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Microgeographic Genetic Variation of the Malaria Vector *Anopheles darlingi* Root (Diptera: Culicidae) from Córdoba and Antioquia, Colombia

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Abstract. *Anopheles darlingi* is an important vector of *Plasmodium* spp. in several malaria-endemic regions of Colombia. This study was conducted to test genetic variation of *An. darlingi* at a microgeographic scale (approximately 100 km) from localities in Córdoba and Antioquia states, in western Colombia, to better understand the potential contribution of population genetics to local malaria control programs. Microsatellite loci: nuclear *white* and cytochrome oxidase subunit I (*COI*) gene sequences were analyzed. The northern *white* gene lineage was exclusively distributed in Córdoba and Antioquia and shared *COI* haplotypes were highly represented in mosquitoes from both states. *COI* analyses showed these *An. darlingi* are genetically closer to Central American populations than southern South American populations. Overall microsatellites and *COI* analysis showed low to moderate genetic differentiation among populations in northwestern Colombia. Given the existence of high gene flow between *An. darlingi* populations of Córdoba and Antioquia, integrated vector control strategies could be developed in this region of Colombia.

INTRODUCTION

Colombia has one of the higher incidences of malaria in the Americas.[1] Areas at greatest risk of malaria transmission include Urabá, the upper Sinú River, and lower Cauca River regions, and the Pacific coast.[2] In 2008, two departments/states in northwestern Colombia, Córdoba and Antioquia, corresponding to the Urabá, Bajo Cauca, and Magdalena Medio regions, reported 35% and 16% of the country's total annual report of malaria cases, respectively.[3] During 2006–2007, municipalities that had the highest levels of malaria transmission in Córdoba were Tierralta, Puerto Libertador, Montelibano, and Valencia,[4] and in Antioquia were El Bagre, Turbo, Tarazá, Caceres, Zaragoza, Necoclí, and San Pedro de Urabá.[5] High prevalences of malaria in these regions have been mainly attributed to species such as *Anopheles nuneztovari* Gabaldón and *Anopheles darlingi* Root.[6–8]

Anopheles darlingi is the most competent vector of human malaria in South America, with predominantly anthropophilic behavior, and relatively high vector capacity.[9–11] This species is widely distributed from southern Mexico to northern Argentina, but it is absent in Nicaragua and Costa Rica, and it was recently reported for the first time in Panama.[12] Population genetics studies of malaria vectors are potentially important because they can generate significant information about gene-flow patterns and population differentiation within a geographic region, and gene flow between populations may influence a vector's capacity and bionomic aspects of human-mosquito contact.[13] The prediction of the dispersal of genes important for insecticide resistance or refractoriness to the parasite[14] could improve vector control strategies.[15] *Anopheles darlingi* has been incriminated as a competent vector of all human *Plasmodium* species present in the Americas, *P. falciparum* Welch, *P. vivax* Grassi and Felleti, variants VK210 and VK247, and *P. malariae* Laveran.[10,16–18] The importance of *An. darlingi* as a human malaria vector in the Americas has resulted in several population structure studies conducted throughout its range, based on morphologic and molecular markers.[11,19,20]

Anopheles darlingi populations from Central and South America demonstrate significant morphologic and behavioral variation.[21] An earlier study on *An. darlingi* populations from seven countries in Central and South America that used isozymes, random amplified polymorphic DNA (RAPD), morphology, and ribosomal DNA internal transcribed spacer 2 (ITS2) sequences found evidence of geographic partitioning according to the RAPD–polymerase chain reaction (PCR) patterns. However, all populations were separated by relatively short genetic distances of ITS2 sequences, and the authors concluded that *An. darlingi* was a single species throughout its geographic range.[11] More recent population genetics studies that used cytochrome oxidase subunit I (*COI*)[19] and microsatellite (MS) loci[20] detected a higher level of differentiation between *An. darlingi* populations from Central and South America, providing some support for previous studies that proposed that *An. darlingi* may be a complex of species.[21–23] This discrepancy between results is likely an effect of the greater sensitivity of the DNA markers, especially DNA sequencing analyses, to detect genetic variability,[24] and concerted evolution that may be homogenizing the ITS2 sequences.[25]

There is no exclusive pattern explaining the population differentiation recently detected among the *An. darlingi* populations studied. For example, the isolation by distance model explained the differentiation among samples from Venezuela, Brazil, and Bolivia by using a restriction enzyme assay of DNA mitochondrial (mtDNA);[26] in the analysis of seven locations in Amapá, Pará, and Mato Grosso States from Brazil by using MS genotyping;[27] and in a study of nine localities from central and western Amazonian Brazil where *An. darlingi* populations at distances < 152 km showed high gene flow.[28] High significant genetic differentiation has been detected between Central America and Amazonian populations of *An. darlingi* (pairwise genetic differentiation [F_{ST}] = 0.1859–0.3901, $P < 0.05$), and this differentiation appears to be influenced mainly by physical distance among populations and by differences in

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effective population sizes.[19,20] Analyses based on the nuclear *white* gene suggested that two different lineages are present in *An. darlingi* populations throughout Central and South America; the northern lineage was found in Central America, parts of Colombia and Venezuela, and the southern lineage was found in Brazil and Peru, and both genotypes/lineages co-occur in localities in Venezuela, Peru, and Bolivia.[20,29,30]

The distribution of *An. darlingi* in Colombia appears to be irregular and interrupted by the Andes mountains; it has been found below an altitude of 500 meters and it is considered the most important malaria vector in the Urabá, Bajo Cauca, and Magdalena Medio regions and in Llanos Orientales, Amazonia, and Orinoquia.[6] Prior studies on *An. darlingi* populations from northwestern (Córdoba), western (Chocó) and southern Colombia (Meta), using amplified fragment length polymorphism (AFLP) and RAPDs estimated F_{ST} values that were congruent with high gene flow among western populations (Córdoba and Chocó) and suggested that the overall genetic differences observed (RAPD $F_{ST} = 0.084$, AFLP $F_{ST} = 0.229$) could be attributed to the biogeographic characteristics of each particular region.[31,32]

Previous studies have made significant contributions to the population structure, evolutionary processes and demographic history of *An. darlingi*. However, these studies assessed populations at a large geographic scale. Thus, we considered that detailed studies at the microgeographic level with samples collected from localities in Antioquia and Córdoba states could provide additional information of historical and current distributions of *An. darlingi*, of particular interest in zones with the highest levels of malaria transmission in Colombia. Analyses were based on eight MS markers,[20,28,33] 802 basepairs of mtDNA *COI*,[19] and restriction enzyme analysis to detect *An. darlingi white* gene lineages.[29,30]

For many years, insecticidal control has been used as an important tool to decrease vector densities in different states in Colombia.[34,35] This control makes it relevant to estimate if *An. darlingi* effective population sizes are being maintained at a low enough level to significantly reduce human–vector contact and determine whether there are signs of a bottleneck caused by frequent application of insecticides in Córdoba and Antioquia. Our study addressed four questions. 1) Which *white* gene genotype(s) are present in Córdoba and Antioquia? 2) Are *An. darlingi COI* haplotypes from Antioquia and Córdoba more genetically related to populations from Central or South America? 3) What is the level of genetic differentiation or population structure among *An. darlingi* populations from localities with the highest levels of malaria transmission in Antioquia and Córdoba? 4) Is there a signature of bottlenecks, perhaps influenced by the historical use of insecticide in these populations?

MATERIALS AND METHODS

Sampling strategy and DNA extraction. Mosquitoes genotyped were adult female *An. darlingi* collected during May 2006–July 2008 from Córdoba (Valencia [VAL], Montelibano [MTL] and Puerto Libertador [PLT]) and Antioquia (El Bagre [BAG] and Zaragoza [ZAR])[8] (Figure 1). The protocol for collection procedures was reviewed and approved by the Bioethics Committee for Human Research at University Research Center (SIU) of University of Antioquia. All

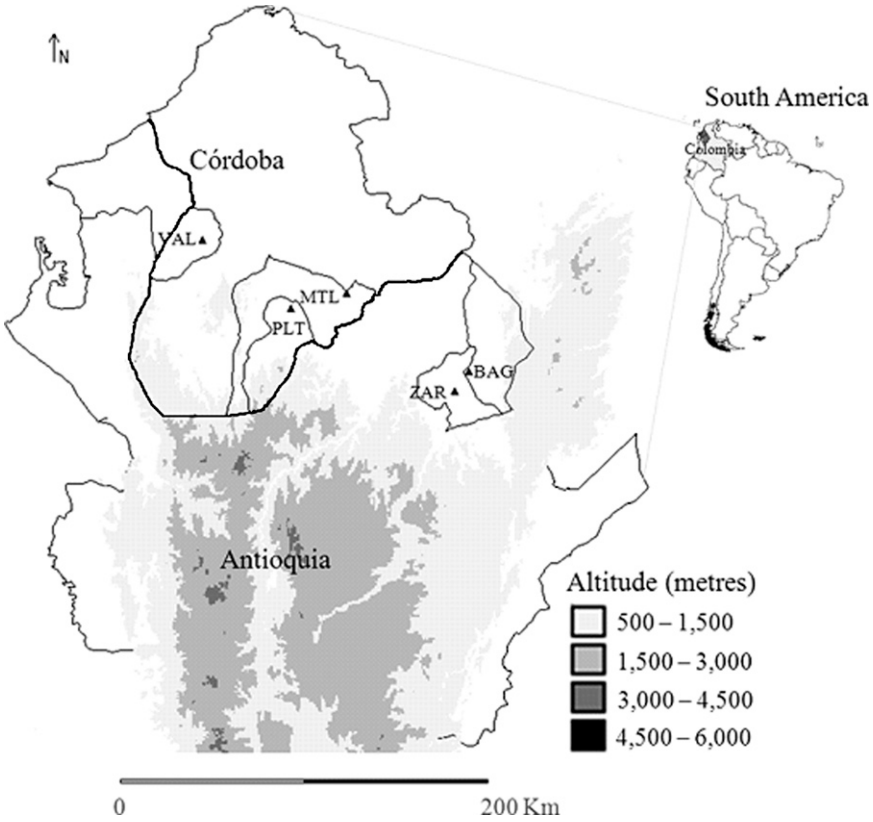

FIGURE 1. Distribution of collection localities for *Anopheles darlingi* in Antioquia and Córdoba, Colombia. The blue line shows the political division between Córdoba and Antioquia states.

collection sites were in the Magdalena biogeographic Province.[36] For MS analysis, specimens collected from VAL (n = 2) were not genotyped and specimens from ZAR (n = 8) that were genotyped were combined with samples from BAG whenever comparisons of population pairs were performed because otherwise the small sample size would not enable calculation of reliable results. For *white* and *COI* genes, all samples available per site or those randomly selected were analyzed (Table 1). *Anopheles darlingi* mosquitoes were identified by using a morphologic key[7] and were confirmed as *An. darlingi* by using an ITS2–PCR–restriction fragment length polymorphism (RFLP)–based assay.[37,38] DNA was extracted from individual mosquito abdomens by using the DNeasy Blood and Tissue Kit (QIAGEN, Duesseldorf, Germany).

***White* gene genotyping.** The PCRs for the *white* gene were performed by using PuReTaqTM Ready-To-Go™ PCR beads (GE Healthcare, Little Chalfont, United Kingdom) containing 10 µM of each primer (WF and W2R) in 25-µL volumes. An RFLP assay using enzyme *Taq*αI, which recognizes the sequence 5′-TCGA-3′,[30] was used with the nuclear *white* gene PCR product (approximately 800 basepairs) of each *An. darlingi* specimen to determine whether populations from Antioquia and Córdoba were closest to the northern and/or the southern lineage.[29,30] To verify the RFLP patterns observed by electrophoresis, an *in silico* test was implemented in Geneious software version 4.7.[39] Some PCR products from each locality were purified by using CentriSpin 40 columns (Princeton Separations, Adelphia, NJ) and sequenced in both directions by the New York State Department of Health Wadsworth Center Applied Genomic Technologies Core Facility. Nucleotide sequences were compiled and edited by using Sequencher version 3.0 (Gene Codes Corporation, Ann Arbor, MI). Eleven *white* gene sequences of *An. darlingi* obtained in this study are available in GenBank (accession nos. GQ285656–GQ285666).

***COI* PCR amplification and sequencing.** A 1,300-basepair segment of mtDNA *COI* gene was targeted for amplification by PCR by using PuReTaq Ready-To-Go PCR beads containing 10 µM of each primer (UEA3 and UEA10)[40] in 25-µL reaction volumes.[19] The PCR products were purified by using ExoSAP-IT (USB Corporation, Cleveland, OH), and the fragments were sequenced in both directions by the New York State Department of Health Wadsworth Applied Genomic Technologies Core Facility. Sequences were compiled and edited by using Sequencher version 3.0. We obtained an 802-basepair overlapping region from sequencing in both directions. Multiple alignment was performed by using ClustalX,[41] and unique haplotypes were determined by using DAMBE version 4.5.68.[42] Identical sequences were considered to be a single haplotype and are available in GenBank (accession nos. GQ285644–GQ285655).

Microsatellite genotyping. Eight dinucleotide MS markers (*ADC01*, *ADC02*, *ADC28*, *ADC29*, *ADC107*, *ADC110*, *ADC137*, and *ADC138*) previously isolated for *An. darlingi*[33] were genotyped as described.[20,28,33] The MS genotyping procedure of 118 *An. darlingi* specimens was performed by the New York State Department of Health Wadsworth Applied Genomic Technologies Core Facility. Data were analyzed using GeneMapper® Software version 4.0 (Applied Biosystems, Foster City, CA), and Peak scanner software version 1.0 (Applied Biosystems) was then used to verify the uniformity of allele size calls in the complete dataset.

Descriptive statistics and population differentiation test. Indices of genetic diversity for *An. darlingi* samples (by population and overall) were determined by using haplotype frequencies of mtDNA *COI* sequences and allelic frequencies of MS loci. Haplotype and nucleotide diversities were generated by using DnaSP version 4.50.2[43] and Arlequin version 3.11 software.[44] MICRO-CHECKER 2.2.3 software[45] was used to detect potential errors that may occur at each MS locus during genotyping or the interpretation of the data such as null alleles, stuttering, and large allele dropout. Allele and genotype frequencies of the amplified alleles were then compared and adjusted if necessary. Number of alleles (*Na*), expected heterozygosity (H_e), observed heterozygosity (H_o), allele richness (*Rs*), and Hardy-Weinberg equilibrium (HWE) were estimated for MS loci by using GenAlEx version 6.1[46] and FSTAT version 2.9.3.2.[47] Statistical significance for HWE and linkage disequilibrium (LD) for each pair of loci was assessed by using exact probability tests available in GENEPOP version 4.0.[48] The sequential Bonferroni procedure[49] was applied whenever multiple comparisons were carried out simultaneously. Inbreeding index (F_{IS}) and the number of migrants per generation (*Nm*) for each population were estimated in Arlequin. The (F_{ST}) and its significance was tested by permutation tests (10,000 replicates).

Effective population size (*Ne*) was estimated for each population by two methods: heterozygosity excess (HE) and LD by using NeEstimator software version 1.3.[50] Analysis of molecular variance (AMOVA) was used to examine population variation among and within populations by using Arlequin software. Geographical coordinates and distances of each sampling location were obtained using Google Earth® software.[51] The program SAMOVA 12.02 (Spatial Analysis of Molecular Variance) was used to identify possible genetic barriers between populations without prior information

TABLE 1

Anopheles darlingi sample size and molecular data, Colombia*

Locality (abbreviation)	Coordinates	No. of specimens analyzed by *white* gene PCR-RFLP	No. of *white* gene sequences analyzed	No. of *COI* sequences analyzed	No. of specimens analyzed by MS
Córdoba					
Valencia	8°15′N, 76°8′W	2	–	2	–
Montelibano	7°59′N, 75°25′W	23	3	20	27
Puerto Libertador	7°54′N, 75°40′W	43	3	31	43
Antioquia					
El Bagre	7°35′N, 74°49′W	8	3	27	40
Zaragoza	7°29′N, 74°51′W	57	2	8	8†
Total		133‡	11	88	118

* PCR-RFLP = polymerase chain reaction–restriction fragment length polymorphism; *COI* = cytochrome oxidase subunit I.
† Samples included only for summary of polymorphism statistics.
‡ *white* gene northern lineage.

of the sampling locations as is necessary for AMOVA.[52] Additional tests for assignment and exclusion of individuals were estimated in GeneClass 2.0 software.[53] A dendogram based on MS genetic distances was constructed by using the unweighted pair group method arithmetic average (UPGMA) cluster analysis in TFPGA program version 1.3[54] to test genetic relationships among different populations.

Demographic inference and neutrality tests. To measure deviations from the null hypothesis of constant population size and random mating, neutrality tests were conducted with *COI* sequences by using the *D* value of Tajima,[55] the D and F values of Fu and Li,[56] and the F_s value of Fu[57] with DnaSP and Arlequin software. Confidence intervals (CIs) were tested by 10,000 coalescent simulations. To test *COI* haplotype distribution, a statistical parsimony-based method, which calculates the maximum number of mutational connections between pairs of *COI* sequences by the 95% parsimony criterion was estimated in TCS software.[58] The MS analysis was also conducted to detect signs of population bottleneck(s) expected as a result of the frequency of insecticidal use in Córdoba and Antioquia. Estimates of expected heterozygosity were calculated for each sample across all loci assuming the stepwise mutation model, the infinite alleles model, and the two-phase model. Statistical significance of the deviation from mutation-drift equilibrium (MDE) was assessed by using the sign test available in Bottleneck version 1.2.02.[59] If a significant number of loci show $He > Heq$, this finding indicates that the population recently experienced a bottleneck. Conversely, $He < Heq$ may suggest population expansion.

Phylogenetic relatedness with populations from Central and South America. More complex evolutionary scenarios are poorly described with only a phylogenetic tree. However, phylogenetic networks are useful tools for inferring different types of reticulate events such as hybridization, horizontal gene transfer, recombination, or gene duplication and loss, therefore, enabling a more comprehensive evaluation of the relationships between haplotypes in the populations.[60] In this study, a neighbor-net network approach was estimated for the complete data set from Colombia and *An. darlingi COI* sequences available in GenBank (accession nos. DQ298209–DQ298244, DQ076235, and DQ076236) from several sites of Central and South America,[19,61] by using SplitsTree4 version 4.10.[60,62] A *COI* gene sequence of *An.* (*Nyssorhynchus*) *albimanus* Wiedemann (GenBank accession no. FJ015205) was included as the outgroup.[63] The neighbor-net network combines aspects of the neighbor-joining and SplitsTree from inferred distance matrices. Thus, it combines taxa into progressively larger and larger overlapping clusters (similar to neighbor-joining), constructs networks rather than trees, and has the advantage of being time efficient and appropriate for the preliminary analyses of complex phylogenetic data.[60,62]

RESULTS

The *white* gene RFLP assay applied to 133 *An. darlingi* specimens from Córdoba and Antioquia and the *in silico* analyses of 11 *white* gene sequences showed that the northern lineage was exclusively distributed in this region (Table 1 and Figure 2).

Genetic diversity at the intra-population level. An 802-basepair sequence of the *COI* gene at position 1,820–2,621 of the *An. gambiae* s.s. Giles mitochondrion complete genome

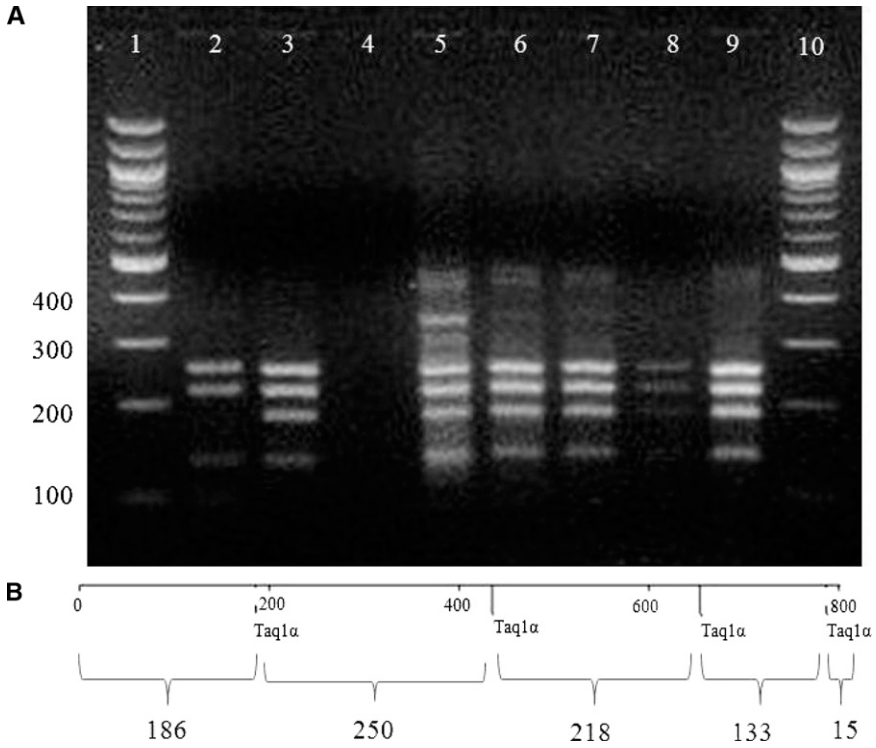

FIGURE 2. Analysis of the *white* gene by polymerase chain reaction (PCR)–restriction fragment length polymorphism. **A**, Band patterns corresponding to *white* gene lineages on a 1.5% agarose gel. Lanes 1 and 10, molecular mass marker; lane 2, pattern of southern lineage of *Anopheles darlingi* from Bolivia; lane 3, pattern of northern lineage of *An. darlingi* from Belize; lane 4, PCR negative control; lanes 5–9, *An. darlingi* samples from Córdoba and Antioquia (5 = MTL, 6 = PLT, 7 = BAG, 8 = VAL, 9 =ZAR). **B**, *In silico* test based on *white* gene DNA sequences to verify patterns observed in the agarose gel (250, 218, 186, 133, and 15 basepairs).

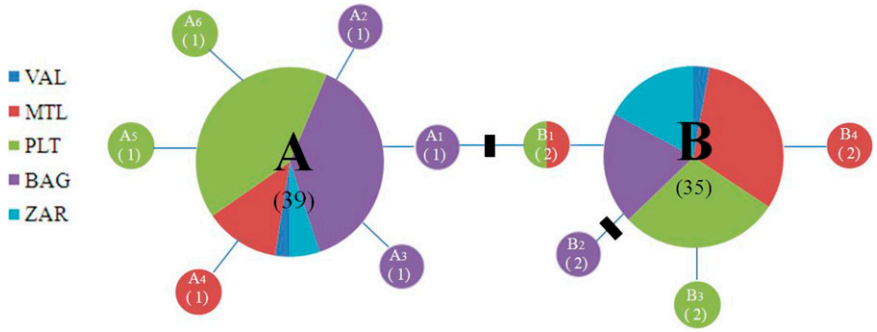

FIGURE 3. Parsimony-based haplotype networks of 12 cytochrome oxidase subunit I haplotypes representing 88 *Anopheles darlingi* mosquitoes. The color of the circles and numbers in parentheses depict the origin and total frequency of each haplotype, respectively. The black bar represents one mutational step. This figure appears in color at www.ajtmh.org.

and aligned with RefSeq NC 002084,[64] was analyzed for 88 *An. darlingi* mosquitoes from five localities (Figure 1 and Table 1). *COI* sequences alignment identified 13 variable sites; 8 were parsimony informative. Nucleotide and haplotype diversity values were similar in all populations from Córdoba and Antioquia, except for VAL and ZAR, which had low sample sizes. There were 12 unique haplotypes, 2 shared between all the localities (named A and B in order of frequency), and 1 shared between MTL and PLT; the others were exclusive to a particular geographic location (Figure 3 and Table 2).

All MS loci were polymorphic in all collection sites and analysis in Micro-checker (95% CIs for Monte Carlo simulations) did not suggest evidence of scoring error caused by stuttering or large allele dropout. Several loci showed evidence of null alleles, as suggested by the general excess of homozygotes for most allele size classes. Analyses for MS datasets from each population indicated significant homozygote excess at loci: *ADC01* (MTL, PLT), *ADC29* and *ADC138* (all populations), *ADC107* (PLT, BAG, and ZAR). Similarly, these populations were not at HWE with loci *ADC01*, *ADC29*, *ADC107*, and *ADC138* showing signs of null alleles. Deviation from HWE caused by heterozygote deficits ($P < 0.05$ after Bonferroni correction) was detected in all loci except *ADC137* (Table 3). No linkage disequilibrium was detected between MS loci after Bonferroni correction ($P < 0.05$).

The number of alleles per locus ranged from 2 to 18: locus *ADC138* showed the lowest value and *ADC01* the highest. Allelic richness per locus ranged from two in ZAR and BAG (*ADC138*) to eight in MTL and BAG (*ADC01*). In general, all localities showed a similar average allelic richness, the lowest value was 5.649 (MTL) and the highest was 6.415 (BAG). The expected heterozygosity across all samples ranged from 0.311 (MTL) to 0.892 (MTL and BAG), and the average expected heterozygosity ranged from 0.720 (MTL) to 0.784 (BAG) (Table 3). The frequency of null alleles at each locus was estimated and adjusted (whenever it was significant, $P > 0.05$), in the data set used for population genetic analysis. In addition, analysis was conducted that excluded information for loci *ADC01*, *ADC29*, *ADC107*, and *ADC138* because those loci showed significant null allele frequencies. However, there were no significant differences between these results and those obtained by using the whole data set.

Under LD, the average *Ne* calculated was 65.8 individuals (95% CI = 41.5–140.8) for MTL, 463.5 individuals (95% CI = 160–α) for PLT, and an infinite number of individuals (95% CI = 338.6–α) for BAG. The average *Ne* for both populations of Córdoba was 4,851 individuals (95% CI = 415–∞), and for both populations of Antioquia there was an infinite number of individuals (95% CI = 1,023–∞). In agreement, the average N_e estimated for each population under HE showed an infinite number of individuals.

Genetic differentiation among populations. To conduct comparisons of population pairs using *COI* and MS loci, we combined samples from BAG and ZAR (Table 1); these sites are only 11 km apart and the preliminary *Nm* estimate detected infinite migrants between them. The F_{ST} estimates indicated low genetic differentiation (MS $F_{ST} = 0.01202$ and *COI* $F_{ST} = 0.02228$) among Córdoba and Antioquia populations. Comparisons between MTL and BAG/ZAR showed the highest degree of differentiation when *COI* and MS data were used. Overall, F_{ST} and *Nm* values indicated low genetic differentiation and high gene flow, respectively, among *An. darlingi* populations from Córdoba and Antioquia (Table 4).

AMOVA conducted with all populations in one group (samples from BAG/ZAR combined as one population) and based on *COI* haplotype frequencies showed 2.23% ($P = 0.14$) of the

TABLE 2

Description of *COI* shared haplotypes and statistics of genetic polymorphisms for *Anopheles darlingi* from Córdoba and Antioquia, Colombia*

Population	*COI* haplotypes	*S*	*h*	*Hd* (SD)	*Pi* (SD)
Valencia	**A(1)**, **B(1)**	4	2	1.000 ± 0.500	0.00499 ± 0.00249
Montelibano	**A(5)**, **A4(1)**, **B(11)**, B1(1), B4 (2)	6	5	0.653 ± 0.093	0.00261 ± 0.00045
Puerto Libertador	**A(16)**, A5(1), A6 (1), **B(10)**, B1(1), B3(2),	7	6	0.643 ± 0.064	0.00281 ± 0.00024
Córdoba	**A(22)**, A4(1), A5(1), A6 (1), **B(22)**, B1(2), B3(2), B4 (2)	9	8	0.663 ± 0.041	0.00286 ± 0.00013
El Bagre	**A(15)**, A1(1), A2(1), A3(1), **B(7)**, B2(2)	8	6	0.638 ± 0.081	0.00287 ± 0.00043
Zaragoza	**A(2)**, **B(6)**	4	2	0.429 ± 0.169	0.00214 ± 0.00084
Antioquia	**A(17)**, A1(1), A2(1), A3(1), **B(13)**, B2(2)	8	6	0.639 ± 0.053	0.00294 ± 0.00025
All sites	**A(39)**, **B(35)**	13	12	0.650 ± 0.033	0.00288 ± 0.00012

* *COI* = cytochrome oxidase subunit I; *S* = number of segregating sites; *h* = number of haplotypes; *Hd* = haplotype diversity; *Pi* = nucleotide diversity; SD = standard deviation. Numbers in parentheses are the frequencies of haplotypes by state or locality. Haplotypes in **bold** are shared between Córdoba and Antioquia populations.

TABLE 3
Summary of polymorphism statistics for microsatellite loci analyses for *Anopheles darlingi* from Córdoba and Antioquia, Colombia*

Populations/loci		*ADC01*	*ADC02*	*ADC28*	*ADC29*	*ADC107*	*ADC110*	*ADC137*	*ADC138*	All loci
MTL	N	27	27	27	26	20	27	27	25	26
	Na	15	9	6	10	6	6	11	5	9
	Rs	8.684	5.453	5.367	7.399	3.137	4.783	6.540	3.831	5.649
	He	0.892	0.702	0.808	0.869	0.311	0.772	0.840	0.567	0.720
	Ho	0.519†	0.704	0.778	0.346†	0.250‡	0.630	0.852	0.160†	0.530
	F_{IS}	0.434	0.016	0.056	0.614	0.221	0.203	0.004	0.728	0.283
PLT	N	42	43	43	43	40	43	40	41	42
	Na	14	8	13	14	10	7	9	6	10
	Rs	7.975	5.494	7.001	7.577	4.478	4.509	6.277	3.610	5.865
	He	0.889	0.738	0.859	0.858	0.577	0.705	0.829	0.485	0.742
	Ho	0.595†	0.698	0.814	0.465†	0.300†	0.698	0.825	0.171†	0.571
	F_{IS}	0.341	0.066	0.065	0.467	0.489	0.022	0.017	0.655	0.243
BAG	N	39	40	40	38	36	39	40	35	38
	Na	18	12	10	16	17	5	10	3	11
	Rs	8.535	6.360	7.204	7.683	7.796	4.727	6.661	2.356	6.415
	He	0.892	0.758	0.873	0.839	0.838	0.786	0.850	0.436	0.784
	Ho	0.821	0.675†	0.825‡	0.368†	0.333†	0.692‡	0.900	0.086†	0.588
	F_{IS}	0.093	0.122	0.067	0.570	0.611	0.132	–0.046	0.808	0.263
ZAR	N	8	8	8	8	8	8	7	8	8
	Na	7	6	8	7	8	5	7	2	6
	Rs	6.625	5.500	7.600	6.725	7.358	4.875	7.000	2.000	5.960
	He	0.820	0.672	0.844	0.813	0.758	0.750	0.796	0.375	0.728
	Ho	0.875	0.500	0.875	0.375§	0.375†	0.875	1.000	0.000§	0.609
	F_{IS}	0	0.317	0.030	0.584	0.553	–0.101	–0.183	1	0.228
All sites	N	29	30	30	29	26	29	29	27	29
	Na	21	12	13	20	23	7	11	7	9
	Rs	8.292	5.818	6.864	7.814	5.918	4.727	6.584	3.459	6.185
	He	0.873	0.717	0.846	0.845	0.621	0.753	0.829	0.466	0.744
	Ho	0.702	0.644	0.823	0.389	0.315	0.724	0.894	0.104	0.574

* MTL = Montelibano; PLT = Puerto Libertador; BAG = El Bagre; ZAR = Zaragoza; N = number of mosquitoes; *Na* = number of alleles; *Rs* = allele richness; H_e = expected heterozygosity; H_o = observed heterozygosity, F_{IS} = inbreeding coefficient. All sites = mean values over loci or collecting sites. Significant differences from H_e according to exact tests against Hardy-Weinberg proportions after the sequential Bonferroni correction are indicated.
† $P < 0.001$.
‡ $P < 0.05$.
§ $P < 0.01$.

total variance among populations and 97.77% within populations. The MS data showed 1.31% ($P = 0.002$) variation among populations and 98.69% within populations. These results are consistent with the SAMOVA analysis based on MS and *COI*, in not detecting genetic barriers among populations from Córdoba and Antioquia. The UPGMA dendogram based on MS genetic distances showed that the BAG/ZAR population was closer to that of PLT than to that of MTL (Figure 4).

Results of the assignment statistics showed that 78% (92 of 118) of the individuals were correctly assigned to their original reference site. All individuals from ZAR were correctly assigned, and MTL, PLT, and BAG showed 93%, 72%, and 70% correct assignment, respectively. Mis-assignments occurred as follows: 1 individual from MTL was assigned to PLT and 1 to BAG; 6 individuals from PLT were assigned to MTL, 5 individuals to ZAR, and 1 individual to BAG; and 6 individuals from BAG were assigned to PLT, 5 individuals to ZAR and 1 individual to PLT.

Demographic inference and neutrality tests. None of four neutrality tests (*D* value of Tajima, D and F values of Fu and Li, and F_s value of Fu) found populations from Antioquia and Córdoba having statistically significant values. The *COI*-based network showed two most common interior haplotypes, A (n = 39) and B (n = 35), were found in all collection sites, and they included 84% of total sequences analyzed and differed only by one mutational step. In addition, haplotype B1 was shared between MTL and PLT (Figure 3 and Table 2). Only a few haplotypes from both regions were tip alleles, showing high demographic stability and free gene flow between

TABLE 4
Estimates of pairwise genetic differentiation (F_{ST}) and gene flow (*Nm*) among populations of *Anopheles darlingi* from Córdoba and Antioquia, Colombia*

	COI			MS		
Populations	MTL	PLT	BAG/ZAR	MTL	PLT	BAG/ZAR
MTL	_	7	11		37	33
PLT	0.06		α	0.01†		42
BAG/ZAR‡	0.08	–0.02		0.01496†	0.01173†	

* Values above the diagonal are N_m values and values below the diagonal are F_{ST} values. *COI* = cytochrome oxidase subunit I; MS = microsatellite; MTL = Montelibano; PLT = Puerto Libertador; BAG = El Bagre; ZAR = Zaragoza; α = infinite value.
† $P < 0.05$.
‡ Samples from BAG and ZAR were combined for these analyses values.

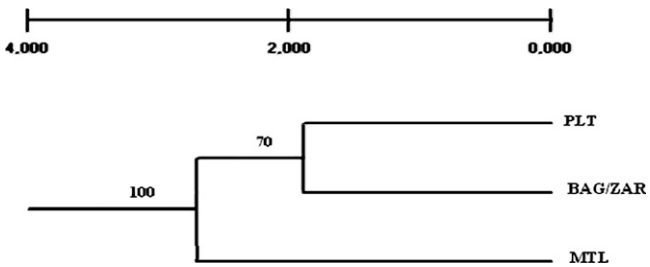

FIGURE 4. Unweighted pair group method arithmetic average dendogram based on microsatellite data for populations of *Anopheles darlingi* analyzed from Córdoba and Antioquia. Samples from BAG and ZAR were combined for this analysis.

populations from Antioquia and Córdoba, which supported demographic equilibrium in these populations. Although the heterozygote test based on MS data under the infinite alleles model, the two-phase model, and the stepwise mutation model showed some loci with heterozygote excess. Overall, estimates of the distribution of the heterozygosity expected under the assumption of MDE were not statistically significant. Once all loci were processed, the allele frequency distribution established for each population was the normal L-shaped distribution, which indicates that the allele frequency distribution in populations from Antioquia and Córdoba is as expected under MDE, and they can be considered stable populations.[65]

Phylogenetic relatedness with populations from Central and South America. Phylogenetic neighbor-net network (Figure 5) based on *COI* haplotypes from Central and South America and those from the present study illustrated marked complexity and ambiguity in the signal. Because this *An. darlingi* phylogenetic network represents various feasible topologies (areas full of boxes), indicating the relationships among specimens from different sites from Central and South America, the result suggests that a more intense sampling should be conducted to gain a clearer picture of the general population structure and evolutionary history of this species through its distribution range.[63] Haplotype M found by Mirabello and Conn[19] in Nechi, a locality in Antioquia, was identical to haplotype B found widely dispersed in the present work (Figures 3 and 5). Variations were noticeably greater between Central American (including Colombia) sequences and those from Brazil and Peru. Thus, the Central American and Colombian cluster compared with Amazonian South American cluster identified by Mirabello and Conn[19] were represented clearly, supporting their analysis and conclusions.

DISCUSSION

Our analyses showed that *An. darlingi* populations from Córdoba and Antioquia in Colombia were exclusively of the *white* gene northern lineage. In agreement with previous findings,[29,30] *white* gene lineages may co-occur only southeast of the Andes Mountains in the Llanos Orientales, Amazonia, and Orinoquia regions. Thus, the collection of additional data in southern and southeastern Colombia should be undertaken to formally test this hypothesis.

Both *COI* and MS data showed moderate to low genetic differentiation between *An. darlingi* populations from the localities with higher malaria transmission in Antioquia and Córdoba. Similar to our data, *An. darlingi* studies conducted at the microgeographic level (within approximately 150 km) in the Peruvian Amazon Region[66] and in the Brazilian Amazon Region,[15,67] have shown that *An. darlingi* populations were highly homogeneous, and there were no clear correlations among genetic and geographic distance.[15]

As in previous reports,[20,28,33] the MS markers used in the present work have not been physically mapped to *An. darlingi* chromosomes. Their location in relation to chromosome inversions is unknown; thus, neutrality cannot be assumed.[68] The MS loci were highly polymorphic, and populations from Córdoba

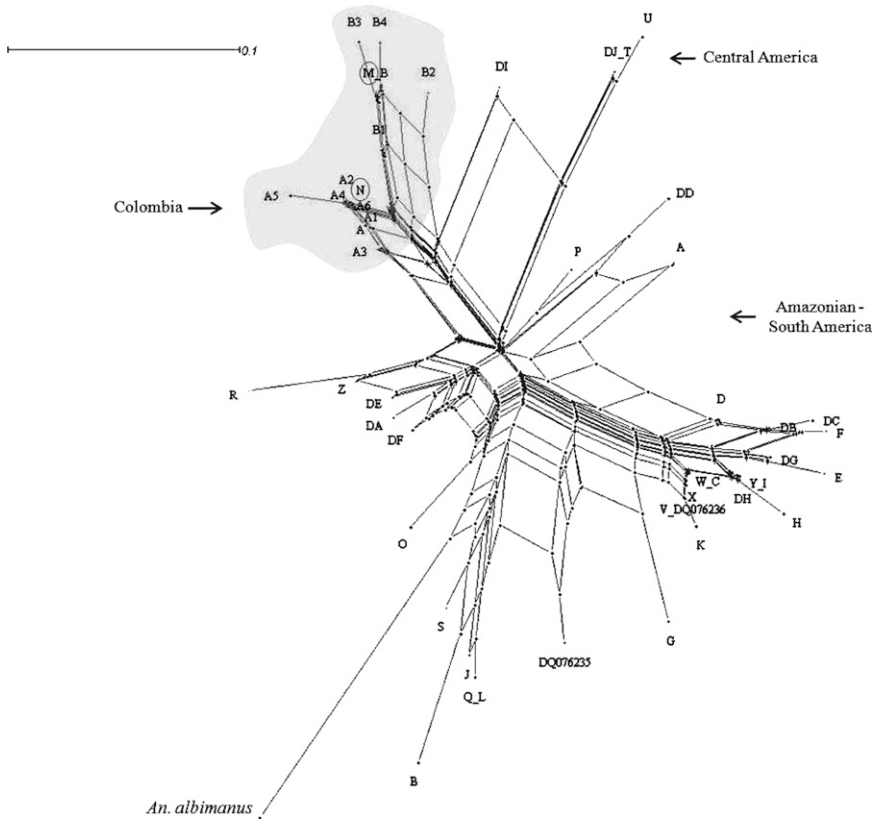

FIGURE 5. Neighbor-Net network for 45 *Anopheles darlingi* cytochrome oxidase subunit I haplotypes from Central and South America. Haplotypes from Córdoba and Antioquia populations are included in the gray section, and shared haplotypes M and N from Mirabello and Conn[19] are circled. The line for *An. albimanus* was shortened for illustration purposes.

and Antioquia had relatively high levels of molecular diversity, as demonstrated by the number of *COI* haplotypes and MS alleles. The levels of *COI* nucleotide diversity for all populations analyzed were higher than those previously estimated for *An. darlingi* from Nechí in Antioquia, a locality near BAG and ZAR.[19] This finding may indicate that genetic diversity for *An. darlingi* from Nechí, Colombia could had been underestimated because of the small sample size analyzed.

In the *An. darlingi* populations from Córdoba and Antioquia, no LD was detected but significant heterozygote deficits were identified in 53.13% of the HWE tests. Some potential causes of decreased heterozygosity relative to expected HW proportions are selection against heterozygotes, inbreeding, Wahlund effect over space or time, null alleles, and/or allele dropout in old samples.[69] Inbreeding as the potential origin of heterozygote deficits is not considered because a pattern affecting all loci homogeneously was not identified. In addition, there was no evidence of subpopulation structure or of large allele dropout. Thus, the heterozygote deficits observed seem to be mainly influenced by null allele incidence.

Overall, populations studied in Córdoba were genetically closer, as were populations from Antioquia. Consequently, cluster analyses based on MS data, such as SAMOVA, UPGMA and sample assignment tests, showed that substantial gene flow exists between these populations. Thus, samples from Córdoba and Antioquia could be considered as a single population. Localities included in this study are part of the Magdalena biogeographic Province,[36] which comprises western Venezuela and northwestern Colombia and it is characterized by temperatures > 24°C, plains (altitude < 500 meters), fragmented forest, presence of livestock, and active agriculture and mining.[70,71] Mining excavations had not been reported as traditional breeding sites for *An. darlingi* in Colombia. However, in the Amazon region, human settlements and mining activities have been associated with an increase in *An. darlingi* densities.[72]

The level of *COI* nucleotide diversity was similar among populations from Córdoba and Antioquia, and most haplotypes were shared between regions (Figure 3), which indicated that *An. darlingi* populations from these two states in Colombia share a common demographic history.[73] In general, neutrality tests and parsimony-based haplotype networks of *COI* and heterozygosity tests based on MS data showed demographic equilibrium with no signals of a bottleneck in populations from Córdoba and Antioquia, even though such signals were predicted after intense insecticide use. A high initial (prior to widespread insecticide use) mosquito-effective population size, combined with high gene flow, may have precluded regional bottlenecks from occurring. Population characteristics detected seem not to be exclusively based on geographic proximity, but may be highly influenced by a common demographic history and high rates of migration favored by the concurrence of ecologic and geographic conditions among regions.

In addition, the high gene flow detected could explain the important role of *An. darlingi* as a malaria vector in both states. At the same time, the migration capacity of *An. darlingi* and large effective population sizes could increase the risk for malaria transmission in areas near its current distribution in Colombia. Previous work on *An. albimanus*, another important malaria vector in Colombia, detected moderate to low genetic differentiation among populations from the Caribbean and the Pacific regions, including sites approximately 200 km apart.[74] Data for *An. albimanus* and *An. darlingi* suggest that populations of these important malaria vectors may be difficult to control, i.e., both species show high effective populations sizes and high gene flow between their populations.

Nucleotide diversity values from populations in Colombia were higher than those from Guatemala and Belize,[19] suggesting that populations from Colombia may be older than the Central American ones. Although the neighbor-net analyses based on *COI* haplotypes from Central and South America, which represents the relationship between specimens from different sites, showed significant ambiguity and complexity in the data, *COI* haplotypes from northwestern Colombia clustered more closely to populations from Central America in comparison to those from Brazil, Peru, and French Guiana (Figure 5). Our data agree with previous work conducted on *An. darlingi* populations from three regions in western and southeastern Colombia, in localities from Antioquia, Chocó, and Meta,[31,32] although those studies did not examine the same molecular markers we examined in our study. Our data also showed that high gene flow among *An. darlingi* populations from western Colombia was confirmed in Córdoba and Antioquia.

In this work and in previous studies of *An. darlingi* that used MS markers, nuclear genes, or mtDNA,[19,20,75] collections were not conducted in eastern and southern Colombia. Therefore, we suggest that additional sampling is necessary, mainly in southern Colombia and also in Panama, to test previous hypotheses of migratory routes of this important vector throughout its range in America. Also, it would be important to elucidate patterns of gene flow between Colombia and Panama, where *An. darlingi* was recently reported.[12]

Studies such as the present one, which were based on various molecular markers and focused on a particular malaria vector and a highly affected malaria region, can provide answers to issues of vector control procedures. The analysis at the microgeographic level of *An. darling* from Antioquia and Córdoba showed that these are panmictic populations, and although current malaria vector control policies are conducted independently by each state in Colombia,[76] our data suggest a change of strategy. This study provides more than a simple picture of the genetic diversity of *An. darlingi*, suggesting the existence of high gene flow between *An. darlingi* populations of Córdoba and Antioquia. Therefore, we suggest that measures to decrease mosquito densities should be applied in coordination among authorities from both states.

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