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Mitochondrial DNA Detects a Complex Evolutionary History with Pleistocene Epoch Divergence for the Neotropical Malaria Vector *Anopheles nuneztovari* Sensu Lato

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Abstract. Cryptic species and lineages characterize *Anopheles nuneztovari* s.l. Gabaldón, an important malaria vector in South America. We investigated the phylogeographic structure across the range of this species with cytochrome oxidase subunit I (*COI*) mitochondrial DNA sequences to estimate the number of clades and levels of divergence. Bayesian and maximum-likelihood phylogenetic analyses detected four groups distributed in two major monophyletic clades (I and II). Samples from the Amazon Basin were clustered in clade I, as were subclades II-A and II-B, whereas those from Bolivia/Colombia/Venezuela were restricted to one basal subclade (II-C). These data, together with a statistical parsimony network, confirm results of previous studies that *An. nuneztovari* is a species complex consisting of at least two cryptic taxa, one occurring in Colombia and Venezuela and the another occurring in the Amazon Basin. These data also suggest that additional incipient species may exist in the Amazon Basin. Divergence time and expansion tests suggested that these groups separated and expanded in the Pleistocene Epoch. In addition, the *COI* sequences clearly separated *An. nuneztovari* s.l. from the closely related species *An. dunhami* Causey, and three new records are reported for *An. dunhami* in Amazonian Brazil. These findings are relevant for vector control programs in areas where both species occur. Our analyses support dynamic geologic and landscape changes in northern South America, and infer particularly active divergence during the Pleistocene Epoch for New World anophelines.

INTRODUCTION

Anopheles (*Nyssorhynchus*) *nuneztovari* sensu lato (s.l.) Gabaldón is distributed from eastern Panama through northern South America,[1] and in Brazil, it is limited to the Amazon region[1] (Figure 1). This species is an important malaria vector in Colombia and Venezuela, where it exhibits high anthropophily, endophagic-exophagic and exophilic behaviors and a peak biting time around midnight.[2–4] In Amazonian Brazil and Peru, *An. nuneztovari* has been found infected with *Plasmodium* sp.[5–9] Although most Brazilian populations are exophagic and mainly zoophilic, with a peak biting time around sunset, in some localities in Pará state, anthropophagic and endophagic behaviors have been encountered,[10] and it has been incriminated as a local vector in Amapá State.[9] Overall, *An. nuneztovari* s.l. from Amazonian Brazil seems not to sustain malaria transmission or to be incapable of causing malaria outbreaks in the absence of the primary vector *Anopheles darlingi* Root.[7,11]

Approximately half of the anopheline vector species belong to sibling/cryptic species complexes.[12] The presence of complexes can cause heterogeneous patterns of malaria transmission, sometimes resulting in reduced vector control effectiveness or even in failure.[13] The main factors that affect the capacity of a vector to transmit malaria parasites are abundance, anthropophily, infection rate and females longevity,[14] which may vary between cryptic species, lineages or genetically structured populations.

South America is particularly rich in species diversity.[15] This is also true for anophelines, especially in northern South America, where there are closely related species groups, species complexes and species with deep genetic differentiation,[16] and the most studied species have diverged during the Pleistocene Epoch. With the advent of the use of multiple molecular genetic markers, several new species have been described and others have been resurrected from synonymy. Examples include *An. triannulatus* Neiva and Pinto, *An. albitarsis* Lynch-Arribálzaga, *An. oswaldoi* Peryassú, and *An. nuneztovari*.[11,12,17,18] Some of these species are implicated in local malaria transmission, adding to the importance of such discoveries.

On the basis of differences in host preference and biting behavior, Elliott[2] first proposed that populations of *An. nuneztovari* s.l. from Amazonian Brazil and Colombia/Venezuela comprised two allopatric races, possibly cryptic species. This hypothesis was corroborated by cytogenetic results.[19,20] Subsequently, morphologic, isoenzymatic, and molecular studies found significant differentiation between the two allopatric races, but no notable evidence to support a species complex.[21–27] However, within Amazonian Brazil, internal transcribed spacer 2 (ITS2)[22] and mitochondrial DNA (mtDNA)–restriction fragment length polymorphism (RFLP) [26] analyses indicated multiple lineages, and a lack of gene flow between the central and eastern regions.[27] A recent study using nuclear *white* gene sequences described five lineages in *An. nuneztovari* s.l.: two in Colombia and Venezuela and three in Amazonian Brazil and Bolivia.[28] In contrast to previous studies, Mirabello and Conn[28] detected deep genetic differentiation among lineages, together with sympatry at five localities in Bolivia, Brazil, and Venezuela.

The morphologic studies of Bergo and others[29] suggested a new species in Amapá State, Brazil. On the basis of male genitalia and phylogenetic analyses of ITS2, cytochrome oxidase subunit I (*COI*) gene, and *white* gene sequences of samples from Amazonian Brazil, Calado and others[18] resurrected *An. goeldii* Rozeboom and Gabaldón from earlier synonymy with *An. nuneztovari* and proposed distinctive geographic distributions: *An. goeldii* in Amazonian Brazil, and *An. nuneztovari* s.s. in Colombia and Venezuela. However, Gabaldón[3] initially resurrected *An. goeldii* when he revised the morphologic differences between *An. goeldii* and *An. nuneztovari*, which are described below.

The discrepancy between results from various studies is likely caused by multiple factors. These factors include undersampling in some key geographic regions, lack of progeny rearing of

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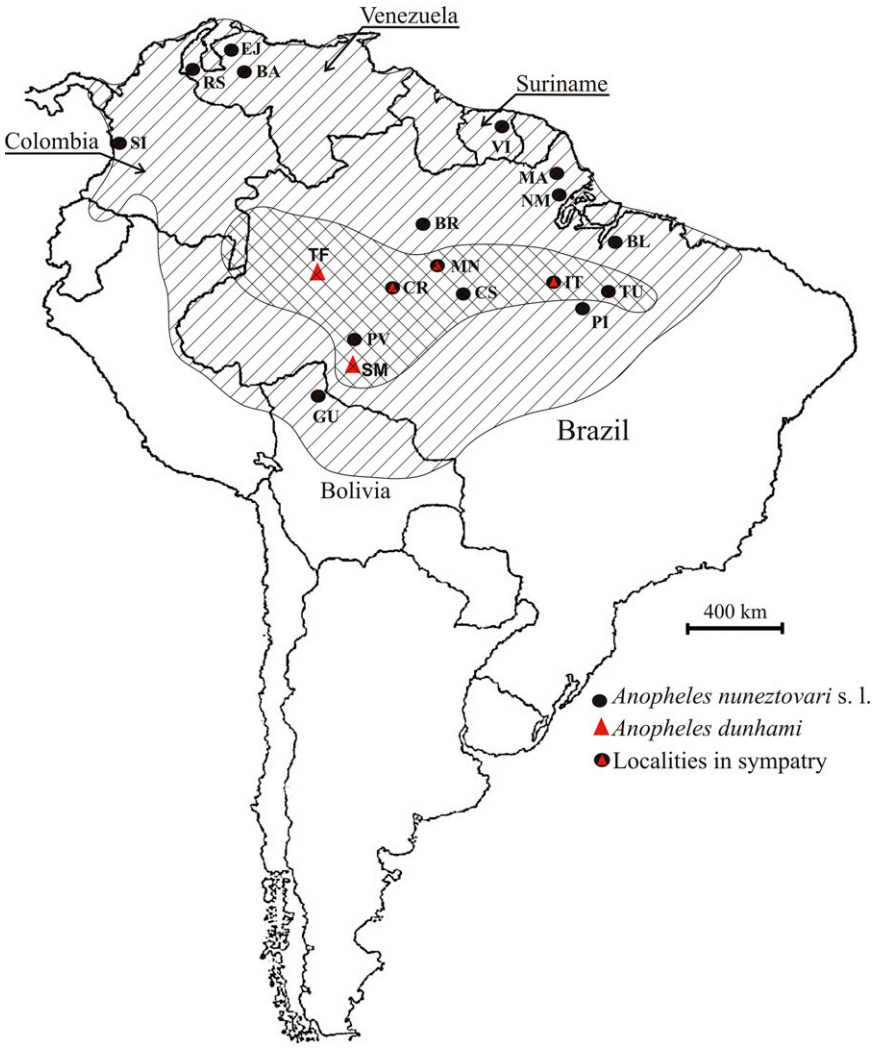

FIGURE 1. Geographic distribution of *Anopheles nuneztovari* s.l. (**larger shaded area**) and *An. dunhami* (**smaller shaded area**), and the collection sites of these species in northern South America. See Table 1 for locality abbreviations.

field-collected specimens or crossing experiments between suspected taxa, distinct modes and rates of evolution of the various molecular markers used, and a recent temporal framework for evolutionary processes within *An. nuneztovari* s.l.[30]

CURRENT TAXONOMIC SITUATION

Anopheles nuneztovari was originally described from San Carlos, Cojedes State, in western Venezuela, on the basis of on male genitalia.[31] Rozeboom and Gabaldón[32] described *An. goeldii* from morphology of eggs, larvae, females, and males collected in Belterra (Fortlandia), on the Tapajós River, in Pará State, Brazil. The synonymy of *An. goeldii* with *An. nuneztovari* was proposed by Floch and Abonnenc,[33] and accepted by Lane[34] and Faran,[1] although Faran believed that *An. goeldii* could be a valid species. The revision of Gabaldón[3] supported the taxonomic status of *An. goeldii* on the basis of differences in the male mesosome leaflets (leaflets = small spines), which are long and strongly sclerotized in *An. nuneztovari* and short and weakly sclerotized in *An. goeldii*. In larvae, the spiracular plate arms are absent or rudimentary in *An. nuneztovari* and longer and visible in *An. goeldii*.[3] Currently, *An. goeldii* is a valid species.[18]

Another closely related species, *An. dunhami* Causey, collected in Tefé, Amazonas State, Brazil, was described as a new taxon[35] by using the mesosome of male genitalia to differentiate it from *An. nuneztovari* and *An. goeldii.* The egg, larvae, and adult color pattern of *An. dunhami* are similar to that of *An. goeldii*.[35] In 1953, Lane[34] synonymized *An. dunhami* with *An. nuneztovari* without comment. Subsequently, *An. dunhami* was resurrected as a distinct species on the basis of morphology[36] and multiple markers.[37] Several molecular markers can now be used to reliably separate *An. dunhami* and *An. nuneztovari* s.l.[18,37–39] Recent records have extended the geographic distribution of *An. dunhami* to include Tabatinga,[37] Coari[38] and Parintins,[18] Amazonas State, Brazil, and Amazonian Colombia,[39] overlapping with the closely related species *An. nuneztovari* s.l. (Figure 1).

We investigated the phylogeographic structure of *An. nuneztovari* s.l. from 17 localities in northern South America (Amazonian Brazil, Bolivia, Colombia, Suriname, and Venezuela) to estimate the number of clades and levels of divergence by using an 873-basepair fragment of the mtDNA *COI* gene. Within Amazonian Brazil, we included 11 localities, 5 of which have not been studied (MA, PI, BR, CS, CR; Figure 1), to address three questions. 1) How many lineages exist within

Amazonian Brazil? 2) What is the level of genetic differentiation among them? 3) Is there evidence of reciprocal monophyly, particularly between populations from Colombia/Venezuela (anthropophilic and endophagic-exophagic) versus those from Amazonian Brazil (predominantly zoophilic and exophagic)? Knowledge of the occurrence and geographic distribution of potential species or lineages is relevant to better understand the involvement of each in malaria transmission throughout the range of *An. nuneztovari* s.l.

MATERIALS AND METHODS

Mosquito collection and identification. Samples of *An. nuneztovari* s.l. were collected from 11 sites in Amazonian Brazil (MA, NM, BL, IT, TU, PI, MN, BR, CS, CR, PV); 1 each in Bolivia (GU), Colombia (SI), and Suriname (VI); and 3 in Venezuela (BA, RS, EJ) (Figure 1). The information on the collection sites, including abbreviations, co-ordinates,[40] and sample sizes are shown in Table 1. The BA site is near the type locality of *An. nuneztovari* in Venezuela. Specimens were captured between 6:00 PM and 9:00 PM feeding on cattle, resting in stables, or when landing on humans. The standard human landing catch protocol was reviewed and approved by the Institutional Review Board of the National Institute of Research of the Amazon, the Brazilian Ministry of Science, Technology and Innovation, Brazil by the U.S. New York State Department of Health Institutional Review Board. The mosquitoes analyzed from IT, MN, PV, VI, GU, RS and EJ were wild-caught females, and the specimens from the remaining localities were the offspring (F_1) of progeny broods reared to adulthood. All mosquitoes were morphologically identified[1] and immediately stored at –80°C until analysis.

DNA extraction, polymerase chain reaction, and sequencing. Total DNA was extracted from individual mosquitoes by using a phenol-chloroform method.[41] The primers used to amplify the 873-basepair *COI* fragment and the polymerase chain reaction amplification conditions are described by Joy and Conn.[42] After visualization by electrophoresis on 1% agarose gels, the polymerase chain reaction products were cleaned by using CentriSpin 40 columns (Princeton Separations Freehold, NJ) and sequenced at the Wadsworth Center Applied Genomics Technology Core (New York State Department of Health, Albany NY). All fragments were sequenced in both directions.

Statistical analysis. The *COI* sequences were edited by using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI) and imported together into programs for statistical analyses. The 873-basepair fragment analyzed corresponds to positions 639–1512 of the mitochondrial genome of *Drosophila yakuba* Burla.

TABLE 1

Collection sites and haplotype frequency of *Anopheles nuneztovari* sensu lato from South America, and *Anopheles dunhami* and *Anopheles darlingi* from Brazil*

Locality, state (abbreviation)	Co-ordinates (latitude, longitude)	No.	Haplotype frequency	Clades/subclades
Brazil				
Arquipelágo do Maruanum, Amapá (MA)	0.29333, –51.27361	2	H1(2)	I
Nova Mazagão, Amapá (NM)†	–0.11667, –51.30750	9	H2(3), H3(1), H4(1), H5(1), H6(1), H7(1), H8(1)	I, II-A
Belém, Pará (BL)	–1.44611, –48.49722	3	H9(3)	I
Itaituba, Pará (IT)†	–4.25000, –55.96111	7	H10(1), H11(1), H12(1), H13(1), H14(1), H15(1), H16(1)	I, II-B
Tucuruí, Pará (TU)	–3.70639, –49.46500	10	H9(2), H17(3), H18 (1), H19(2), H20(2)	I
Comunidade Pitinga, Pará (PI)	–4.25167, –51.00361	4	H21(2), H22(2)	I
Manaus, Amazonas (MN)†	–3.09333, –59.93083	10	H15(1), H23(1), H24(1), H25(1), H26(2), H27(1), H28(2), H29(1)	I, II-A, II-B
BR-174 Highway, Km 206, Amazonas (BR)†	–1.29083, –60.40722	10	H3(2), H26(3), H27(1), H28(1), H30(1), H31(1), H32(1)	I, II-B
Castanho, Amazonas (CS)†	–3.84417, –60.37389	8	H15(2), H26(2), H33(1), H34(1), H35(1), H36(1)	I, II-A, II-B
Coari, Amazonas (CR)	–4.09611, –63.13944	4	H37(3), H38(1)	II-B
Porto Velho, Rondônia (PV)	–8.80750, –63.91639	5	H10(5)	I
Suriname				
Victoria, Brokopondo (VI)†	5.08333, –54.96667	9	H24(1), H39(7), H40(1)	I, II-A
Bolivia				
Guayaramirín, Beni (GU)	–10.85000, –65.35000	2	H41(1), H42(1)	II-C
Colombia				
Sitronela, Buenaventura, Valle (SI)	3.83139, –77.07972	10	H43(2), H44(1), H45(4), H46(1), H47(2)	II-C
Venezuela				
Barinas, Barinas (BA)	8.61667, –70.18333	9	H41(5), H48(3), H49(1)	II-C
Rio Socuavó, Zúlia (RS)	8.90000, –72.63333	9	H42(7), H50(2)	II-C
El Juval, Trujillo (EJ)	9.55000, –70.60000	2	H51(1), H52(1)	II-C
Anopheles dunhami, Brazil				
Itaituba, Pará (IT)	–4.25000, –55.96111	1	H53(1)	
Manaus, Amazonas (MN)	–3.09333, –59.93083	1	H54(1)	
Coari, Amazonas (CR)	–4.09611, –63.13944	4	H55(2), H56(1), H57(1)	
Tefé, Amazonas (TF) type locality	–3.33889, –64.62722	1	H58(1)	
São Miguel, Rondônia (SM)	–8.60000, –63.81667	1	H59(1)	
Anopheles darlingi, Brazil				
Manaus, Amazonas (MN)	–3.09333, –59.93083	2	H60(1), H61(1)	

* No. = sample size; H1–H52 = haplotypes of *An. nuneztovari* s.l.; H53–H59 = haplotypes of *An. dunhami;* H60 and H61 = haplotypes of *An. darlingi*. Values in parentheses are numbers of individuals observed for each haplotype. Underlined haplotypes are shared among localities.

† Locations with two or three sympatric clades/subclades.

Eight of 121 specimens of this study, morphologically identified as *An. nuneztovari* s.l., were molecularly clustered as *An. dunhami* on the basis of sequence comparison of one descendent of a progeny from the type locality,[35] which had been identified as *An. dunhami* by using multiple markers.[37]

A statistical parsimony network was generated by using TCS 1.21[43] at the 95% confidence level, and homoplasies were resolved using the rules in Crandall and Templeton.[44] Phylogenetic relationships were inferred by maximum-likelihood (ML) analysis implemented in the Treefinder Program[45] on the basis of the Generalized Time Reversible GTR + G + I Proportion model selected by jModelTest,[46] with 1,000 replicates. *Anopheles dunhami* and *An. darlingi* sequences were used as the outgroup. Bayesian inference (BI) analysis was performed with Mr. Bayes version 3.1[47] using the model TPM2uf + I + G determined by the jModelTest.[46] The settings were two simultaneous independent runs of the Markov Chain Monte Carlo for 20 million generations, sampling every 1,000 generations with a burn in of 50%. *Anopheles dunhami* sequences were included in the ingroup, and *An. darlingi* sequences were used as the outgroup.

Intra-population diversity measures for each sample and for haplotype clade and subclades were implemented in DnaSP 4.0[48] and Arlequin 3.1.[49] The haplotype and nucleotide diversities were not calculated for the samples MA, GU, and EJ because of small sample size (n = 2).

Neutrality tests were calculated in DnaSP 4.0.[48] The *D* test of Tajima[50] and the *D* and *F* tests of Fu and Li[51] were used to test for strict neutrality, and the *F*s test of Fu,[52] and the R_2 test of Ramos-Onsins and Rosas[53] were used to test population size stability. The *F*s and R_2 tests are the most powerful tests for detecting population expansion and genetic hitchhiking, and the *D* and *D* and *F* tests are the most effective tests for detecting background selection. The mismatch distribution was computed for clade I and the three subclades and for the entire data set in Arlequin 3.1[49] with 1,000 permutations. To quantify the smoothness of the mismatch distribution, we calculated the raggedness (*r*) in Arlequin 3.1.[49]

Sequence divergence among *An. nuneztovari* s.l. haplotypes and between *An. nuneztovari* s.l. and *An. dunhami* haplotypes was estimated by using the uncorrected *p* genetic distance in PAUP 4.0b1.[54] The sequence divergences among clades and subclades were calculated by using the Kimura two-parameter evolutionary model in MEGA 4.0,[55] and the divergence time was estimated assuming two distinct mutation rates: 2.3% estimated for the *COI* gene in insects[56] and 11.4% from the complete *Drosophila* mtDNA genome[57,58] per million years. Haplotype sequences are deposited in GenBank under the accession numbers AF270915 to AF270932, and JN692127 to JN692171.

RESULTS

In *An. nuneztovari* s.l., the 113 sequences contained 63 (7.2%) polymorphic sites, and 52 (6.0%) sites were parsimoniously informative. The mean base composition was rich in A + T (70.2%). Fifty-two haplotypes were observed, with 28 (53.8%) singletons, 14 (26.9%) shared among individuals within localities, and 10 (19.2%) shared between localities (Table 1). The most frequent and ancestral haplotype (n = 7; H26) was detected in three Amazonas State sites (MN, BR, and CS). Haplotypes H3, H10, H24, H41, and H42 were shared between geographically distant localities: NM and BR, IT and PV, MN and VI, GU and BA, and GU and RS, respectively. No shared haplotypes were observed between central (MN, BR, CS, CR, and IT) and eastern (BL, TU, and PI) regions from Amazonian Brazil, or between Amazonian Brazil plus Suriname and Bolivia/Colombia/Venezuela.

All haplotypes were connected in the network in which four clades were distinguished (Figure 2). The haplotypes from Amazonian Brazil plus Suriname were located in I, II-A and

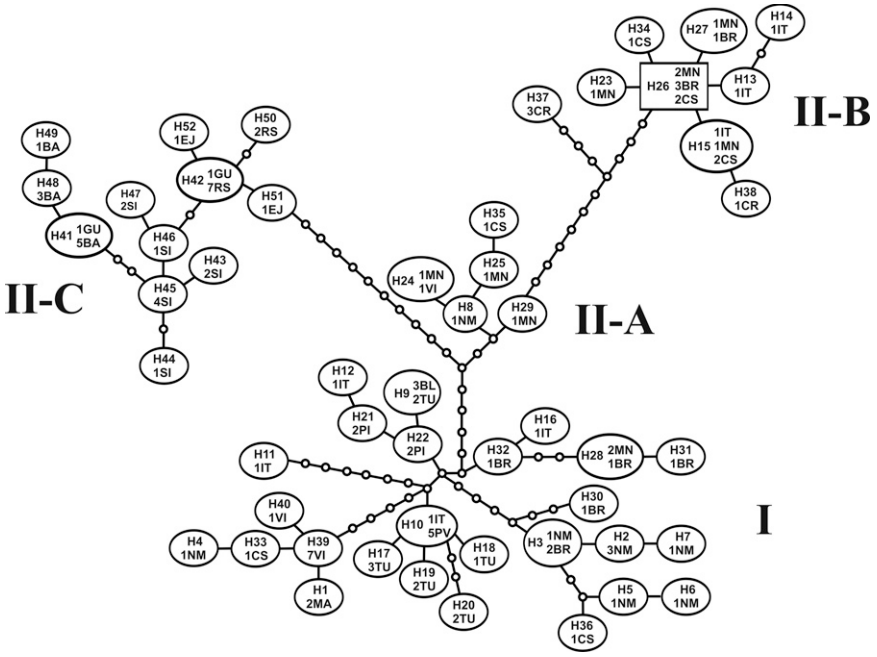

FIGURE 2. Parsimony network of the 52 haplotypes observed in *Anopheles nuneztovari* s.l. H1–H52 = haplotypes. The number of individuals observed in each haplotype is next to the locality abbreviation. Empty smaller circles represent mutational events. See Table 1 for locality abbreviations.

II-B, differing from one another by 9–11 mutational steps. In contrast, haplotypes from Bolivia/Colombia/Venezuela were all in II-C. The ML and BI analyses yielded trees with identical topologies, but varying levels of support. The BI tree and the distribution of the clades are shown in Figures 3 and 4, respectively. Two major monophyletic clades were observed. Clade I, the largest, comprised individuals of all regions analyzed from Amazonian Brazil and Suriname. Clade II, more basal, consisted of three subclades. Subclade II-A included individuals from northeastern and central Amazonian Brazil and Suriname. Suclade II-B was not well supported for ML and included individuals from central Amazonian Brazil. Subclade II-C included individuals from Bolivia/Colombia/Venezuela. Several localities had haplotypes clustered in multiple subclades (Table 1). All *An. dunhami* haplotypes were strongly supported on an isolated branch.

Mean ± SE values of h and π diversities (0.976 ± 0.005 and 0.01347 ± 0.00039, respectively) and K (11.690) were fairly high (Table 2). The highest values of h, π, and K were found in MN, BR, CS, and IT (central Amazonian Brazil), followed by NM (northeastern Amazonian Brazil). Both h and π diversities were highest in clade I (Table 3).

The neutral evolution tests showed negative and non-significant values in most cases, except subclade II-C (positive and non-significant for D test of Tajima) (Table 3). This finding may reflect an excess of rare alleles consistent with either positive selection or an increase in population size. Results of the Fs test of Fu were negative and significant for clade I, subclade II-C, and combined data, indicating significant deviations from equilibrium. Mismatch distribution for clade I, subclades II-A and II-C, and combined data showed unimodal distribution, suggesting populations at non-equilibrium (Figure 5). Subclade II-B had a bimodal distribution and could represent two colonization events. The raggedness index (r) for all groups did not reject the null hypothesis of sudden demographic expansion. However, for the sum of squared deviations goodness of fit test, only subclade II-B rejected the null hypothesis ($P = 0.04$). Consequently, the time since the population expansion was estimated by using $t = \tau/2\mu$, where μ is the mutation rate per site per generation. The *Drosophila* mutation rate of 10^{-8}/site/year and 10 generations/year[59] was used in this calculation. The estimated expansion times for clade I and three subclades were during the Pleistocene Epoch (approximately 8,768–48,293 years ago). Subclade II-B populations expanded more recently, approximately 8,768 years ago.

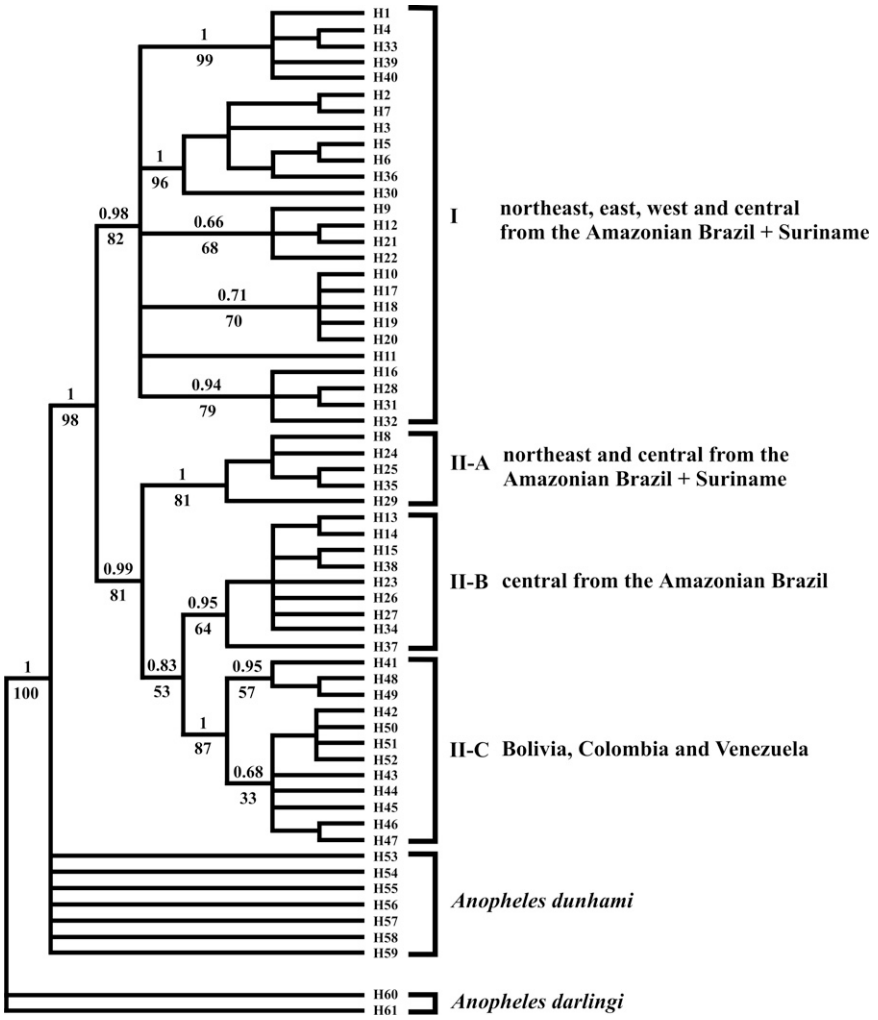

FIGURE 3. Bayesian Inference (BI) topology of the 59 haplotypes of *Anopheles nuneztovari* s.l. and *Anopheles dunhami* inferred under the TPM2uf + I + G model. Numbers on each branch represent posterior probabilities in BI (above branch) and bootstrap proportions in ML (below branch). *Anopheles darlingi* was used as outgroup.

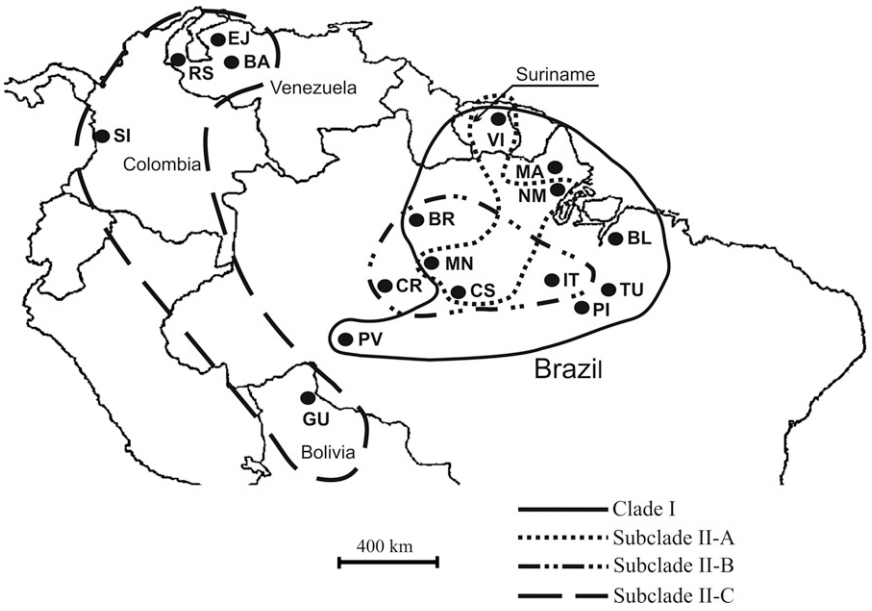

FIGURE 4. Estimated geographic distributions of clade I and subclades II-A, II-B, and II-C of *Anopheles nuneztovari* s.l. See Table 1 for locality abbreviations.

In *An. nuneztovari* s.l., the uncorrected *p* genetic distance among haplotypes was low to moderate (0.12–2.98%) and overlapped with the distance between *An. nuneztovari* s.l. and *An. dunhami* haplotypes (1.6–3.4%; mean = 2.6%). The range of genetic distance was higher within Amazonian Brazil (0.12–2.98%) than between Amazonian Brazil and Bolivia/Colombia/Venezuela (1.03–2.52%). Sequence divergence determined by using the Kimura two-parameter model between clades and subclades was also low (Table 4). However, the mean *K* values among I, II-A, II-B, and II-C were much higher than within them (Tables 3 and 4). Estimated time of divergence among the four groups suggests diversification during the Pleistocene Epoch.

The seven haplotypes observed for *An. dunhami* showed uncorrected *p* genetic distance from 0.12% to 1.83%, and new records for this species were found in IT, MN, and SM. The distance between *An. dunhami* and *An. darlingi* was 8.9%, and the distance between *An. nuneztovari* s.l. and *An. darlingi* was 9.4%.

DISCUSSION

Phylogenetic analyses supported four reciprocally monophyletic clades for *An. nuneztovari* s.l., three represented by Amazon Basin samples (divergence = 1.6–2.1%) distributed in

TABLE 2
Intra-population genetic diversity measures of *Anopheles nuneztovari* sensu lato from South America*

Locality	No.	Ts/Tv	Haplotype observed	NS	*K*	Haplotype diversity (*h*) ± SD	Nucleotide diversity (π) ± SD
Brazil							
MA	2	0/0	1	NA	NA	NA	NA
NM†	9	21/2	7	23	6.861	0.917 ± 0.092	0.00786 ± 0.00236
BL	3	0/0	1	NA	NA	NA	NA
IT†	7	24/5	7	29	12.571	1.000 ± 0.076	0.0146 ± 0.00203
TU	10	9/0	5	9	3.333	0.867 ± 0.071	0.0038 ± 0.00053
PI	4	1/0	2	1	0.667	0.667 ± 0.204	0.00076 ± 0.00023
MN†	10	21/4	8	25	10.133	0.956 ± 0.059	0.01161 ± 0.00164
BR†	10	21/5	7	26	12.244	0.911 ± 0.077	0.01421 ± 0.00150
CS†	8	30/2	6	32	11.893	0.929 ± 0.084	0.01382 ± 0.00354
CR	4	10/0	2	10	5.000	0.500 ± 0.265	0.00573 ± 0.00304
PV	5	0/0	1	NA	NA	NA	NA
Suriname							
VI†	9	13/3	3	16	3.556	0.417 ± 0.191	0.00407 ± 0.00284
Bolivia							
GU	2	4/0	2	4	4.000	NA	NA
Colombia							
SI	10	2/3	5	5	1.578	0.822 ± 0.097	0.00181 ± 0.00041
Venezuela							
BA	9	1/1	3	2	0.778	0.639 ± 0.126	0.00089 ± 0.00023
RS	9	2/0	2	2	0.778	0.389 ± 0.164	0.00089 ± 0.00038
EJ	2	0/2	2	2	2,000	NA	NA
Total	113	9.353/1.588	52	63	11.690	0.976 ± 0.005	0.01347 ± 0.00039

*No. = sample size; Ts/Tv = transitions/transversions; NS = no. of polymorphic sites; *K* = average number of nucleotide differences; NA = not analyzed. See Table 1 for locality abbreviations.
†Locations with two or three sympatric haplotype clade/subclades.

TABLE 3
Summary statistics of polymorphisms and neutrality tests for each haplotype clade in *Anopheles nuneztovari* sensu lato*

Haplotype clade	No.	NS	*K*	*h* ± SE	π ± SE	Tajima *D*	Fu and Li *D*	Fu and Li *F*	Fu *F*s	R_2	*r*	SSD	τ
Clade I	54	39	7.02	0.9560 ± 0.012	0.00804 ± 0.00049	–0.6039	–0.2834	–0.4773	–7.125†	0.0867	0.0083	0.0026	8.432
Subclade II-A	6	5	2.07	0.9333 ± 0.122	0.00237 ± 0.00053	–0.3147	–0.2147	–0.2514	–2.041	0.1863	0.1200	0.0218	2.346
Subclade II-B	21	16	3.28	0.8524 ± 0.055	0.00375 ± 0.00096	–0.9671	–0.1087	–0.4210	–1.298	0.0935	0.0895	0.0432‡	1.531
Subclade II-C	32	13	3.34	0.8891 ± 0.032	0.00383 ± 0.00027	0.1178	–0.3004	–0.1979	–2.452‡	0.1215	0.0245	0.0108	4.307
Total	113	63	3.93	0.976 ± 0.005	0.01339 ± 0.00038	–0.4079	–0.0842	–0.2656	–16.565§	0.912	0.0053	0.0033	14.367

* No. = sample size; NS = no. of polymorphic sites; *K* = Average number of nucleotide differences; *h* ± SE and π ± SE = haplotype and nucleotide diversities, respectively, with respective SEs; R_2 = Ramos-Onsins and Rosas statistics; *r* = raggedness index; SSD = sum of squared deviations; τ = expansion parameter.
† $P < 0.001$.
‡ $P < 0.05$.
§ $P < 0.0001$.

two major clades (I and II) and one represented by Bolivia/Colombia/Venezuela samples (divergence = 1.7%) restricted to basal subclade II-C. The haplotype network also indicated a substantial genetic division between them. Taken together, these results suggest incipient speciation or distinct species within *An. nuneztovari* s.l. This finding is partially congruent with the nuclear *white* gene results of Mirabello and Conn,[28] who hypothesized three clades in the Amazon Basin plus Bolivia and two in Colombia and Venezuela. Clade I and subclade II-C may correspond, respectively, to lineage 1 and lineages 2 and 3 from Mirabello and Conn,[28] whereas subclades II-A and II-B seem not to correspond to lineages 4 and 5.[28] This disagreement is likely caused by distinct modes of inheritance between markers used or the sampling design.

One explanation for the presence of Amazon Basin haplotypes in both major clades could be incomplete lineage sorting caused by recent divergence or retention of ancestral polymorphism. Another possibility could be introgression between populations from northern Amazonian Brazil and southeastern Venezuela[60]; a hypothesis that may be confirmed or refuted by comparative analyses of these samples.

Unlike earlier studies that detected higher levels of differentiation between Amazon Basin and Colombia/Venezuela populations,[18–28] our results identified the deepest divergence and earliest divergence time between clade I and subclade II-B, both within the Amazon Basin. These discrepant results could have been caused by more localities from the central Amazonian Brazil being sampled in the present study. This region may be pivotal because it includes the most divergent subclade (II-B) and the oldest haplotype. These data suggest that additional incipient species may exist in this complex within the Amazon Basin.

Clade I consisted nearly exclusively of Amazonian haplotypes (except CR) and several smaller subdivisions. One of these subdivisions, strongly supported by BI and ML analyses, grouped most individuals from NM (7 of 9) and few from BR (3 of 10) and CS (1 of 8). All of these individuals shared three transitions that were rare or absent in the remaining individuals analyzed. This finding may suggest a potential barrier to gene flow, supporting the findings of Bergo and others[29] of a new putative species in Amapá State. Calado and others[18] proposed that *An. nuneztovari* s.l. from Amazonian Brazil could be *An. goeldii*. A comparative analysis using the small overlapping fragment between our data and those authors[18] suggested that individuals of this clade I subdivision could be *An. goeldii*. As a consequence, this species could exist in northeastern and central Amazonian Brazil. This analysis also indicated that some clade I haplotypes from PI and IT, Pará State, were closely related to *An. goeldii* haplotypes found by Calado and others.[18] If these haplotypes represent *An. goeldii*, it also could exist in the eastern region of Amazonian Brazil, and its populations should be differentiated genetically.

Curiously, *An. nuneztovari* s.l. from some localities in Pará State (eastern region) exhibit accentuated anthropophily and endophagy.[10] However, mosquitoes collected from the central Amazonian region have been mainly zoophagic and exophilic.[7] In the present study and in the study of Scarpassa and others,[27] samples from these two regions had no shared haplotypes and were recognized as distinct lineages by Conn and others,[26] which could indicate a barrier to gene flow. If this suggestion is correct, this finding may explain distinct behavioral patterns of these mosquitoes.

Subclades II-A and II-B samples may represent distinct evolutionary lineages or species with overlapping ranges in central Amazonian Brazil, likely caused by historical fragmentation with differentiation, followed by population expansion and secondary contact. Therefore, in addition to *An. goeldii* as discussed above, other lineages or species could exist in the *An. nuneztovari* complex in this region. Some support for this suggestion comes from a study of male genitalia of specimens from Manaus. Two distinct forms were observed: one was compatible with *An. goeldii* and the other has not yet been determined (Silva HP, Lima CP, Tadei WP, unpublished data).

Subclade II-C, represented by samples from Bolivia/Colombia/Venezuela, is basal and shares no haplotypes with any other geographic region, supporting results of earlier studies.[18–28] Morphologic comparisons showed that the apex of the aedeagus is distinct between samples from the Amazon Basin and Colombia plus Venezuela.[3,18] These data combined with molecular analyses led to the resurrection of *An. goeldii*.[18] In the present study, divergence among haplotypes was low to moderate. However, this finding was consistent with the data of Calado and others[18] and with divergence estimates of other sister-taxa in the *Nyssorhynchus* subgenus.[61] Additionally, populations of *An. nuneztovari* s.l. from the Amazon Basin and Colombia/Venezuela can be differentiated by a fixed inversion on the X chromosome.[19,20] Taken together, our results suggest that subclade II-C samples could be *An. nuneztovari* s.s., which may be in the process of separating from *An. goeldii* and from other lineages or incipient species present in the

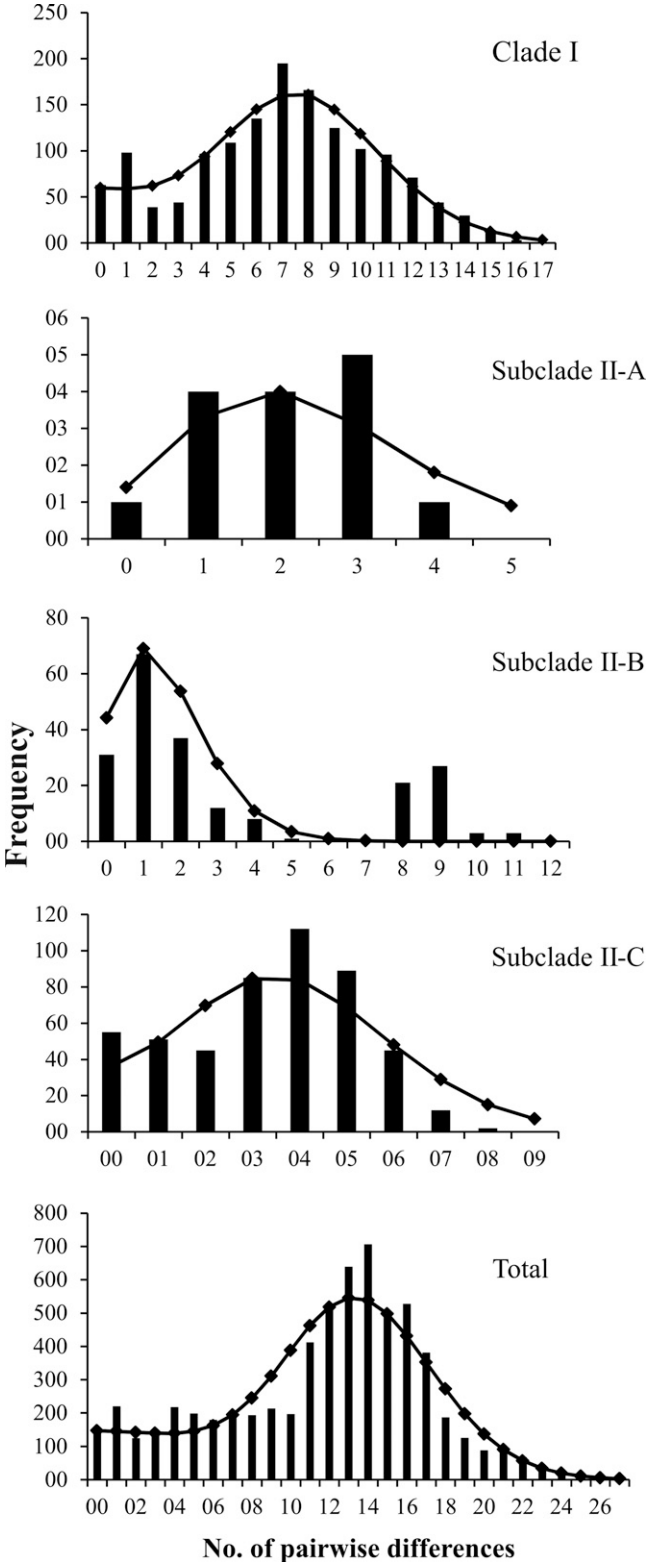

FIGURE 5. Observed mismatch distributions among haplotypes in *Anopheles nuneztovari* s.l. for clade I, three subclades, and combined data. Bars are observed distribution, and the line shows the distribution simulated under a sudden expansion model.

Amazon Basin by an extremely recent evolutionary event.[30] In addition, on the basis of our results and those of previous studies,[22,28] we propose that two species may co-occur in Bolivia, one of which is likely to be *An. nuneztovari* s.s. and the other *An. goeldii* or lineage 5 reported by Mirabello and Conn.[28] Colombia (SI) haplotypes were not shared with Venezuela and/or Bolivia haplotypes. The isolation in SI can reflect the eastern Andes Cordillera acting as a possible physical barrier to dispersal between this and others populations.[24,25,28]

TABLE 4

Average nucleotide divergence based on Kimura two-parameter model, mean number of nucleotide differences (*K*), and estimated time of divergence among haplotypes groups of *Anopheles nuneztovari* s.l.

Haplotype clade	Sequence divergence, %	Mean nucleotide differences (*K*)	Divergence time, million years*	Divergence time, million years†
I vs. II-A	1.6	13.42	0.35	0.70
I vs. II-B	2.1	18.21	0.46	0.92
I vs. II-C	1.7	13.93	0.37	0.75
II-A vs. II-B	1.6	14.86	0.35	0.70
II-A vs. II-C	1.7	14.87	0.37	0.75
II-B vs. II-C	1.7	14.82	0.37	0.75

*Assuming a mutation rate = 2.3%.
†Assuming a mutation rate = 11.4%.

In this study, intra-population diversity measures were fairly high in *An. nuneztovari* s.l., as detected in a previous study in this species,[26] and in other anophelines.[61–63] The samples from the Amazon Basin tended to have higher genetic diversity, in particular nucleotide diversity, than those from Bolivia/Colombia/Venezuela. These differences were congruent with those of previous mitochondrial and nuclear gene studies[22,25–28,64] and can be indicative of cryptic species, or possibly differences in effective population size (*N*e) or other events related to the history of these populations. The highest genetic diversity was observed for localities in the central region of Amazonian Brazil. In general, older populations have a higher diversity than younger populations.[65] On the basis of this criterion and the presence of the oldest haplotype, the central Amazonian Brazil region may represent the ancestral area of this species, as recently proposed for two similarly distributed anophelines.[66,67]

The central region shared more haplotypes with geographically distant localities (NM, PV, and VI, which are approximately 1,100 km apart). Additional evidence indicates that this region may represent the ancestral area. Also, two haplotypes from Bolivia (GU) were shared with more frequent Venezuelan haplotypes (BA and RS, which are approximately 2,000 km apart), but not with PV (approximately 280 km apart). Similarly, ITS2 sequences clustered populations from Bolivia/Colombia/Venezuela into a single group,[22] and this result was also observed with the *white* gene.[28] The sharing of haplotypes across great distances may be the result of isolation, followed by secondary contact in an area known for its complex geologic changes.[68]

Pleistocene epoch divergence has been detected for a wide range of insect species,[56,69] including disease vectors,[28,61,63] suggesting climatic changes as an important force driving the speciation in the Neotropical region. In the present study, the divergence time estimated among all clades and subclades supported diversification during the Pleistocene Epoch. Results of the *F*s test of Fu suggested demographic expansion for clade I, subclade II-C, and combined data. The mismatch distribution depicted populations at non-equilibrium for all groups, although the sum of squared deviations goodness of fit test did not reject the null hypothesis, except for subclade II-B. The departure from equilibrium, the star-like network,

and lowest τ for this subclade indicates that it experienced expansion more recently than the other clades (estimated to have occurred 8,768 years ago). Additionally, the bimodal distribution visualized for subclade II-B suggests that it may have undergone two bottlenecks or colonization events. If this suggestion is correct, the deeper divergence for this subclade could be explained by genetic drift and the ancestral haplotype persisting during these events. Taken together, climatic changes, such as precipitation decrease, more arid conditions, and forest fragmentation, which occurred in the Pleistocene Epoch,[70] likely contributed to speciation in this complex.

These findings may help to clarify the involvement of *An. nuneztovari* complex members in malaria transmission, especially in Amazonian Brazil, where its hypothesized vector competence is still controversial.

The *COI* fragment analyzed clearly separated *An. nuneztovari* s.l. from *An. dunhami*. On the basis of this result, we report three new records for *An. dunhami* in Amazonian Brazil, and its range may be more extensive than previously believed. The low genetic distance found between these two species (mean = 2.6%) and results of previous studies, including multiple markers,[37] isozymes,[38] ITS2, *COI* and *white* gene sequences,[18,39] strongly suggest recent divergence. Given that *An. nuneztovari* s.l. was detected infected with *Plasmodium* species in Amazonian Brazil and it was implicated as a local vector in Amapá State,[9] the accurate identification of these species is important for vector control strategies in areas where they are sympatric.

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REFERENCES

1. Faran ME, 1980. Mosquito studies (Diptera, Culicidae). XXXIV. A revision of the *Albimanus* section of the subgenus *Nyssorhynchus* of *Anopheles*. *Contrib Am Entomol Inst 15:* 1–215.
2. Elliott R, 1972. The influence of vector behavior on malaria transmission. *Am J Trop Med Hyg 21:* 755–763.
3. Gabaldón A, 1981. *Anopheles nuneztovari*: importante vector y agente de malaria refractaria en Venezuela. *Bol Dir Malariol Saneam Amb XXI:* 28–38.
4. Gutiérrez LA, González JJ, Gomes GF, Castro MI, Rosero DA, Luckhart S, Conn JE, Correa MM, 2009. Species composition and natural infectivity of anthropophilic *Anopheles* (Diptera: Culicidae) in the states of Córdoba and Antioquia, northwestern Colombia. *Mem Inst Oswaldo Cruz 104:* 1117–1124.
5. Arruda M, Carvalho MB, Nussenzweig RS, Maracic M, Ferreira AW, Cochrane AH, 1986. Potential vectors of malaria and their different susceptibility to *Plasmodium falciparum* and *Plasmodium vivax* in northern Brazil identified by immunoassay. *Am J Trop Med Hyg 35:* 873–881.
6. Hayes J, Calderon G, Falcon RI, Zambrand V, 1987. Newly incriminated anopheline vector of human malaria parasites in Junin Department, Peru. *J Am Mosq Control Assoc 3:* 418–422.
7. Tadei WP, Dutary-Thatcher B, Santos JM, Scarpassa VM, Rodrigues IB, Rafael MS, 1998. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. *Am J Trop Med Hyg 59:* 325–335.
8. Póvoa MM, Wirtz RA, Lacerda RN, Miles MA, Warhurst D, 2001. Malaria vectors in the municipality of Serra do Navio, State of Amapá, Amazon Region, Brazil. *Mem Inst Oswaldo Cruz 96:* 179–184.
9. Galardo AC, Arruda M, Couto ARA, Wirtz R, Lounibos LP, Zimmerman RH, 2007. Malaria vector incrimination in three rural riverine villages in the Brazilian Amazon. *Am J Trop Med Hyg 76:* 461–469.
10. Tadei WP, Mascarenhas BM, Podestá MG, 1983. Biologia de Anofelinos Amazônicos. VIII. Conhecimentos sobre a distribuição de espécies de *Anopheles* na região de Tucuruí-Marabá (Pará). *Acta Amazon 13:* 103–140.
11. Marrelli MT, Floeter-Winter LM, Malafronte RS, Tadei WP, Lourenço-de-Oliveira R, Flores-Mendonza C, Marinotti O, 2005. Amazonian malaria vector anopheline relationships interpreted from ITS2 rDNA sequences. *Med Vet Entomol 19:* 208–218.
12. Krzywinski J, Li C, Morris M, Conn JE, Lima JB, Póvoa MM, Wilkerson RC, 2011. Analysis of the evolutionary forces shaping mitochondrial genomes of a neotropical malaria vector complex. *Mol Phyl Evol 58:* 469–477.
13. Van Bortel W, Trung H, Sochantha T, Keokenchan K, Roelants P, Backeljau T, Coosemans M, 2004. Ecoethological heterogeneity of the members of the *Anopheles minimus* complex (Diptera: Culicidae) in southeast Asia and its consequences for vector control. *J Med Entomol 41:* 366–374.
14. Lounibos LP, Conn JE, 2000. Malaria vector heterogeneity in South America. *Am Entomol 46:* 238–249.
15. Wallace AR, 1853. *A Narrative of Travel on the Amazon and Rio Negro*. London: Reeve and Co.
16. Harback RE, 2004. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. *Bull Entomol Res 94:* 537–553.
17. Silva do Nascimento TF, Lourenço-de-Oliveira R, 2002. *Anopheles halophylus*, a new species of the subgenus *Nyssorhynchus* [*sic*] (Diptera: Culicidae) from Brazil. *Mem Inst Oswaldo Cruz 97:* 801–811.
18. Calado DC, Foster PG, Bergo ES, Santos CLS, Galardo AK, Sallum MA, 2008. Resurrection of *Anopheles goeldii* from synonymy with *Anopheles nuneztovari* (Diptera: Culicidae) and a new record for *Anopheles dunhami* in the Brazilian Amazon. *Mem Inst Oswaldo Cruz 103:* 791–799.
19. Kitzmiller JB, Kreutzer RD, Tallaferro E, 1973. Chromosomal differences in populations of *Anopheles nuneztovari*. *Bull World Health Organ 48:* 435–445.
20. Conn J, Puertas YR, Seawright JA, 1993. A new cytotype of *Anopheles nuneztovari* from western Venezuela and Colombia. *J Am Mosq Control Assoc 9:* 294–301.
21. Hribar LJ, 1994. Geographic variation of male genitalia of *Anopheles nuneztovari* (Diptera: Culicidae). *Mosq Syst 26:* 132–144.
22. Fritz GN, Conn J, Cockburn AF, Seawright JA, 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2

from populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Mol Biol Evol 11:* 406–416.
23. Linley JR, Lounibos LP, Conn J, Duzak D, Nishimura N, 1996. A description and morphometric comparison of eggs from eight geographic populations of the South American malaria vector *Anopheles* (*Nyssorhynchus*) *nuneztovari* (Diptera: Culicidae). *J Am Mosq Control Assoc 12:* 275–292.
24. Scarpassa VM, Tadei WP, Suarez MF, 1996. Allozyme differentiation among allopatric populations of *Anopheles nuneztovari* (Diptera: Culicidae). *Braz J Genet 19:* 265–269.
25. Scarpassa VM, Tadei WP, Suarez MF, 1999. Population structure and genetic divergence in *Anopheles nuneztovari* (Diptera: Culicidae) from Brazil and Colombia. *Am J Trop Med Hyg 60:* 1010–1018.
26. Conn JE, Mitchell SE, Cockburn AF, 1998. Mitochondrial DNA analysis of the neotropical malaria vector *Anopheles nuneztovari*. *Genome 41:* 313–327.
27. Scarpassa VM, Geurgas S, Azeredo-Espin AM, Tadei WP, 2000. Genetic divergence in mitochondrial DNA of *Anopheles nuneztovari* (Diptera: Culicidae) from Brazil and Colombia. *Genet Mol Biol 23:* 71–78.
28. Mirabello L, Conn JE, 2008. Population analysis using the nuclear white gene detects pliocene/pleistocene lineage divergence within *Anopheles nuneztovari* in South America. *Med Vet Entomol 22:* 109–119.
29. Bergo ES, Souto RN, Galardo AK, Nagaki SS, Calado DC, Sallum MA, 2007. Systematic notes on *Anopheles* meigen (Diptera: Culcidae) species in the state of Amapá, Brazil. *Mem Inst Oswaldo Cruz 102:* 373–376.
30. Queiroz K, 2007. Species concepts and species delimitation. *Syst Biol 56:* 879–886.
31. Gabaldón A, 1940. Estudios sobre anofelinos. Serie I. 1. Descripcion de *Anopheles* (*Nyssorhynchus*) *nunez-tovari* n. sp. y consideraciones sobre una sub-division del grupo *Nyssorhynchus* (Diptera: Culicidae). *Publ Div Malariol 5:* 3–7.
32. Rozeboom LE, Gabaldón A, 1941. A summary of the "*tarsimaculatus*" complex of *Anopheles* (Diptera: Culicidae). *Am J Hyg 33:* 88–100.
33. Floch H, Abonnenc E, 1946. Sur *A. nuneztovari* et *A. pessoai* en Guyane Française table d'identification des *Nyssorhynchus guyanais*. *Inst Pasteur de la Guyane et du Territoire de L'Inini 126:* 1–5.
34. Lane J, 1953. *Neotropical Culicidae. Volume. 1* São Paulo: Universidade de São Paulo.
35. Causey OR, 1945. Description of *Anopheles* (*Nyssorhynchus*) *dunhami*, a new species from the Upper Amazon Basin. *J Natl Malar Soc 4:* 231–235.
36. Peyton EL, 1993. *Anopheles* (*Nyssorhynchus*) *dunhami*, resurrected from synonymy with *Anopheles nuneztovari* and validated as a senior synonym of *Anopheles trinkae* (Diptera: Culicidae). *Mosq Syst 25:* 151–156.
37. Lounibos LP, Wilkerson RC, Conn JE, Hribar LJ, Fritz GN, Danoff-Burg JA, 1998. Morphological, molecular, and chromosomal discrimination of cryptic *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae) from South America. *J Med Entomol 35:* 830–838.
38. Trindade DB, Scarpassa VM, 2002. Genetic differentiation and diagnostic loci among *Anopheles* (*Nyssorhynchus*) *rangeli, An.* (*Nys.*) *nuneztovari* and *An.* (*Nys.*) *dunhami*. (Diptera: Culicidae) from the Brazilian Amazon. *J Med Entomol 39:* 613–620.
39. Ruiz F, Linton YM, Ponsonby DJ, Conn JE, Herrera M, Quinones ML, Vélez ID, Wilkerson RC, 2010. Molecular of topotypic specimens confirms *Anopheles* (*Nyssorhynchus*) *dunhami* Causey (Diptera: Culicidae) in the Colombian Amazon. *Mem Inst Oswaldo Cruz 105:* 899–903.
40. Foley D, 2010. A spreadsheet mapping approach for error checking and sharing collection point data. *Biodiversity Inf 7:* 137–142.
41. Sambrook J, Russell DW, 2001. *A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
42. Joy D, Conn JE, 2001. Molecular and morphological phylogenetic analysis of an insular radiation in Pacific black flies (*Simulium*). *Syst Biol 50:* 18–38.
43. Clement M, Posada D, Crandall KA, 2000. TCS: a computer program to estimate gene genealogies. *Mol Ecol 9:* 1657–1660.
44. Crandall KA, Templeton AR, 1993. Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics 134:* 959–969.
45. Jobb G, 2008. *Treefinder version of October 2008*. Munich, Germany. Distributed by the author. Available at: www.treefinder.de.
46. Posada D, 2008. jModelTest: phylogenetic model averaging. *Mol Biol Evol 25:* 1253–1256.
47. Ronquist F, Huelsenbeck JP, 2003. *Mr. Bayes 3:* Bayesian phylogenetic inference under mixed models. *Bioinformatics 19:* 1572–1574.
48. Rozas J, Sánches-DelBarrio JC, Messeguer X, Rozas R, 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics 19:* 2496–2497.
49. Excoffier L, Laval G, Schneider S, 2006. *An Integrated Software Package for Population Genetics Data Analysis,* Version 3.01. Computational and Molecular Population Genetics Laboratory, Institute of Zoology, University of Berne, Switzerland. Available at: http://cmpg.unibe.ch/software/arlequin3.
50. Tajima F, 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphisms. *Genetics 123:* 585–595.
51. Fu YX, Li WH, 1993. Statistical tests of neutrality of mutations. *Genetics 133:* 693–709.
52. Fu YX, 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics 147:* 915–925.
53. Ramos-Onsins SE, Rozas J, 2002. Statistical properties of new neutrality tests against population growth. *Mol Biol Evol 19:* 2092–2100.
54. Swofford DL, 2003. *PAUP (Phylogenetic Analysis Using Parsimony) and Others Methods*, Version 4. Sunderland, MA: Sinauer Associates.
55. Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software, version 4.0. *Mol Biol Evol 24:* 1596–1599.
56. Brown A, 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proc Natl Acad Sci USA 91:* 6491–6495.
57. Tamura K, 1992. The rate and pattern of nucleotide substitution in *Drosophila* mitochondrial DNA. *Mol Biol Evol 9:* 814–825.
58. Hasan AU, Suguri S, Fujimoto C, Itaki RL, Harada M, Kawabata M, Bugoro H, Albino B, Tsukahara T, Hombhanje F, Masta A, 2008. Phylogeography and dispersion pattern of *Anopheles farauti* senso stricto mosquitoes in Melanesia. *Mol Phyl Evol 46:* 792–800.
59. Walton C, Handley JM, TunLin W, Collins FH, Harbach RE, Baimai V, Butlin RK, 2000. Population structure and population history of *Anopheles dirus* mosquitoes in southeast Asia. *Mol Biol Evol 17:* 962–974.
60. Moreno J, Rubio-Palis Y, Sánchez V, Mariany D, 2004. Primer registro de *Anopheles* (*Nyssorhynchus*) *nuneztovari* Gabaldón, 1940 (Diptera: Culicidae) en el estado Bolívar, Venezuela y sus implicaciones eco-epidemiológicas. *Entomotropica 19:* 55–58.
61. MacKeon SN, Lehr MA, Wilkerson RC, Ruiz JF, Sallum MA, Lima JB, Póvoa MM, Conn JE, 2010. Lineage divergence detected in the malaria vector *Anopheles marajoara* (Diptera: Culicidae) in Amazonian Brazil. *Malar J 9:* 271.
62. Scarpassa VM, Conn JE, 2006. Molecular differentiation in natural populations of *Anopheles oswaldoi* sensu lato (Diptera: Culicidae) from the Brazilian Amazon, using sequences of the *COI* gene from mitochondrial DNA. *Genet Mol Res 5:* 493–502.
63. Loaiza JR, Scott ME, Bermingham E, Rovira J, Conn JE, 2010. Evidence for Pleistocene population divergence and expansion of *Anopheles albimanus* in southern Central America. *Am J Trop Med Hyg 82:* 156–164.
64. Onyabe DY, Conn JE, 1999. Intragenomic heterogeneity of a ribosomal DNA spacer (ITS2) varies regionally in the neotropical malaria vector *Anopheles nuneztovari* (Diptera: Culicidae). *Insect Mol Biol 8:* 435–442.
65. Kambhampati S, Rai KS, 1991. Mitochondrial DNA variation within and among populations of the mosquito *Aedes albopictus*. *Genome 34:* 288–292.

66. Pedro MP, Sallum MA, 2009. Spatial expansion and population structure of the neotropical malaria vector, *Anopheles darlingi* (Diptera: Culicidae). *Biol J Linn Soc Lond 97:* 854–866.
67. Pedro MP, Uezu A, Sallum MA, 2010. Concordant phylogeographies of 2 malaria vectors attest to common spatial and demographic histories. *J Hered 54:* 1–10.
68. Roussetti DF, Mann de Toledo P, Góes AM, 2005. New geological framework for Western Amazonia (Brazil) and implications for biogeography and evolution. *Quat Res 63:* 78–89.
69. Dick CW, Roubik DW, Gruber KF, Bermingham E, 2004. Long-distance gene flow and cross-Andean dispersal of lowland rainforest bees (Apidae: Euglossini) revealed by comparative mitochondrial DNA phylogeography. *Mol Ecol 13:* 3775–3785.
70. Mayle FE, Beerling DJ, Gosling WD, Bush MB, 2004. Responses of Amazonian ecosystems to climatic and atmospheric carbon dioxide changes since the last glacial maximum. *Philos Trans R Soc Lond B Biol Sci 359:* 499–514.