances was controlled (partial correlation = 0.01, $P = 0.63$). These results suggest that, despite the different ecological environments, at the micro-geographic level of this study, there is high homogeneity among populations.

We also performed a hierarchical analysis of variance developed for molecular data (AMOVA), which confirms the very low level of differentiation among the nine populations ($F_{ST} = 0.015$, $P < 0.0001$). The differentiation among populations within the ecologic groups (rural villages and shrub areas) is also low ($F_{SC} = 0.017$, $P < 0.0001$), but no significant differentiation was observed among the ecologic groups (ru-

TABLE 2
Expected Heterozygosity (H_e) values of the “populations” of *Anopheles darlingi*

Population	Expected heterozygosity	95% confidence intervals
Vil-Zungarococha	0.28	0.27–0.30
Vil-Santa Clara	0.27	0.27–0.30
Vil-San Gerardo	0.29	0.28–0.30
Shrub-San Jose	0.29	0.28–0.30
Vil-Varillal	0.30	0.28–0.31
SecFor-Monte Calvario	0.28	0.28–0.30
Vil-El Dorado	0.30	0.28–0.31
Shrub-San Gerardo	0.28	0.28–0.30
PriFor-Tiberias Pintuyacu I	0.29	0.28–0.30
All populations	0.30	0.29–0.30

TABLE 3
Estimation of F_{ST} statistics (above the diagonal) in population of *Anopheles darlingi* from nine areas around Iquitos*

Population	1	2	3	4	5	6	7	8	9
1 Vil-Zungarococha	0.017	0.019	0.021	0.018	0.020	0.017	0.010	0.017	0.014
2 Vil-Santa Clara	12.8	0.019	0.011	0.016	0.022	0.021	0.018	0.022	0.023
3 Vil-San Gerardo	11.6	22.2	0.018	0.013	0.026	0.030	0.015	0.019	0.011
4 Shrub-San Jose	13.7	15.5	18.6	0.015	0.022	0.015	0.011	0.010	0.017
5 Vil-Varillal	12.0	11.0	9.2	11.4	0.019	0.015	0.017	0.017	0.016
6 SecFor-Monte Calvario	14.6	11.5	8.0	16.1	16.8	0.018	0.008	0.018	0.019
7 Vil-El Dorado	24.6	13.5	16.5	21.7	14.2	29.7	0.013	0.013	0.014
8 Shrub-San Gerardo	14.8	11.0	13.2	24.4	14.2	13.4	18.3	0.016	0.011
9 PriFor-Tiberias Pintuyacu I	17.8	10.6	22.7	14.4	14.9	13.2	18.3	22.7	0.016

* N_M values (the number of migrants per generation) are below the diagonal and the average genetic distance are on the diagonal.

ral villages, shrub areas, secondary forest, and primary forest; $F_{CT} = 0.000$, $P = 0.84$).

DISCUSSION

The migration of humans into previously unpopulated areas of Iquitos and the subsequent deforestation have been associated with a new distribution of *An. darlingi* in these areas.² In the more rural areas of Iquitos, *An. darlingi* was notable for its absence in surveys prior to 1991, but it is now the most important malaria vector. We aimed to determine whether there was an association between the variability of invading *An. darlingi* populations and habitat. Particularly, we tested the hypothesis that mosquito populations in recently cleared forest habitats were more uniform than populations in secondary and primary forest. Using RAPD-PCR, we found no significant population structure and, therefore, no evidence that cleared forest has facilitated the differentiation of any specific *An. darlingi* subpopulation.

This study also used newly developed primers to identify *An. darlingi* to confirm that microscopy correctly identified this mosquito species. The identities of 270 adult females of

the species *An. darlingi*, as determined by morphologic analysis, were reconfirmed with a PCR test. The PCR had a 100% sensitivity and specificity compared with the morphologic analysis, which is the gold standard for species identification. The availability of our highly efficient and cheap test, based on a single PCR reaction, permits the identification of *An. darlingi* individuals even when specimens are damaged.

The design of the reported primers was based on the sequence of the ribosomal DNA regions 5.8S and 28S (between which the ITS2 are encountered). We confirmed that our primers were good candidates for the identification of *An. darlingi*. Marrelli and others used this same region for the identification of multiple *anopheline* mosquito species from the Brazilian Amazon from groups of morphologically similar species.¹⁰ Also, Garros and others demonstrated that the ITS2 fragment is able to successfully discriminate between different *Anopheles* species. This assay has the advantage of permitting the identification of at least 13 *Anopheles* species within the subgenus *Celia* from Africa and Asia,³⁸ however, it cannot be done like our assay, as a simple PCR test.

In this study, the RAPD-PCR identified 65 RAPD loci, a comparable number to those identified in previous studies:

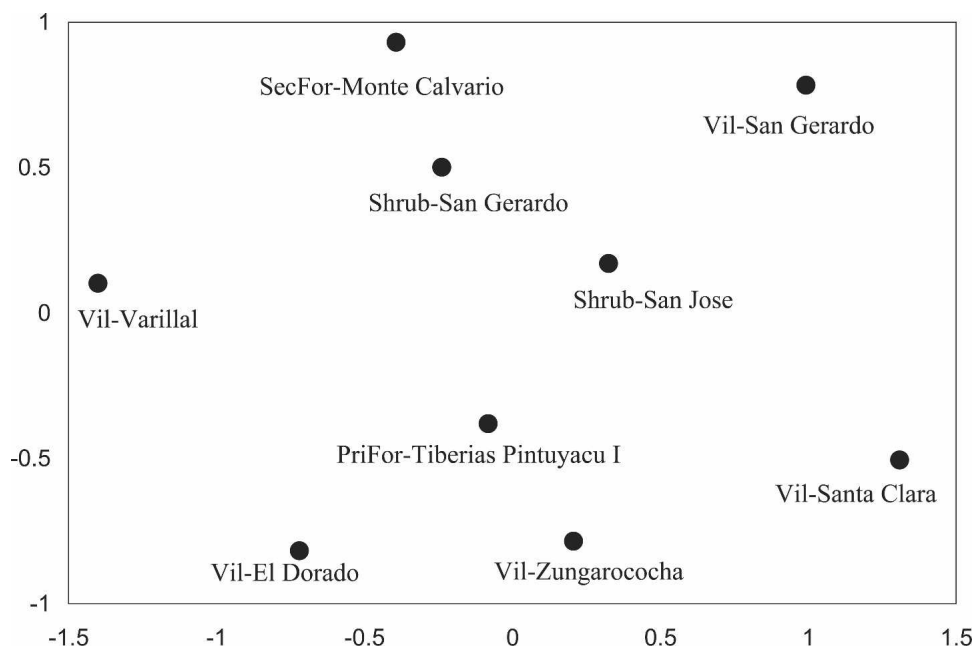


FIGURE 4. Synthetic representation of the F_{ST} genetic distance matrix, obtained by the non-metric Multidimensional Scaling (MDS).

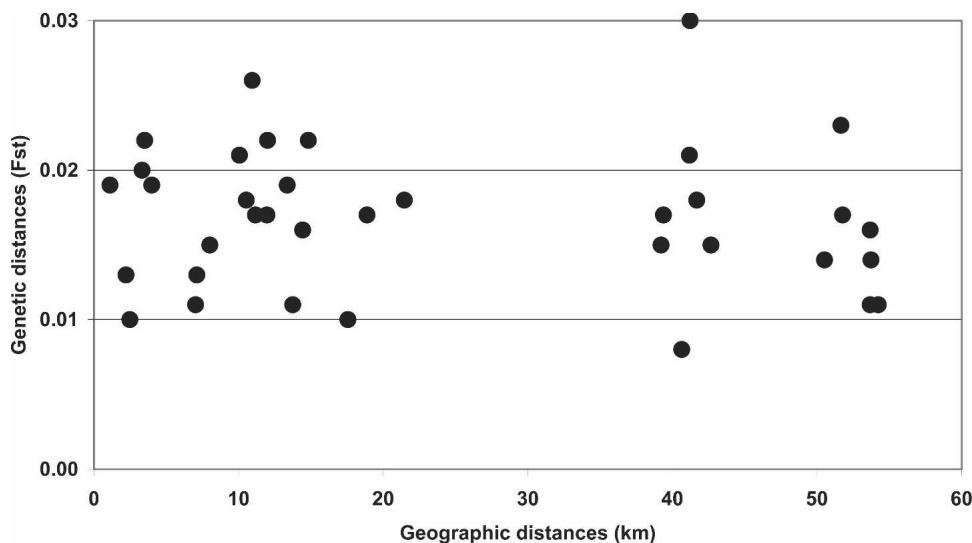


FIGURE 5. Scatter plot of genetic distances (F_{ST}) on geographic distance in kilometers.

(46–65).^{10,12,19,39} The within-population diversity (i.e., expected average heterozygosity across loci) observed in our samples was comparable to previous studies on various *Anopheles* species in different South and Central American populations.¹⁷ Using various molecular markers, other investigators found similar values of heterozygosity. In three populations of *An. nuneztovari* from Colombia, the within-population diversity was 0.343,¹¹ and for the same species in four populations from Brazil and two from Colombia the within-population diversity ranged from 0.087 to 0.116.²¹ In Brazil, a within-population diversity for *An. darlingi* of 0.171 was found,²⁰ and in the same species in seven countries of Central and South America a range of 0.063 to 0.122 was found.¹³

The level of differentiation among populations in the studied area was very low (θ_B , an F_{ST} estimator, is 0.0125). In particular, there was no evidence of a higher differentiation among the cluster of populations of deforested areas along the Iquitos-Nauta highway and *An. darlingi* populations from the primary forest (Tiberias Pintuyacu I) and the secondary forest of Monte Calvario, which are located at more than 50 km with respect to the Iquitos-Nauta highway. These results suggest that enough gene flow exists among these populations to maintain homogeneity across the area. These results were consistent with other studies in different parts of the world that did not show a strong population structure among mosquito populations for different species, even at distances as great as 100 and 200 km.^{39,40}

Posso and others¹¹ evaluated the degree of genetic differentiation between populations of *An. nuneztovari* in Colombia using RAPD-PCR.¹¹ They found that populations separated by 325 km show higher migration rates than populations separated by only 250 km and divided by a geographic barrier. At a continental level, Maguin and others¹³ found that RAPD-PCR isolated populations of *An. darlingi* geographically and detected a small genetic distance between them, whereas ITS2 grouped all of them together as identical. Malafrente and others also found by means of ITS2 that the populations of *An. darlingi* from different states of Brazil were identical.¹⁶ However, Conn and others demonstrated that between the populations of *An. darlingi* from different countries

of South America there exists a significant correlation between the genetic and geographic distances, and this is reflected in the different phenotypes.¹⁸

If the studied populations of *An. darlingi* maintain a high level of gene flow across an area such as that considered in this study, it implies that genetic effects of human intervention for the control of mosquito populations will spread across areas encompassing deforested environments as well as the adjacent forest. For instance, if use of insecticides in villages raises frequencies of alleles conferring insecticide resistance, our study suggests that these alleles can spread even to the near (i.e. 50–60 km) primary and secondary forest. Conversely, if populations are highly homogeneous, they likely show the same level of genetic susceptibility to insecticides, and the same eradication program can be applied to different villages and the surrounding forested areas.

In this study, we showed high homogeneity among nine populations of *An. darlingi* separated by 20–60 km located in different habitats from the areas surrounding the city of Iquitos. This result suggests implementation of strategies to combat these populations because one epidemiologic unit would permit the control of the principal malaria vector in this study area. In this context, this study is a contribution to malaria programs that are striving to eradicate the vector in these areas.

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