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Evidence for Pleistocene Population Divergence and Expansion of *Anopheles albimanus* in Southern Central America

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Abstract. The micro-geographic structure of *Anopheles albimanus* was studied in southern Central America using partial sequences of the mtDNA cytochrome oxidase subunit I gene (COI). Analysis of molecular variance supported significant genetic structure between populations from Costa Rica and western Panama versus those from central-eastern Panama ($\Phi_{CT} = 0.33$), whereas the within group divergence was shallow and statistically insignificant ($\Phi_{ST} = 0.08$). Furthermore, a statistical parsimony network depicted three divergent groups of haplotypes that were not evenly distributed across the study area. Our findings are in partial agreement with previous studies, yet they do not support physical barriers to gene flow or contemporary isolation by distance in this region. Instead, three co-occurring groups of *An. albimanus* may be the result of multiple introductions, most likely caused by historical fragmentation and subsequent secondary contact. In addition, the molecular signature of population expansion of *An. albimanus* was detected in central-eastern Panama approximately 22,000 years ago (95% confidence interval [CI] 10,183–38,169). We hypothesize that the population structure of *An. albimanus*, as determined by our COI locus analysis, is the result of late Pleistocene climatic changes in northern South America.

INTRODUCTION

Malaria prevalence has decreased overall during the last decade in Central America.[1] However, Panama suffered a major outbreak from 2001 to 2005, which represented 60% of the total number of malaria cases reported in the past 35 years.[2] The main vector in Panama is *Anopheles albimanus*; nevertheless, six other *Anopheles* species involved in malaria transmission elsewhere in the Americas have been recorded in Panama.[3] Malaria is currently under control, but an influx of non-immune travelers into endemic areas, the presence of drug-resistant *Plasmodium falciparum*,[4] and the recent discovery of *Anopheles darlingi* in eastern Panama,[5] are expected to worsen the situation. Studies on the population structure of malaria vectors are important to determine the extent of genetic exchange among populations and to predict the spread of genes conferring insecticide resistance.[6,7] In theory, focal control interventions against mosquitoes would only be effective if mosquito immigration from outside the target area is low. In addition, such studies may provide key information on lineage divergence, discrete populations, and timing of demographic phenomena, all of which can influence the involvement of vectors in malaria transmission.[8–10]

Anopheles albimanus is a malaria vector in the subgenus *Nyssorhynchus* with considerable ecological adaptability and broad, mostly coastal, geographic distribution in the Neotropics.[11] Generally, *An. albimanus* is crepuscular, zoophilic, exophagic, exophilic, and seasonally abundant.[12] However, heterogeneity in environmental factors, host availability, and vector control strategies across the species range may lead to population differentiation. Although several studies have shown that *An. albimanus* is a single taxon,[13–16] there is evidence for significant intraspecies variation in host-feeding behavior, *Plasmodium* susceptibility, longevity, and insecticide resistance.[17–19] These differences may affect the ability to transmit *Plasmodium* sp. and the response to vector control strategies. For example, in Belize, *An. albimanus* is regarded as a secondary vector because it seldom enters human habitations, and prefers to feed on domestic animals.[19,20] However, domestic animals are rarely encountered in some areas of Central America, where indigenous people reside and malaria is endemic. In these situations, high numbers of *An. albimanus* and exposure to mosquito bites caused by poor housing construction may enhance its importance as a vector.[3,5]

Previous research on the population genetics of *An. albimanus* using mitochondrial DNA (mtDNA) ND5 sequences found low to moderate differentiation between Central and South American populations, attributed mainly to isolation by distance (IBD).[21] In contrast, analysis of microsatellite loci using the same sample localities showed a small effect of distance, but uncovered differences in allele frequencies between Costa Rica and eastern Panama.[22] The discrepancy in allele frequencies may be attributed either to the mountain range that crosses Costa Rica and western Panama, which may act as a barrier to gene flow, or to population contraction in Panama caused by intense insecticide use.[22] Nonetheless, the analysis of only four populations in Costa Rica and Panama in the latter studies may have produced inaccurate results for the IBD analysis, and demographic history (e.g., stability of the population size) and natural selection (e.g., neutrality), which are known to influence inferences of population structure in *Anopheles* mosquitoes,[8,10] were not explored in these studies.

Intensive chemical and physical larval control (e.g., source reduction) was carried out in Panama during and after the construction of the Panama Canal (1904–1914). Likewise, Panama has used nationwide residual insecticide spraying (IRS) with DDT, propoxur, fenitrothion, deltamethrin, and cyflutrin since 1957 as the main control method for malaria vectors. To date, little is known about the impact of these control strategies on the population structure of *An. albimanus* and other malaria vectors. The mtDNA has proven to be extremely useful in identifying the population structure and demographic history in malaria vectors because of its lack of recombination and

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high levels of variability, and it has been used extensively to study anopheline mosquitoes in the Neotropics, allowing for comparison across species and geographic areas.[8,21–25] In this study, we use partial sequences of the mitochondrial gene, cytochrome oxidase subunit I (COI), and extensive sampling throughout southern Central America, to address the following questions: 1) Is there genetic differentiation in *An. albimanus* at this micro-geographic scale?; and if so 2) What are the main factors driving this differentiation (e.g., geographic distance, physical barriers to dispersal, natural selection, and/or demographic phenomena)?

MATERIALS AND METHODS

Mosquito collection. Females of *An. albimanus* were collected outdoors by human landing catches and using Centers for Disease Control (CDC) miniature light traps (model 2848, BioQuip Products, Inc., Rancho Dominguez, CA) from 16 localities spanning roughly 1,100 km of a northwestern-southeastern transect across southern Central America (Figure 1; Table 1). The landing catch protocol was reviewed and approved by a Human Ethics Review Board at McGill University, in Canada and the Instituto Commemorativo Gorgas de Estudios de la Salud (ICGES), in Panama City. All mosquitoes were killed with chloroform, identified to species level in the field with the morphological key of Wilkerson and Strickman,[26] and stored in desiccant within 24 hours of capture. Samples were hand carried to the United States and stored at −80°C at the Griffin Laboratory until molecular work was performed.

Molecular procedures. Eight to 25 mosquitoes were analyzed from each locality and samples from different days, collectors, and sampling methods were randomly included in the DNA analysis. We followed the procedures used by Mirabello and Conn[25] for DNA isolation and polymerase chain reaction (PCR) amplification. We used the primers: UAE3/UAE10 (5′ TATAGCATTCCCACGAATAAATAA-3′/5′ TCCAATGCACTAATCTGCCATATTA-3′)[27] to amplify a 1,300-bp fragment of the COI gene. The PCR products were cleaned using CentriSpin 40 columns (Princeton Separations,

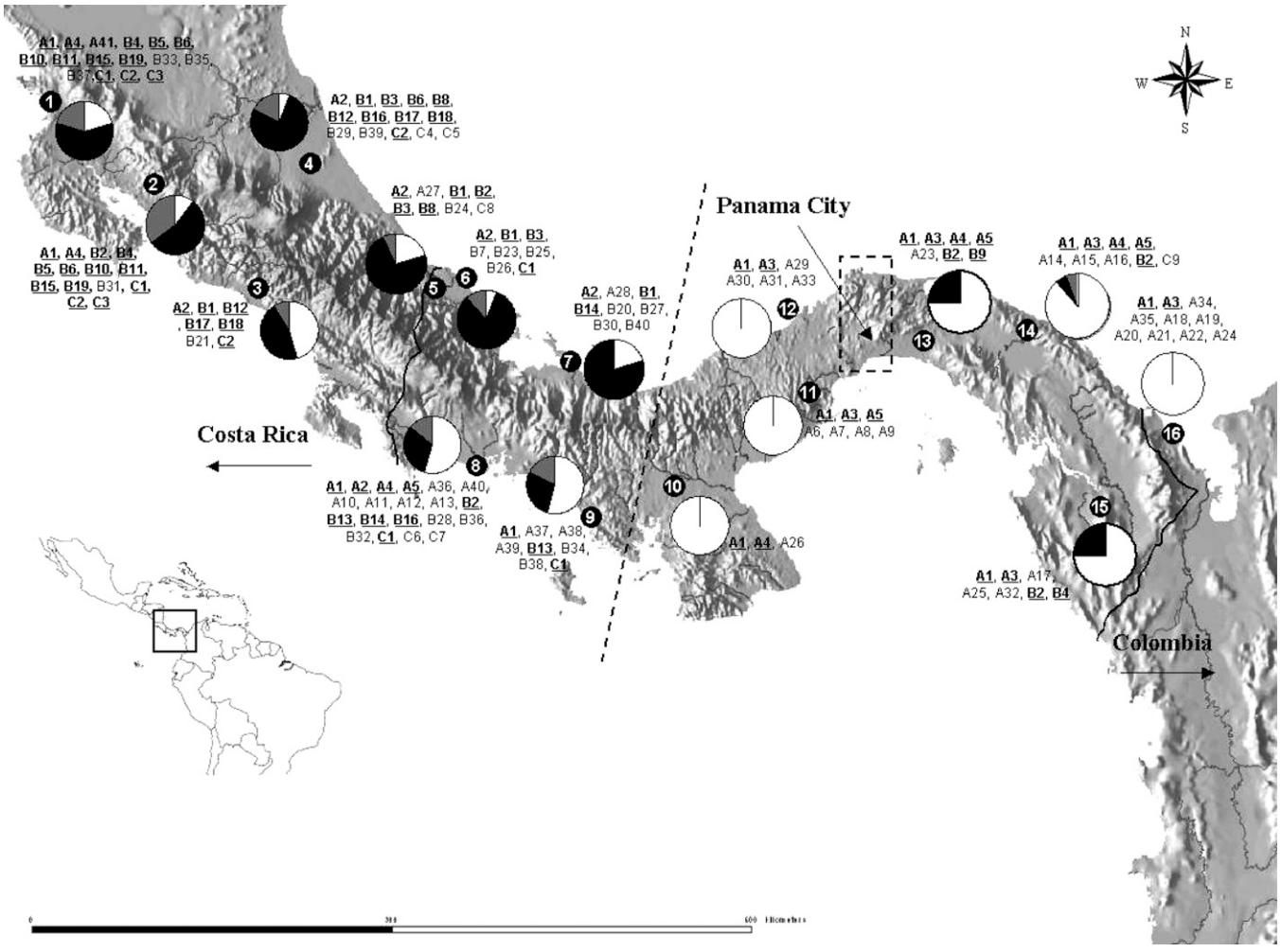

FIGURE 1. Geographic distribution of the COI haplotypes in Central America. The letters correspond to the haplotype(s) observed for each locality. The circled numbers correspond to the localities in Table 1, positioned on the map according to the longitude and latitude of the site. Underlined bold letters depict shared haplotypes, and plain letters depict private haplotypes. Circles represent the distribution of groups A (white), B (black), and C (shaded) in the network. The dotted rectangle shows the area of the former Panama Canal Zone. Dashed line divides Costa Rica and western Panama (localities 1–9), from central-eastern Panama (10–16). Inset map depicts the geographic position of the study area.

TABLE 1

Anopheles albimanus collection localities and haplotype frequency*

Locality	n	Latitude/longitude	Frequency
1. Soley	21	10.98333 N, −85.68333 W	**A1**(2), **A4**, A41, **B4**(2), **B5**, **B6**, **B10**, **B11**, **B15**, **B19**, B33, B35, B37, **C1**(4), **C2**, **C3**
2. Palo Verde	21	10.36667 N, −85.13333 W	**A1**, **A4**, **B2**, **B4**(3), **B5**, **B6**, **B10**, **B11**, **B15**, **B19**, B31, **C1**(4), **C2**(2), **C3**
3. Dominical	11	9.31333 N, −83.95133 W	**A2**, **B1**(4), **B12**, **B17**(3), **B18**, B21, **C2**
4. Matina	19	10.25331 N, −83.51667 W	**A2**(2), **B1**(2), **B3**, **B6**, **B8**, **B12**(2), **B16**, **B17**, **B18**, B29, B39, **C2**, C4, C5
5. Puente Blanco	15	9.45337 N, −82.66661 W	**A2**(2), A27, **B1**(3), **B2**(3), **B3**(3), **B8**, B24, C8
6. California	17	9.48333 N, −82.48333 W	**A2**, **B1**(8), **B3**, B7(2), B23, B25, B26, **C1**(2)
7. Rio Diablo	10	9.06667 N, −81.75333 W	**A2**, A28, **B1**(2), **B14**, B20 (2), B27, B30, B40
8. La Barqueta	22	8.35661 N, −82.48333 W	**A1**(3), **A2**, **A4**, **A5**, A36, A40, A10, A11, A12, A13, **B2**, **B13**, **B14**, **B16**, B28, B36, B32, **C1**, C6, C7
9. Las Lajas	12	8.11667 N, −81.73363 W	**A1**(4), A37, A38, A39, **B13**, B34, B38, **C1**(3)
10. Las Colomas	9	7.68333 N, −80.93333 W	**A1**(6), **A4**, A26
11. Santa Clara	16	8.46667 N, −80.31661 W	**A1**(8), **A3**, **A5**, A6, A7, A8, A9
12. Rio Indio	24	9.15111 N, −80.44441 W	**A1**(17), **A3**(2), A29, A30, A31, A33
13. Bayano	25	9.13333 N, −78.33367 W	**A1**(18), **A3**, **A4**, **A5**, A23, **B2**, B9(5)
14. Ukupa	17	9.11667 N, −77.93333 W	**A1**(5), **A3**(2), **A4**(4), **A5**, A14, A15, A16, **B2**, C9
15. Yaviza	8	0.98333 N, −77.51667 W	**A1**(2), **A3**, A17, A25, A32, **B2**, **B4**
16. Zapsurro	18	0.61667 N, −77.31667 W	**A1**(9), **A3**, A34, A35, A18, A19, A20, A21, A22, A24

* Frequency = haplotype frequency; only those with $N = 2$ or more are in parentheses following haplotype designation; underlined bold letters are shared haplotypes, and plain letters are private haplotypes in that population; n = mosquitoes per locality. Haplotypes labeled A1–A41; B1–B41, and C1–C9 belong to groups A, B, and C in the network, respectively. Costa Rica and western Panama (localities 1–9), central-eastern Panama (10–16).

Freehold, NJ), and sequencing was carried out by the Wadsworth Center Genomics Core Facility (New York State Department of Health). Sequences were unambiguously aligned with Sequencher 3.0 (Gene Codes Corp, Ann Arbor, MI), grouped together by site, and trimmed in PAUP, version 4.0.[28] Sequence fragments of 824 bp (only the overlap between the two primers), spanning positions 1873–2698 of the mitochondrial genome of *An. gambiae*, were used to determine unique haplotypes in MacClade, version 3.0.[29] The DNA sequences were checked for stop codons and confirmed as that of COI by translation into amino acid sequences using *Anopheles quadrimaculatus* mtDNA genetic code in MEGA 4.0.[30]

Structure of genetic variation. The program TCS version 1.12[31] was used to calculate the number of mutational steps necessary to link any two haplotypes at the 95% confidence level (CI), as determined in ParsProb 1.1.[32] A statistical parsimony network represented genealogical relationships among *An. albimanus* haplotypes, and homoplasy was resolved based on the algorithm estimation rules described in Crandall and Templeton[33] and summarized in Uthicke and Benzie.[34] Analysis of molecular variance (AMOVA) was carried out in Arlequin 3.11.[35] First, we grouped the sequences of *An. albimanus* as western (1–9) and central–eastern localities (10–16) (Figure 1; Table 1) to test for congruence with previous studies.[21,22] Second, we grouped the sequences from localities in the Pacific (1, 2, 3, 8, 9, 10, 11, and 15) and the Atlantic coasts (4, 5, 6, 7, 12, 13, 14, and 16) (Figure 1; Table 1) to test for genetic divergence caused by potential ecological differences (e.g., precipitation and/or tide regimen). Hierarchical AMOVA tests using both haplotype frequencies and the number of mutations between haplotypes (Φ) partitioned the genetic variance within and among populations within groups (Φ_{SC}, Φ_{ST}) and among groups (Φ_{CT}).

The Mantel analysis was used to test the null hypothesis of the independence of the geographic and genetic distance by a pairwise matrix of linearized genetic distances, estimated by F_{ST}, and ln geographic distance among all populations. We also assessed the effect of the Central American Cordillera on genetic differentiation by testing whether genetic distance between populations on either the Atlantic or the Pacific coast correlates better with geographic distance than with direct-line distances. This procedure was carried out using a third matrix that included a categorical variable in the partial Mantel test.[36] The significance of the Mantel test was determined by permutations of $N = 10{,}000$ through a non-parametric procedure implemented in the IBD web service version 3.15.[37]

Demographic history. Neutrality tests of Tajima's D,[38] Fu and Li's F^* and D^*,[39] were used to test for strict neutrality (e.g., the majority of mutations are selectively neutral), whereas Fu's F_S[40] and R_2[41] were used to test for population size stability.[42] These statistics detect deviations from the pattern of polymorphism expected from a neutral model of evolution in a similar fashion, but use the information in the sample differently. Fu's F_S[40] and R_2[41] are among the most powerful tests to detect population expansion and genetic hitchhiking, whereas Tajima's D,[38] Fu and Li's F^* and D^*,[39] are the most effective tests to detect background selection. Neutrality tests were calculated in DnaSP, version 4.50.02[43] for each population and for the haplotype groups defined in the parsimony network. Significance of neutrality tests was assessed by 10,000 coalescence simulations in DnaSP, version 4.50.02.[43] In addition, haplotype and nucleotide diversities[44] were computed in Arlequin 3.11[35] for each population and for the groups in the parsimony network.

The mismatch distribution was performed to distinguish between an exponentially growing population depicting a smooth unimodal distribution and a stable population that has reached mutation drift-equilibrium depicting a multimodal, or erratic distribution, caused by stochastic lineage loss.[45] To quantify the smoothness of the mismatch distribution,[46] we calculated the raggedness (r) statistic in DnaSP, 4.50.02.[43] The mismatch distribution was calculated in Arlequin 3.11[35] for the entire data set and for the haplotype groups in the parsimony network. Net divergence (Da)[44] among populations from western (1–9) and central-eastern Panama (10–16) (Figure 1; Table 1) was estimated in MEGA 4.0[30] using the Tamura and Nei model.[47] Standard errors (SE) were estimated by the bootstrap method using 10,000 replicates. Divergence time (T) between

groups of populations was estimated as ($Da = 2\ \mu t_D$) where $2\ \mu$ is the divergence rate,[8] and the 95% CIs for the divergence times were calculated as ±1.96 SE of the net distances.

RESULTS

In total, 265 female mosquitoes (Table 1) from 16 localities were sequenced for an unambiguous alignment of 824 bp (both directions). Sequences were AT-rich (70.01% A and T bases) as encountered in the COI gene of other anopheline species.[8,25] There were no nonfunctional genes (e.g., pseudogenes) as shown by the absence of stop codons, the prevalence of synonymous substitutions, low pairwise divergence, and clear electropherograms. Sequences were deposited into GenBank (accession nos.: FJ516463–FJ516553). The total number of segregating sites was 85, with 85% transitions and 15% transversions, the majority of which were silent substitutions. Ninety-one haplotypes were detected, 27 (29.6%) were shared among localities, and 64 (70.3%) were singletons (Figures 1 and 2).

Structure of genetic variation. The statistical parsimony network illustrates the relationship among haplotypes of *An. albimanus*. All haplotypes differed by less than 12 mutational steps, so they could be connected parsimoniously. Three groups, each separated by 7 mutational steps, suggested substantial haplotype partition (Figure 2). Group A was star-shaped with short branches and an excess of singleton mutations, the signal of a demographic expansion, background selection, or selective sweep.[40,44,48] This group contained haplotype A1, which appears to be the most ancestral owing to its high frequency (75/265 sequences), wide geographic distribution (12/16 localities), and interior position in the network.[48] In addition, 82.4% of the haplotypes in group A were distributed exclusively in central-eastern Panama (Figure 1; Table 1). In contrast, group B had longer branches, more missing haplotypes, fewer singleton mutations, and 90.2% of its haplotypes were encountered exclusively in Costa Rica and western Panama (Figure 1; Table 1). Group C included only 9.8% of the total haplotypes in the parsimony network, which is star-shaped with short branches and only two missing haplotypes (Figure 2). Similar to group B, 88.8% of the haplotypes in group C were restricted to Costa Rica and western Panama (Figure 1; Table 1). These three haplotype groups represent a substantial genetic division in the data set, showing roughly 0.8% sequence divergence and an average number of 7.5 nucleotide differences (k) among them.

Hierarchical AMOVA tests attributed 33.99% ($P = 0.0005$) of the total genetic variance to the western versus eastern group comparison, thus partially supporting previous findings,[21,22] whereas the Pacific versus Atlantic group comparison accounted only for 6.93% ($P = 0.711$) of the total variance and was not statistically significant (Table 2). Furthermore, the

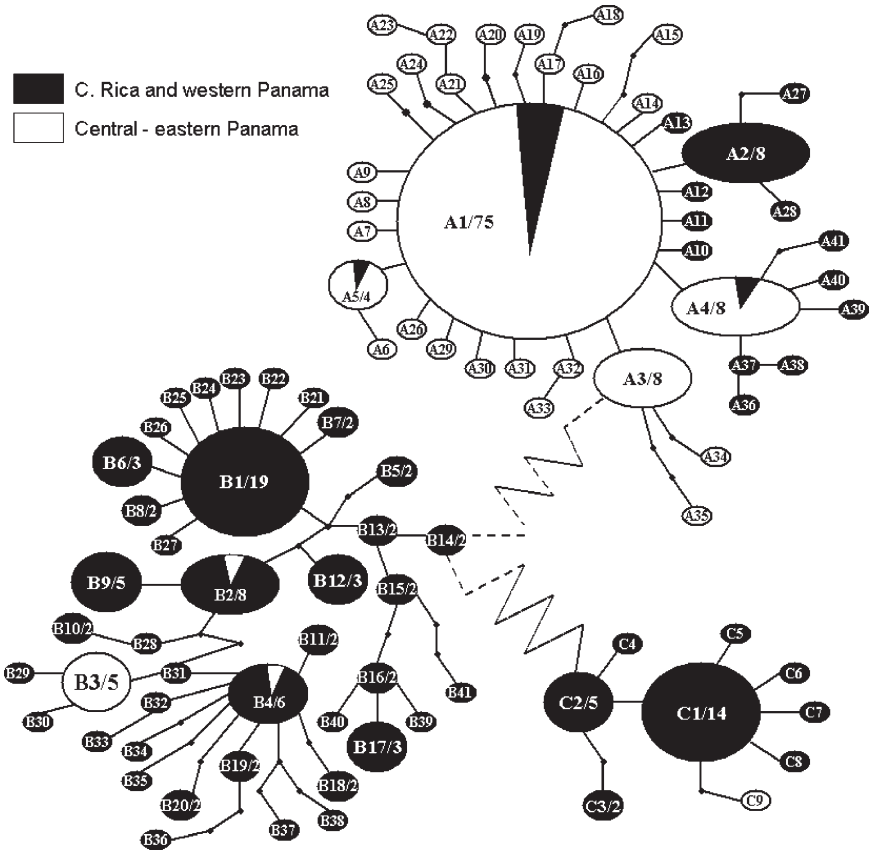

FIGURE 2. Statistical parsimony network of 91 COI haplotypes of *Anopheles albimanus*. The letters correspond to the haplotypes observed for the 16 localities in Table 1. The solid black dots represent missing haplotypes. Dotted lines depict 7 mutational steps between groups A (labeled A1–A41); B (B1–B41); and C (C1–C9). The size of the circle is proportional to the frequency of the haplotype, which is indicated following the haplotype code, and the color indicates the geographic distribution.

TABLE 2

Comparative results of a hierarchical analysis of molecular variance (AMOVA), using Φ statistics from haplotype frequencies and genetic divergence among populations of *Anopheles albimanus*. (a) Costa Rica and western Panama (localities 1–9) vs. central-eastern Panama (10–16); (b) Pacific (1–3,8–11,13,15) vs. Atlantic (4–7,12,14,16) coasts.

	Source of variation	Total variation (%)	Fixation index	*P*
(a)	Within populations	60.45	$\Phi_{SC} = 0.39$	< 0.0001***
	Among populations within groups	5.57	$\Phi_{ST} = 0.08$	< 0.121
	Among groups	33.99	$\Phi_{CT} = 0.33$	< 0.0005***
(b)	Within populations	73.07	$\Phi_{SC} = 0.26$	< 0.00021***
	Among populations within groups	30.24	$\Phi_{ST} = 0.29$	< 0.0011**
	Among groups	6.93	$\Phi_{CT} = -0.003$	< 0.711

* $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$.

Mantel analysis to test for IBD among all populations was statistically insignificant ($R^2 = 0.0214$, $P = 0.577$) suggesting little input of direct-line geographic distance as an explanatory factor of the observed spatial pattern of genetic diversity. The correlation coefficient using a third categorical variable to test the effect of geographic distance on the genetic distance after controlling for the effect of the Central American Cordillera was not statistically significant ($R^2 = 0.0714$, $P = 0.377$).

Demographic history. Overall values of haplotype and nucleotide diversities were higher (ranging from 0.77 to 0.98 and 0.008 to 0.01, respectively) for populations in Costa Rica and western Panama than for populations in central-eastern Panama (ranging from 0.45 to 0.96 and 0.001 to 0.006, respectively) (Table 3). By group, nucleotide diversity was highest in B, followed by lower and similar values for A and C (Table 4). The mismatch distributions for the entire data set (all populations) and for B alone depicted multimodal curves, and did not fit a model of sudden population expansion, thus suggesting a stationary population at equilibrium. In contrast, A and C were unimodal and fit the model of sudden population expansion (Figure 3). In support of these findings, the mismatch distribution for A was strongly biased toward low divergence values with a mode of three nucleotide changes, suggesting a relatively recent expansion event (Figure 3).

TABLE 3

Summary statistics for polymorphisms of *Anopheles albimanus**

By locality	H/n	h (SD)	π (SD)
1. Soley	16/21	0.96 (0.031)	0.01 (0.002)
2. Palo Verde	15/21	0.95 (0.032)	0.01 (0.001)
3. Dominical	7/11	0.87 (0.089)	0.01 (0.001)
4. Matina	15/19	0.97 (0.020)	0.01 (0.002)
5. Puente Blanco	8/15	0.90 (0.046)	0.01 (0.001)
6. California	8/17	0.77 (0.099)	0.008 (0.001)
7. Rio Diablo	8/10	0.95 (0.053)	0.01 (0.001)
8. La Barqueta	20/22	0.98 (0.020)	0.01 (0.001)
9. Las Lajas	8/12	0.89 (0.071)	0.01 (0.001)
10. Las Colomas	3/9	0.55 (0.016)	0.001 (0.001)
11. Santa Clara	9/16	0.76 (0.011)	0.004 (0.001)
12. Rio Indio	6/24	0.49 (0.111)	0.001 (0.001)
13. Bayano	4/25	0.45 (0.105)	0.004 (0.003)
14. Ukupa	9/17	0.87 (0.051)	0.004 (0.001)
15. Yaviza	7/8	0.96 (0.077)	0.006 (0.001)
16. Zapsurro	10/18	0.76 (0.108)	0.002 (0.002)

* H = unique haplotypes; n = number of individuals; h = haplotype diversity; SD = standard deviation; π = nucleotide diversity. Costa Rica and western Panama (localities 1–9), central-eastern Panama (10–16).

The average number of nucleotide differences (*k*) for A, B, and C was 1.31, 4.83, and 1.10, respectively; suggesting higher diversity and perhaps equilibrium only in B. The raggedness statistic for the entire data set and for A and B was low and not significant providing further support for population expansion. In contrast, C showed a higher and significant value for the raggedness statistic, therefore rejecting the model of sudden population expansion (Figure 3).

Neutrality tests of Tajima's *D*,[38] Fu and Li's *F** and *D**,[39] and Fu's F_S[40] showed negative and significant values for the entire data set, and for A (Table 4), thus allowing the rejection of either strict neutrality or population size stability, because of either background selection, selective sweep, or demographic expansion. The R_2 test[41] was also low and significant for A further supporting the departure from equilibrium most likely because of demographic expansion (Table 4). The time since expansion for A was calculated using $t = \tau/2u$, where $u = \mu \times$ number of base pairs sequenced × generation time, and μ is the mutation rate.[44,45] The *Drosophila* mutation rate of 10^{-8}/site/year[49] and 10 generations/year[8] were used in this calculation. The estimate of τ, from the raggedness calculation, is 3.39 for A. Therefore, the time since expansion for *An. albimanus* in central-eastern Panama is approximately 22,000 years ago (95% CI 10,183–38,169), in the late Pleistocene.

DISCUSSION

Pattern of genetic structure. The role of *An. albimanus* as a primary malaria vector of *Plasmodium vivax* and *P. falciparum* has been historically acknowledged; yet, its capacity to transmit malaria parasites varies across its range.[3,19,20] Furthermore, evidence for cryptic species in *An. albimanus* have been unsubstantiated so far[13–16] and population differentiation seems to be low overall.[19,20] The shallow population structure of *An. albimanus* in the Americas may be attributed to long-term effective population size caused by high dispersal capability, opportunistic feeding behavior, and ecological plasticity. Nevertheless, this study shows considerable geographic structure for *An. albimanus* across Costa Rica and the Isthmus of Panama, with a genetic discontinuity within less than 300 km.

Our results partially support previous findings on the population structure of *An. albimanus* using the mtDNA ND5 gene and microsatellites, which depicted differences in allele and haplotype frequencies between Costa Rica and eastern Panama.[21,22] The authors in these studies invoked isolation by distance, physical barriers to gene flow, and/or population contraction in Panama resulting from intense insecticide use as the likely causes of population differentiation. However, neither IBD nor barriers to dispersal across Costa Rica and Panama were supported by our results. The correlation coefficient and the Mantel test remained low and statistically insignificant despite several analyses, and 27 (29.6%) haplotypes were shared among populations and 11 (13.5%) were shared between the Atlantic and Pacific coasts of Costa Rica and western Panama where the mountain range reaches its maximum altitude (roughly 3,000 m). These findings indicate that the Central American Cordillera has not restricted the dispersal of *An. albimanus* in the past. They also show that the spatial pattern of haplotype diversity depicted by the COI gene is not caused by restricted gene flow as a result of geographic distance.

TABLE 4
Summary statistics for polymorphisms and neutrality tests of *Anopheles albimanus*

By groups	H/n	h (SD)	π (SD)	F_S	D_T	F^*	D^*	R_2	r
Group A	41/139	0.70 (0.043)	0.001 (0.0002)	–53.61***	–2.5**	–6.30*	–7.22*	0.018***	0.017
Group B	41/99	0.94 (0.014)	0.005 (0.0002)	–26.36	–1.36	–2.30	–2.29	0.072	0.012
Group C	9/27	0.71 (0.085)	0.001 (0.0003)	–4.57	–1.69	–2.42	–2.24	0.062	0.066
Total	91/265	0.907 (0.015)	0.008 (0.0002)	–73.26***	–1.42**	–4.07*	–6.26*	0.041**	0.011

* $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$.
H = unique haplotypes; n = number of individuals; h = haplotype diversity; SD = standard deviation; π = nucleotide diversity.
F_S = Fu's F_S statistic; D_T = Tajima's D; F^* = Fu and Li's F test; D^* = Fu and Li's D test; R_2 = Ramos-Onsins and Rozas R_2 test; r = raggedness statistic. See the statistical parsimony network of *An. albimanus* for group definitions.

Mountain ranges (cordilleras) have been identified as barriers to gene flow for some American malaria vectors. The Green Mountains in the eastern United States are believed to restrict dispersal of *Anopheles punctipennis*, and some lineages of *Anopheles nuneztovari* s.l. were partially separated by the eastern Andean Cordillera.[24,50] Because these two *Anopheles* species have specific ecological requirements, and limited geographic distributions, mountain ranges are likely to restrict their dispersal. In contrast, *An. albimanus* is capable of flying up to 32 km, and has been found as high as 1,941 m[12,18]; thus, low mountain ranges are unlikely to play a substantial role in its geographic structure. A positive and significant relationship between geographic and genetic distances was reported for *An. nuneztovari* s.l. and *An. darlingi* in South America using nuclear markers: the *white* gene,[7,9] and microsatellites.[7,51,52] However, in most of these cases the correlation was weak, and not detected when different lineages and geographic regions were analyzed separately.[7,9,50] Furthermore, IBD was not detected in *An. nuneztovari* s.l. with analysis of mtDNA restriction fragment length polymorphisms (RFLPs)[53] or in *An. darlingi* using the COI gene.[25] Given that neither *An. darlingi* nor *An. nuneztovari* s.l. are at mutation-drift equilibrium,[9] misleading IBD may be the result of historical fragmentation, initial colonization pathway mimicking the IBD pattern, or inappropriate sampling.[54]

Mosquito control during the construction of the Panama Canal was carried out in the former Canal Zone and few control measures were undertaken in the surrounding areas. However, we observed reduced diversity in several localities 95 to 410 km from the former Canal Zone (Figure 1). Even though IRS is efficient in controlling endophilic *Anopheles* species, the exophagic and exophilic behavior displayed by *An. albimanus* in Central America[19,20] make the efficacy of IRS uncertain even in highly susceptible populations. Moreover, avoidance behavioral responses against DDT exhibited by Panamanian populations of *An. albimanus* may have lessened the physical contact between this species and sprayed surfaces.[55] For the previous reasons, we believe that intensive mosquito control alone does not explain the lower diversity and different haplotype composition seen in localities of central-eastern Panama as hypothesized by Molina-Cruz and others.[22]

Nevertheless, a lower mutation rate of the COI gene indicates that this marker is not as sensitive as microsatellites to detect more recent demographic events.[22] However, the larger

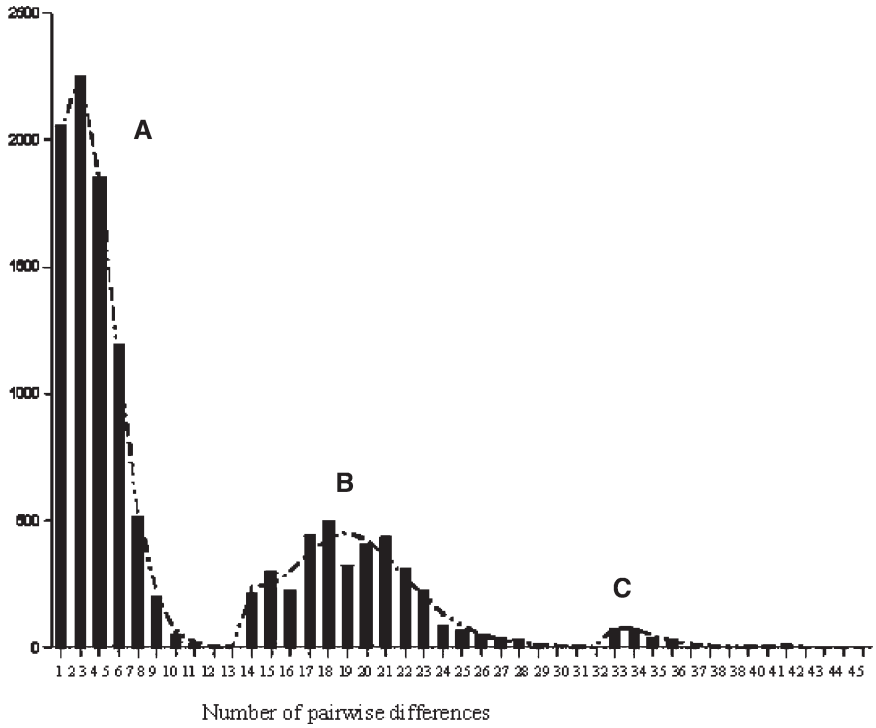

FIGURE 3. Mismatch distribution for groups A, B, and C of *Anopheles albimanus*. Bars are observed distribution, and the dashed line shows the distribution simulated under a model of sudden expansion.

effective population size and longer coalescent time of microsatellites (e.g., nuclear genome), suggest that population contraction in Panama because of mosquito control is unlikely, as insecticide treatment is a recent phenomenon and microsatellites are lagging indicators of population processes.[56] The general agreement of previous microsatellite and mtDNA ND5 gene studies,[21,22] and rather similar results in our COI gene analysis, could indicate that the processes causing genetic differentiation in *An. albimanus* are genome-wide effects, and that they occurred at a much earlier time frame than mosquito control in Panama. The high prevalence and wide distribution of *An. albimanus* during the last 35 years in Panama also argue against a recent bottleneck caused by insecticide treatment.[3,5]

Demographic history. The unimodal and statistically insignificant mismatch distribution under the demographic expansion model and 4 of 5 negative and significant neutrality tests in group A, indicate that *An. albimanus* is not at equilibrium, because of a past bottleneck, background selection, population expansion, or selective sweep in central-eastern Panama. The neutrality tests cannot distinguish between demographic phenomena and selection as the causes of departure from equilibrium. Therefore, we performed the McDonald-Kreitman neutrality test (MK) in DnaSP, version 4.50.02[43] using all the sequences of *An. albimanus* from central-eastern Panama and 52 COI sequences of *An. triannulatus* from the same localities as an outgroup (unpublished data). The MK considers the evolution of a protein-coding gene in two closely related species in which nucleotide differences can be classified as either synonymous or non-synonymous, and it was used to investigate the influence of natural selection on the COI sequences. The MK test yielded a statistically insignificant P value of 0.870 in the Fisher's exact test; therefore, the departure from equilibrium observed in *An. albimanus* from eastern Panama is not a result of directional selection, but more likely to an expansion.

The observed haplotype partition seems to be more related to the demography of *An. albimanus* and a complex history of dispersal events throughout southern Central America. The genetic signature of population historical processes can remain for long periods of time, obscuring the true geographical population structure that may exist.[57] In general, populations from Costa Rica and western Panama showed higher diversity, with a decreasing trend in the nucleotide diversity from Costa Rica toward eastern Panama. Although this may be interpreted as a single founder event followed by sequential population bottlenecks in the colonizing front, and range expansion from Costa Rica into eastern Panama, an alternate interpretation is the co-occurrence and haplotype mixing of three maternal lineages (A, B, and C) in populations of Costa Rica and western Panama, and the predominance of only A in populations of central-eastern Panama. Furthermore, B and C depicted a decreasing trend in haplotype diversity from Costa Rica toward eastern Panama, whereas A also decreased in haplotype diversity, but in the opposite direction (Figure 1). This pattern may represent historical geographic fragmentation in eastern Panama and a subsequent re-invasion by two different source populations (B and C) from the north and one source population from Colombia (A). The fact that old and predominant haplotypes from Costa Rica and western Panama gave rise to haplotypes found in eastern Panama and vice versa, further supports a pattern of recent regional secondary contact.

Taken together, these findings suggest a discontinuous colonization pattern of *An. albimanus* throughout Costa Rica and the Isthmus of Panama as the most likely explanation for the observed distribution of genetic diversity. The dispersal of different groups of mosquitoes (multiple introductions) could explain three co-occurring and genetically differentiated maternal lineages in Costa Rica and Panama, which may have arrived at different times, with a separate demographic history and therefore an already significant genetic structure between them.[58] The divergence time (t_D) from the net nucleotide substitution per site (Da = 0.004 ± 0.001, 95% CI) between western and central-eastern populations of *An. albimanus* placed the time since divergence around 200,000 years ago (235,000 ± 165,000, 95% CI) in the late Pleistocene.

Anopheles albimanus is believed to have originated in the Caribbean islands and subsequently invaded the American continent, but no definitive information on the colonization path exists.[22] Group B seems to be the oldest owing to its long branches and missing haplotypes in the parsimony network, and higher nucleotide diversity, which may support a north-south colonization path throughout Costa Rica and Panama; however, this disagrees with the coalescent theory, which predicts that group A is the oldest because of haplotype A1.[48] Nevertheless, given the non-equilibrium scenario and likely past geographic fragmentation of *An. albimanus* in the present study, it is possible that the COI gene does not have enough resolution to infer the original colonization pathway of *An. albimanus* throughout southern Central America. Because of the haploid nature and maternal mode of inheritance, mtDNA genes have 4-fold smaller effective population size than nuclear markers, and therefore the COI gene may have been affected by more recent demographic phenomena during the Pleistocene, thus erasing more ancient signals of colonization events.

Pollen data from marine records of the Panama Basin and the Pacific slope of the northern Andes show cold and humid conditions between 39,410 and 28,120 years ago with moderately high sea levels and less precipitation. In contrast, the coldest and driest conditions and the lowest sea levels occurred between 28,120 and 14,500 years ago.[59] These environmental changes extend through the population expansion time proposed for *An. albimanus* in this study. *Anopheles albimanus* is highly opportunistic, able to develop in a wide variety of breeding habitats[18]; however, it is more abundant in coastal plains, close to the shore, and at less than 100 m.[11] Therefore, a combination of Pleistocene climatic oscillation, most likely involving temperature, precipitation, and sea level changes in eastern Panama, seems to be a plausible explanation for the population expansion of *An. albimanus*.

The entire data set also concurs with population expansion, yet B and C showed a less clear signal. These two groups are distributed mostly in Costa Rica and western Panama where mountains and upland plains might have served as a refuge providing alternative and more stable breeding sites during glacial periods. It is noteworthy that haplotype A1 was not recorded from the Atlantic coast of Costa Rica and western Panama. Pleistocene glaciations exposed most of the Gulf of Panama, located in the narrowest strip of land of central Panama,[60] and this may have facilitated re-invasion of this expanding haplotype through the Pacific coast, whereas re-invasion through the Atlantic coast might have been more irregular.

CONCLUSIONS

In the present study, the COI gene uncovered significant genetic structure at a micro-geographic scale. The genetic differentiation in *An. albimanus* appears to be caused by Pleistocene historical fragmentation, re-colonization, and population expansion, rather than to contemporary geographic structure, insecticide treatment, and selective pressures on the COI locus. Other studies carried out in fresh water fishes, the pseudoscorpion *Cordylochernes scorpioides* and the tungara frog *Physalaemus pustulosus*, have supported a complex biogeographical history in the Isthmus of Panama where significant genetic differentiation is seen at short distances, and waves of colonization, extinctions, and re-invasions appear to be the main forces shaping the distribution of genetic diversity.[61–63] Our results have to be viewed with caution as they may represent divergence only in the mitochondrial genome and therefore a nuclear marker will be required to test these findings. In addition, more research will be needed to determine whether these three maternal lineages may have different potential roles in malaria transmission.

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