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Research

Phylogenetic Network of Mitochondrial *COI* Gene Sequences Distinguishes 10 Taxa Within the Neotropical Albitarsis Group (Diptera: Culicidae), Confirming the Separate Species Status of *Anopheles albitarsis* H (Diptera: Culicidae) and Revealing a Novel Lineage, *Anopheles albitarsis* J

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Abstract

The Neotropical Albitarsis Group is a complex assemblage of essentially isomorphic species which currently comprises eight recognized species—five formally described (*Anopheles albitarsis* Lynch-Arribalzaga, *An. deaneorum* Rosa-Freitas, *An. janconnae* Wilkerson and Sallum, *An. marajoara* Galvao and Damasceno, *An. oryzalimnetes* Wilkerson and Motoki) and three molecularly assigned (*An. albitarsis* F, G & I)—and one mitochondrial lineage (*An. albitarsis* H). To further explore species recognition within this important group, 658 base pairs of the mitochondrial DNA cytochrome oxidase subunit I (*COI*) were analyzed from 988 specimens from South America. We conducted statistical parsimony network analysis, generated estimates of haplotype, nucleotide, genetic differentiation, divergence time, and tested the effect of isolation by distance (IBD). Ten clusters were identified, which confirmed the validity of the eight previously determined species, and confirmed the specific status of the previous mitochondrial lineage *An. albitarsis* H. High levels of diversity were highlighted in two samples from Pará (= *An. albitarsis* J), which needs further exploration through additional sampling, but which may indicate another cryptic species. The highest intra-specific nucleotide diversity was observed in *An. deaneorum*, and the lowest in *An. marajoara*. Significant correlation between genetic and geographical distance was observed only in *An. oryzalimnetes* and *An. albitarsis* F. Divergence time within the Albitarsis Group was estimated at 0.58–2.25 Mya, during the Pleistocene. The *COI* barcode region was considered an effective marker for species recognition within the Albitarsis Group and a network approach was an analytical method to discriminate among species of this group.

Key words: Albitarsis Group, barcoding, network, cryptic diversity, new lineage

Delineation of taxa within essentially isomorphic cryptic species groups is a significant challenge (de Queiroz 2007), leading to the increased reliance of molecular tools to identify and delimit species (Leache and Fujita 2010, Fujita et al. 2012). To explore relationships between cryptic taxa, tree-based phylogenetic approximations such as maximum parsimony, maximum likelihood, and distance methods are often used. However, gene evolution, which may be reticulate, is not always best represented in this way. To resolve such relationships,

network approaches have been developed to estimate genealogies. These include statistical geometry (Eigen et al. 1988), likelihood networks (Strimmer and Moulton 2000), median networks (Bandelt et al. 2000), and median-joining network approaches (Bandelt et al. 1999). Hart and Sunday (2007) observed a strong association between breaks in network connectivity and species-level separation. The method of Templeton, Crandall, and Sing (1992) (TCS) has been used extensively to infer genealogies when divergences are low (Gerber and Templeton 1996, Vila et al. 1999, Gomes-Zurita et al. 2000). In addition, using a phylogenetic network, Chen et al. (2010) were able to predict species limits within nemertean species complexes (*Cephalothrix rufifrons*, *C. major,* and *C. spiralis*) that lacked sufficient morphological characters to separate individual taxa.

Many investigations have been undertaken to assess the taxonomic status of the component members of the Albitarsis Group, some of which play a significant role in malaria transmission in regions of South America (Klein et al. 1991a,b; Rubio-Palis 1994; Chadee et al. 2006; Povoa et al. 2006; Gutierrez et al. 2010). Based on phylogenetic analyses of mitochondrial protein-coding genes, Foster et al. (2017) suggested the elevation of *Nyssorhynchus, Kerteszia*, *Lophopodomyia*, and *Stethomyia* to a generic level. As the ranking of taxa as genera or subgenera is subjective, the traditional recognition of *Anopheles* as a genus (Harbach 2018) is retaining herein. According to the latest review (Ruiz-Lopez et al. 2012), the Albitarsis Group was understood to comprise eight formally recognized species: *Anopheles albitarsis* Lynch-Arribálzaga, *An. deaneorum* Rosa-Freitas, *An. janconnae* Wilkerson and Sallum (= *An. albitarsis* E) (Lehr et al. 2005, Motoki et al. 2009), *An. marajoara* Galvão and Damasceno, *An. oryzalimnetes* Wilkerson and Motoki (= *An. albitarsis* B) (Motoki et al. 2009), three molecularly determined taxa, *An. albitarsis* F (Brochero et al. 2007), *An. albitarsis* G (McKeon et al. 2010, Krzywinski et al. 2011, Ruiz-Lopez et al. 2012), and *An. albitarsis* I (Gutierrez et al. 2010, Ruiz-Lopez et al. 2012), and the mitochondrial lineage, *An. albitarsis* H (Ruiz-Lopez et al. 2012). Only *An. deaneorum* can reliably be separated from species in the Albitarsis Group and then only in the larval stage, based on seta 3-C of the larvae (Rosa-Freitas 1989). Seta 3-C is branched in *An. deaneorum*, whereas *An. albitarsis*, *An. marajoara*, and *An. oryzalimnetes* exhibit single, simple, or aciculate seta.

The Albitarsis Group has a large distribution in South America. *Anopheles albitarsis* is found in northern Argentina, southern Paraguay, and Brazil (Wilkerson et al. 1995a, Lehr et al. 2005, Li and Wilkerson 2005, Ruiz-Lopez et al. 2012, Foley et al. 2014); *An. albitarsis* F and *An. albitarsis* I are found in northern South America (Colombia, Trinidad and Tobago, and Venezuela) (Brochero et al. 2007, Gutierrez et al. 2010, Ruiz-Lopez et al. 2012); while *An. oryzalimnetes* is widely distributed in the central region of Brazil (Wilkerson et al. 1995a,b; Lehr et al. 2005; Ruiz-Lopez et al. 2012; Foley et al. 2014; herein), and *An. marajoara* in the northern and central regions of Brazil (Ruiz-Lopez et al. 2012, Foley et al. 2014). *Anopheles deaneorum* is present in the western regions of Brazil (Wilkerson et al. 1995b, Li and Wilkerson 2005, Ruiz-Lopez et al. 2012, Foley et al. 2014); *An. janconnae* is restricted to the northern region (Lehr et al. 2005, Povoa et al. 2006, Ruiz-Lopez et al. 2012); *An. albitarsis* G in the Amazon and east region (Ruiz-Lopez et al. 2012, Foley et al. 2014; herein); and *An. albitarsis* H in the central and western regions (Ruiz-Lopez et al. 2012, Foley et al. 2014).

Using *COI* sequences generated from specimens from Brazil, Argentina, Colombia, Paraguay, Trinidad and Tobago, and Venezuela, Ruiz-Lopez et al. (2012) proposed eight species in the Albitarsis Group (*An. albitarsis*, *An. deaneorum*, *An. janconnae*, *An. marajoara*, *An. oryzalimnetes*, and *An. albitarsis* F, G & I), and recognized the novel mitochondrial lineage *An. albitarsis* H. In addition, Foley et al. (2014) examined environmental and ecological divergence events for these nine taxa from additional localities. The present study examines the *COI* sequences from 988 individuals, using the dataset of Ruiz-Lopez et al. (2012) and Foley et al. (2014), including 20 new *COI* sequences, to test whether network analysis further supports Ruiz-Lopez et al.'s eight purported taxa, and whether additional support could be garnered for the taxonomic status of *An. albitarsis* H.

Materials and Methods

Sampling and Data Access

Full specimen records (collection locality, coordinates, specimen identifiers, location of voucher specimens etc.) and all genetic data (edited chromatograms, consensus *COI* sequence files, and corresponding GenBank numbers) are publicly accessible under the project container code MBIAA (*Anopheles albitarsis* complex) on the BOLD website (http://www.boldsystems.org), as part of efforts of the Mosquito Barcoding Initiative (MBI). Detailed collection data are lodged in VectorMap (vectormap.si.edu) where distribution maps can be readily visualized. Where available, voucher specimens and / or DNA extracts of specimens used in this study are stored in the archive collections of the Walter Reed Biosystematics Unit, Smithsonian Institution–National Museum of Natural History, Museum Support Center (MSC), Suitland, Maryland, United States, or in the Frozen Tissue Collection at the Natural History Museum, London, United Kingdom.

Specimens utilized in the molecular study were all morphologically verified as belonging to the Albitarsis Group using the available keys (Linthicum 1988, Gonzalez and Carrejo 2009) and original species descriptions. Specimens sequenced include topotypic material of *An. albitarsis* s.s., *An. deaneorum*, *An. marajoara*, and *An. oryzalimnetes*, and type series material of *An. janconnae*. In this dataset, a total of 988 *COI* sequences generated from specimens collected from all over Latin America over a period of 20 yr by RCW, FRL, MTM, and JEC and collaborators were analyzed. Data used in this analysis 564 *COI* sequences first reported in the phylogenetic treatment of Ruiz-Lopez et al. (2012)—*An. albitarsis s.s.* [JQ615201–JQ615309], *deaneorum* [JQ615310–JQ615345], *An. janconnae* [JQ615346–JQ615441], *An. marajoara* [JQ615442–JQ615511], *An. oryzalimnetes* [JQ615512–JQ615562], *albitarsis* F [JQ614998–JQ615041], *An. albitarsis* G [JQ615042–JQ615146], *An. albitarsis* H [JQ615147–JQ615188], and *An. albitarsis* I [JQ615189–JQ615200]—and 404 specimens included in the spatial evolutionary and ecological vicariance analysis (SEEVA) of Foley et al. (2014)—*An. albitarsis* s.s. [KJ492701–KJ492726, KJ492728–KJ492738, KJ012833], *An. oryzalimnetes* [KJ012000–KJ012004, KJ012015–KJ012065, KJ012834–KJ012861, KJ492780–KJ492894], *An. marajoara* [KJ011926–KJ011999, KJ492772–KJ492775, KJ492777–KJ492779], *An. deaneorum* [KJ492739–KJ492771], *albitarsis* G [KJ012832, KJ492676, KJ492776], *An. albitarsis* H [KJ011904–KJ011925, KJ492677–KJ492696, KJ492698–KJ492700]. To these previously published datasets, we added a further 20 new samples, including 2 *An. oryzalimnetes* [MT231277, MT231279], 16 *An. albitarsis* G [MT231262-MT231273, MT231275, MT231276, MT231278, MT231281], and two specimens of the newly defined *An. albitarsis* J [MT231274, MT231280]), and revisited the taxonomic status of *An. albitarsis* H.

DNA Extraction and Sequencing

A fragment of the mtDNA *COI* gene corresponding to the barcode region (658-bp) was amplified using the LCO1490 and HCO2198 primers of Folmer et al. (1994). The PCR amplification

protocol utilized is detailed explicitly in Ruiz-Lopez et al. (2010). All sequencing reactions were carried out in both directions using ABI Big Dye Terminator Kit v.3.1 (PE Applied Biosystems, Warrington, United Kingdom), analyzed on an ABI Prism 3730—Avant Genetic Analyzer (Applied Biosystems, Foster City, CA), edited using Sequencher version 5.0.1 (Gene Codes Corporation, Ann Arbor, MI) and automatically aligned in CLUSTAL X (Thompson et al. 1997).

Data Analysis

To infer haplotype relationships within the data sets, a statistical parsimony network was performed using TCS 1.21 (Clement et al. 2000). Homoplasies were resolved using the algorithm estimation rules in Crandall and Templeton (1993).

Haplotype and nucleotide diversities were generated using DnaSP version 5.0 (Librado and Rozas 2009). Genetic differentiation was evaluated using the F_{ST} approach with significance determined by permutation test (10,000 replicates). F_{ST} values were generated using Arlequin 3.0 (Excoffier et al. 2005), based on the clusters determined by the phylogenetic network.

The Isolation By Distance Web Service version 3.23 (Jensen et al. 2005) was used to analyze the correlation between genetic distance estimated and geographic distances between collection sites of each species. Significance of the correlation coefficient was assessed applying the Mantel test (10,000 randomizations) (Mantel 1967).

Divergence times within the *COI* genealogy were estimated using the BEAST software package v1.8.0 (Drummond et al. 2012). The sequence data were summarized by making a set of haplotypes of

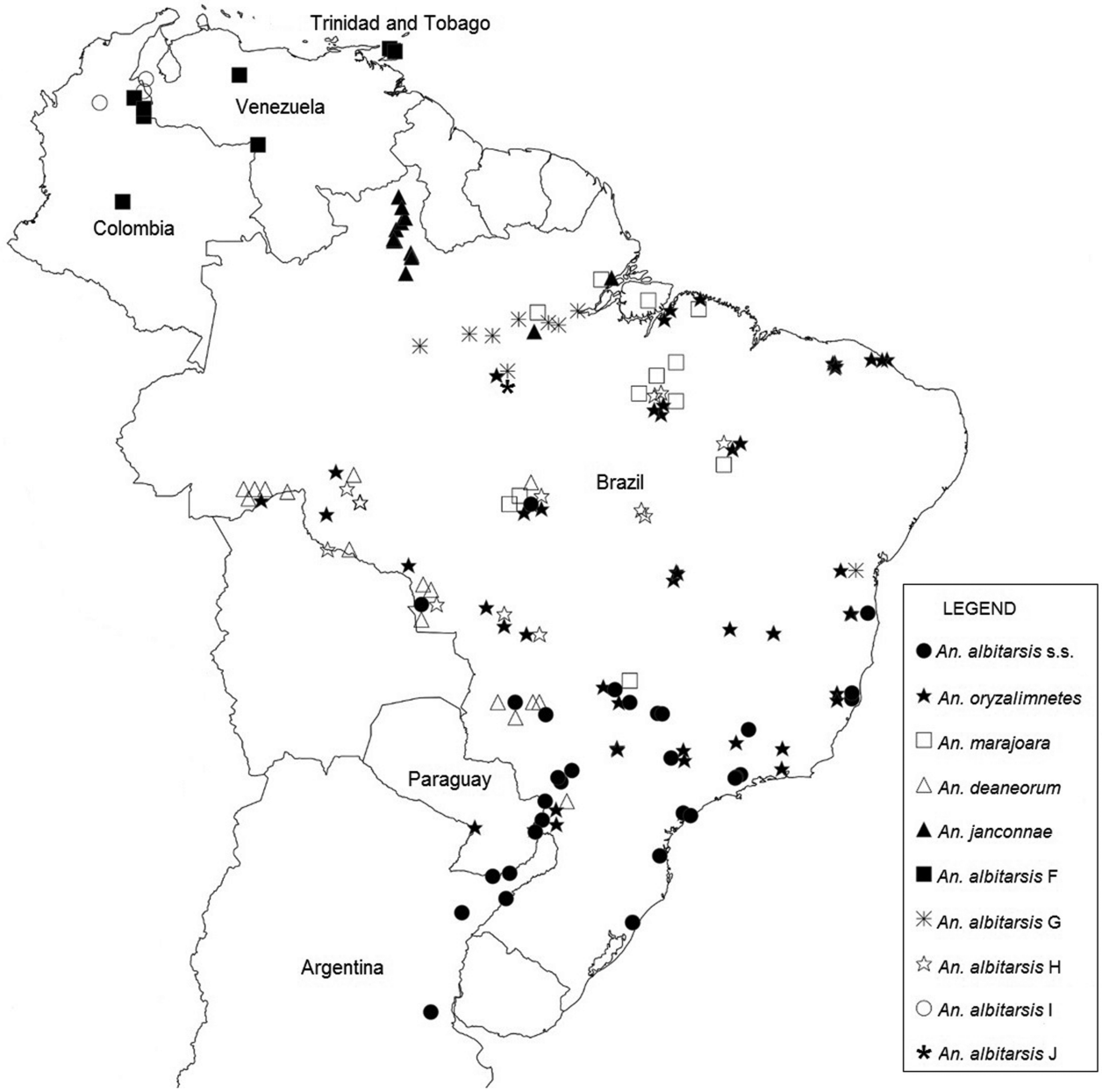

Fig. 1. Map showing the distribution of member species in the Albitarsis Group based on the 988 specimens used in this study.

each taxon. The standard arthropod mtDNA mutation rate of 2.3% per million years (Brower 1994) was set, giving a value of 0.0115 substitutions/site/lineage. The HKY model (Hasegawa et al. 1985) was applied with gamma distribution assuming constant size and relaxed molecular clock. The analysis was repeated five times, with 30 million generations of each run, sampling every 1,000 generations. The analysis results were combined in LogCombiner v1.8.0 with the initial 10% of the trees discarded as burn-in, and the results were averaged using Tracer v1.6 (Rambaut et al. 2013). The consensus tree was compiled with TreeAnnotator v1.8.0 and displayed in FigTree v1.3.1 (Rambaut 2009).

Results

Sample information is described in Fig. 1, Table 1, and Supp Table S1 (online only). Of the 415 haplotypes identified, 107 (26%) were shared within species and 308 (74%) were unique (Supp Table S1 [online only]; Table 2).

Genetic Variation

The statistical parsimony network illustrates the relationship among haplotypes of Albitarsis Group. The ten taxa differed by 8–18 mutational steps and could be connected parsimoniously (Fig. 2). This

Table 1. Origin by country and states of the 988 Albitarsis Group specimens used in this study

Country	*N*	States	Species
Argentina	38	Buenos Aires (21), Corrientes (15), Misiones (2)	*An. albitarsis* s.s.
Brazil	57	Paraná (17), Santa Catarina (4), São Paulo (36)	*An. albitarsis* s.s.
	38	Paraná (7), São Paulo (10), Rio Grande do Sul (4), Espírito Santo (4), Mato Grosso (3), Mato Grosso do Sul (1), Goiás (2), Minas Gerais (6), Bahia (1)	*An. albitarsis* s.s. *An. albitarsis* s.s.
	42	Bahia (4), Ceará (18), Mato Grosso (12), Pará (6), Rio de Janeiro (1), Rondônia (1), São Paulo (4)	*An. oryzalimnetes*
	201	Bahia (2), Ceará (33), Mato Grosso (3), Pará (67), Rondônia (20), São Paulo (12), Acre (2), Espírito Santo (11), Paraná (9), Maranhão (4), Piauí (7), Minas Gerais (18), Goiás (13)	*An. oryzalimnetes* *An. oryzalimnetes*
	69	Amapá (27), Mato Grosso (3), Pará (39)	*An. marajoara*
	86	Amapá (4), Pará (80), Minas Gerais (1), Maranhão (1)	*An. marajoara*
	36	Mato Grosso (2), Rondônia (32), Paraná (2)	*An. deaneorum*
	33	Mato Grosso (10), Acre (13), Mato Grosso do Sul (10)	*An. deaneorum*
	96	Roraima (93), Amapá (2), Pará (1)	*An. janconnae*
	105	Amazonas (57), Pará (48)	*An. albitarsis* G
	19	Pará (18), Bahia (1)	*An. albitarsis* G
	42	Mato Grosso (39), Rondônia (3)	*An. albitarsis* H
	45	Mato Grosso (6), Rondônia (5), Pará (25), Tocantins (8), Maranhão (1)	*An. albitarsis* H
	2	Pará (2)	*An. albitarsis* J
Colombia	10	Meta (2), Norte de Santander (6), Vichada (2)	*An. albitarsis* F
	11	Antioquia (9), Norte Santander (2)	*An. albitarsis* I
Paraguay	14	Alto Paraná (14)	*An. albitarsis* s.s.
	9	Alto Paraná (9)	*An. oryzalimnetes*
Trinidad	19	St. George East, St. Andrew/St. David (19)	*An. albitarsis* F
Venezuela	15	Cojedes (6), Zulia (9)	*An. albitarsis* F
	1	Zulia (1)	*An. albitarsis* I

N = total sample size. Numbers in parenthesis indicate samples size from each state.

Table 2. Summary of haplotypes and diversity measures of the 988 *COI* gene sequences for members of the Albitarsis Group collected in six countries across South America (Argentina, Brazil, Colombia, Paraguay, Trinidad and Tobago, and Venezuela)

Species	*N*	Haplotypes			*h* (SD)	π
		Unique (%)	Shared (%)	Total		
An. albitarsis I	12	5 (71)	2 (29)	7	0.864 (0.0780)	0.00855
An. albitarsis F	44	26 (81)	6 (19)	32	0.970 (0.0160)	0.01244
An. deaneorum	69	49 (86)	8 (14)	57	0.993 (0.0040)	0.01076
An. albitarsis G	124	20 (71)	8 (29)	28	0.802 (0.0260)	0.01040
An. janconnae	96	25 (64)	14 (36)	39	0.920 (0.0180)	0.00890
An. albitarsis H	87	34 (72)	13 (28)	47	0.966 (0.0090)	0.00806
An. marajoara	155	19 (68)	9 (32)	28	0.520 (0.0050)	0.00171
An. oryzalimnetes	252	52 (63)	30 (37)	82	0.970 (0.0030)	0.00687
An. albitarsis s.s.	147	76 (82)	17 (18)	93	0.982 (0.0050)	0.01097
An. albitarsis J	2	2 (100)	–	2	1.000 (0.0500)	0.00354
Total	988	308	107	415	0.982 (0.0020)	0.03129

N = number of individuals analyzed; Unique = unique haplotypes, the number in parentheses indicates the percentage of unique haplotypes from the total haplotypes; Shared = shared haplotypes, the number in parentheses indicates the percentage of shared haplotypes from the total numbers of haplotypes; Total = the total numbers of haplotypes detected; *h* = haplotype diversity; π = nucleotide diversity.

suggests substantial haplotype partitioning among species of the Albitarsis Group. Among the 10 taxa identified, a novel lineage was detected and designated as *An. albitarsis* J (Fig. 2).

Of the 155 specimens of *An. marajoara* confirmed from Pará, Amapá, Mato Grosso, Maranhão, and Minas Gerais states in Brazil, the most commonly observed haplotype was C1 (n = 107), found only in Pará and Amapá (Fig. 2; Supp Table 1 [online only]). The highest percentages (37, 36, and 32%, respectively) of shared haplotypes were found in *An. oryzalimnetes*, *An. janconnae*, and *An. marajoara* (Table 2). Conversely, a relatively high proportion of unique haplotypes (86, 82, and 81%) were noted in *An. deaneorum*, *An. albitarsis* s.s. and *An. albitarsis* F, respectively (Table 2). The most closely connected clusters were *An. albitarsis* H and *An. albitarsis* J, with the fewest mutational steps (eight steps) between them (Supp Table 1 [online only]).

The most diverse species—*An. deaneorum*—showed 57 haplotypes in 69 individuals (75%), and consequently the highest haplotype diversity (h = 0.993); *An. marajoara* presented the lowest haplotype diversity (h = 0.520) with only 28 haplotypes in 155 individuals (Table 2).

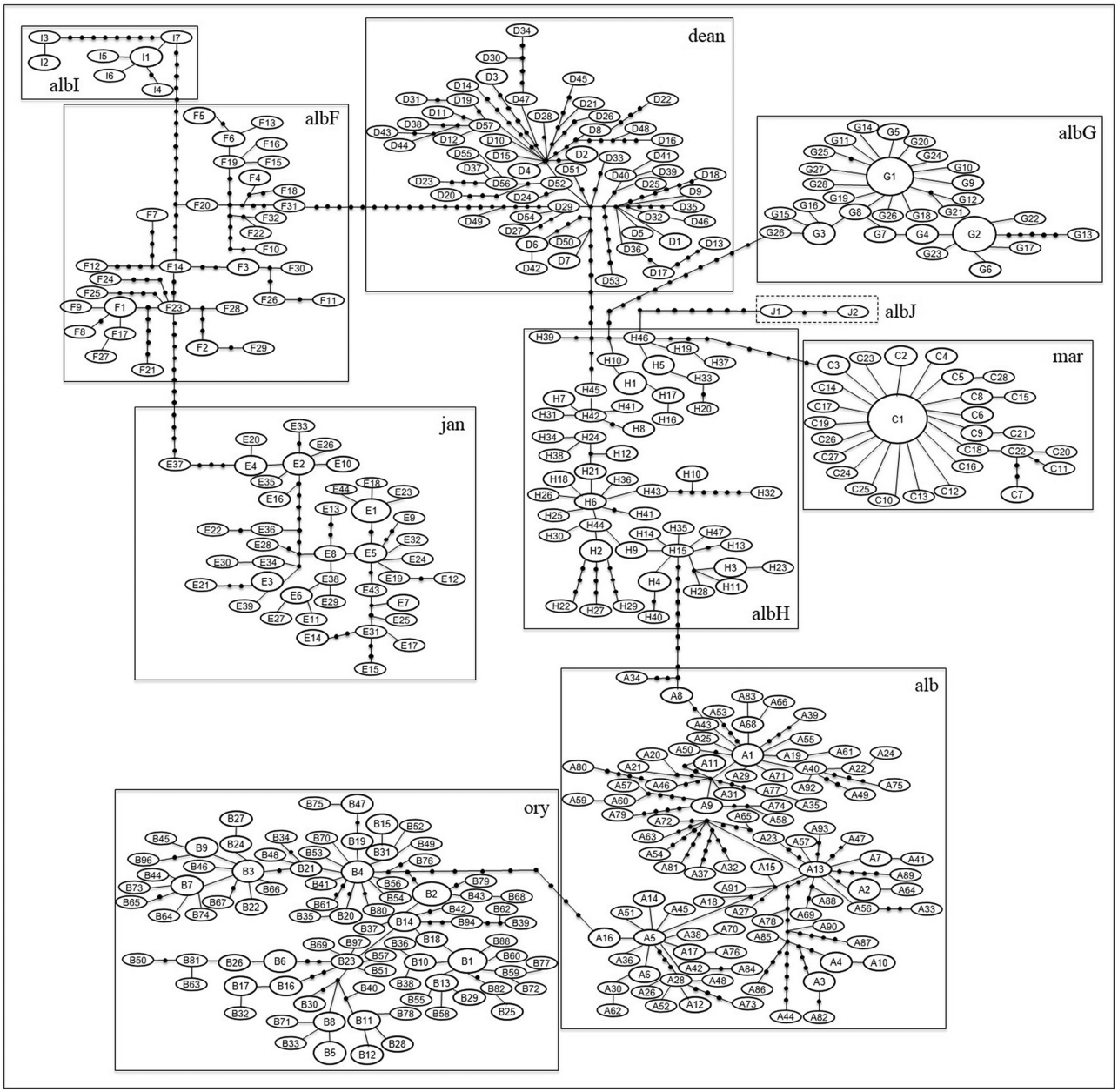

Fig. 2. Phylogenetic network of 415 *COI* haplotypes (658-bp) representing the 988 specimens of the Albitarsis Group collected from six countries across South America (Argentina, Brazil, Colombia, Paraguay, Trinidad and Tobago, and Venezuela). The area of each circle is proportional to the frequency of the haplotype. Black circle represents missing or unsampled haplotypes and each segment connecting haplotypes represents one mutational step. Each box depicts one taxa of the Albitarsis Group and the dashed box indicates a new lineage (albJ). albI = *An. albitarsis* I; albF = *An. albitarsis* F; dea = *An. deaneorum*; albG = *An. albitarsis* G; jan = *An. janconnae*; albH = *An. albitarsis* H; mar = *An. marajoara*; ory = *An. oryzalimnetes*; alb = *An. albitarsis* s.s.; albJ = *An. albitarsis* J.

Table 3. Estimate of genetic differentiation (*FST*) based on pairwise estimates of *COI* haplotype frequencies of ten taxa (988 specimens) in the Neotropical Albitarsis Group

	alb	ory	mar	dea	jan	albF	albG	albH	albI
An. albitarsis s.s. (alb)									
An. oryzalimnetes (ory)	0.0242*								
An. marajoara (mar)	0.2510*	0.2417*							
An. deaneorum (dea)	0.0127*	0.0186*	0.2698*						
An. janconnae (jan)	0.0489*	0.0543*	0.2949*	0.0440*					
An. albitarsis F (albF)	0.0238*	0.0297*	0.2951*	0.0181*	0.0558*				
An. albitarsis G (albG)	0.1075*	0.1106*	0.3450*	0.1061*	0.1405*	0.1200*			
An. albitarsis H (albH)	0.0261*	0.0319*	0.2754*	0.0205*	0.0572*	0.0318*	0.1181*		
An. albitarsis I (albI)	0.0696*	0.0755*	0.3723*	0.0644*	0.1042*	0.0775*	0.1738*	0.0786*	
An. albitarsis J (albJ)	0.0138	0.0226	0.4076	0.0051	0.0612	0.0222	0.1560	0.0260	0.1016

The significance was tested by permutation tests (10,000 replicates).
*Significant *P*-value (<0.001).

Table 4. Correlation between geographic and genetic distances among species of the Albitarsis Group by the Mantel test

Species	*P*-value	R^2
An. albitarsis s.s.	0.4240	5.994
An. oryzalimnetes	**0.0050**	0.0483
An. marajoara	0.7330	0.0267
An. deaneorum	0.7950	0.180
An. janconnae	0.1980	0.0294
An. albitarsis F	0.0550	0.0885
An. albitarsis G	**0.0230**	0.306
An. albitarsis H	0.1340	0.107
An. albitarsis I	0.2550	0.105

The numbers in bold are significant ($P < 0.05$).

Genetic Differentiation

The genetic differentiation, F_{ST}, was significant among all the taxa ($P < 0.001$), except between *An. albitarsis* J and the remaining species (Table 3). The lowest but still significant F_{ST} value was between *An. deaneorum* and *An. albitarsis* s.s. ($F_{ST} = 0.0127$) and the highest significant value was between *An. albitarsis* I and *An. marajoara* ($F_{ST} = 0.3723$) (Table 3). The F_{ST} between *An. albitarsis* J and *An. marajoara* was not significant ($F_{ST} = 0.4076$) (Table 3). A significant positive correlation was found with the Mantel test for *An. oryzalimnetes* ($P = 0.005$, $R^2 = 0.0483$) and *An. albitarsis* G ($P = 0.023$, $R^2 = 0.306$) (Table 4).

Estimation of Divergence Times

A phylogenetic topology is shown in Fig. 3, with the divergence times using the rate of 2.3% per million years under the relaxed molecular clock model. Using BEAST, the estimate of divergence time among species of the Albitarsis Group was 0.58–2.25 million of years ago, in the Pleistocene, with speciation events occurring between 0.58–1.61 Mya (Fig. 3).

Discussion

South American forests were substantially altered by climate and ecological oscillations that occurred in the Pleistocene (Colinvaux and Oliveira 2001). In this epoch, high levels of species divergence have been detected for many insect species (Lee and Li 2012, Li et al. 2012, Schultheis et al. 2012), including malaria vectors (Pedro and Sallum 2009, Loaiza et al. 2010, McKeon et al. 2010, Scarpassa and Conn 2011), suggesting climatic change as an important driver of speciation in the Neotropics. In the present study, the divergence time estimated among all taxa of the Albitarsis Group supported diversification during the Pleistocene.

The haplotype network identified 10 major clusters within the Albitarsis Group *COI* sequences (Fig. 2). Eight are formerly designated species: *An. albitarsis* s.s., *An. oryzalimnetes*, *An. marajoara*, *An. deaneorum*, *An. janconnae*, *An. albitarsis* F, *An. albitarsis* G, *An. albitarsis* I, one herein we confirm as a new species, *An. albitarsis* H, and here we add two samples of a hitherto undetermined lineage—*An. albitarsis* J. The clusters correspond partially to those supported by phylogenetic analyses in Wilkerson et al. (2005) and in Lehr et al. (2005); and totally supported in Ruiz-Lopez et al. (2012) with the additional lineage, *An. albitarsis* J (Fig. 2).

Ruiz-Lopez et al. (2012) first identified *An. albitarsis* H from Mato Grosso and Rondônia states in Brazil. Here we added several additional locations in Mato Grosso and Rondônia, and increased its known distribution to include Pará, Tocantins, and Maranhão states. Based on our haplotype network analysis, *An. albitarsis* H formed a distinct cluster from the remaining undisputed eight species within the Albitarsis Group, and with respect to our novel lineage (*An. albitarsis* J). The *COI* sequences representing *An. albitarsis* H separated from *An. albitarsis* J, *An. marajoara*, *An. deaneorum*, *An. albitarsis* s.s. and *An. albitarsis* G by 8, 9, 9, 11, and 13 mutational steps (Fig. 2), respectively, suggesting substantial haplotype partitioning.

Ruiz-Lopez et al. (2012) indicated highest genetic similarity of *An. albitarsis* H with *An. marajoara* and *An. deaneorum* using K2P distance method (0.020 and 0.024, respectively), but herein, with additional samples, we found that the estimate of genetic differentiation for *An. albitarsis* H from the remaining species of the Albitarsis Group was significant ($F_{ST} = 0.0205$–0.2754; $P < 0.001$; except for *An. albitarsis* J) (Table 3). The highest value was found between *An. albitarsis* H and *An. marajoara*, indicating that adding a further 86 specimens of *An. marajoara* to those examined in Ruiz-Lopez et al. (2012) supported the high value for genetic differentiation observed. The lowest differentiation but still significant value was between *An. albitarsis* H and *An. deaneorum* ($F_{ST} = 0.0205$; $P < 0.001$). In this case, the additional 33 specimens of *An. deaneorum* did not make any difference in the F_{ST} estimates and support the original result of Ruiz-Lopez et al. (2012). The relatively recent divergence between *An. albitaris* H and *An. deaneorum* explain the low differentiation between them. In addition, both species are sympatric in Mato Grosso and Rondônia states in Brazil, and their close relationship

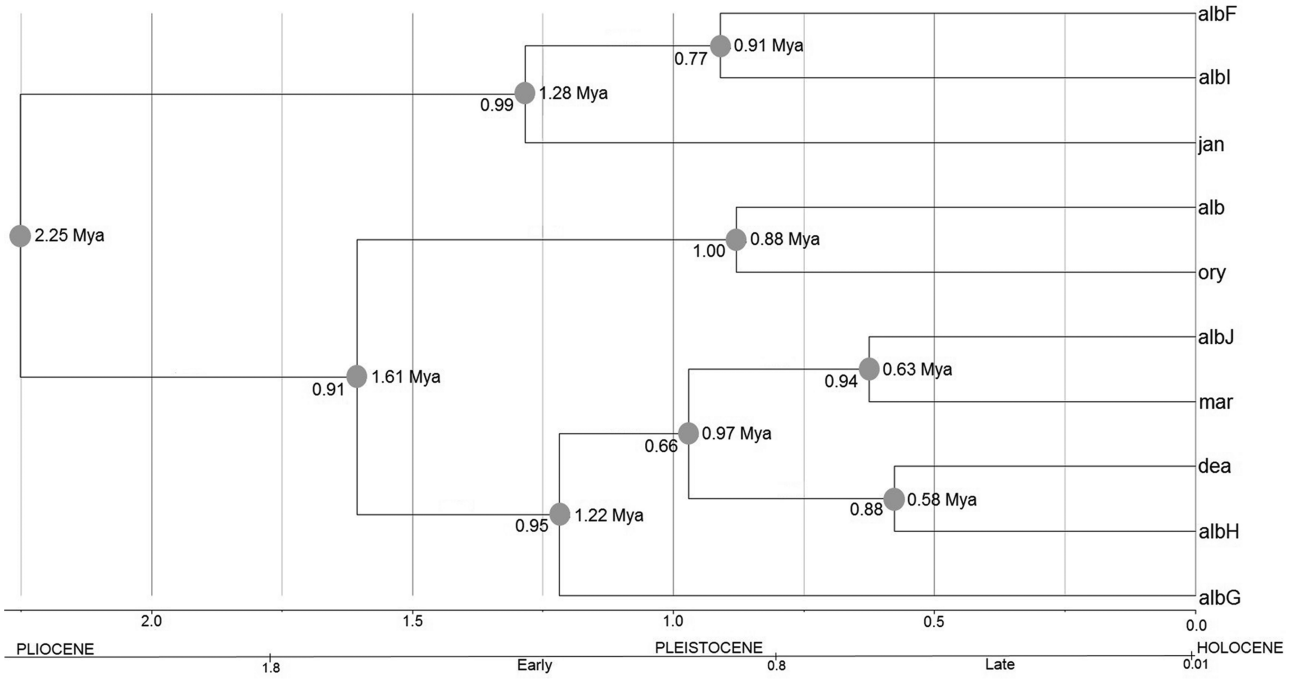

Fig. 3. Phylogenetic consensus tree obtained from *COI* gene sequences (658-bp) from 988 specimens collected in six countries across South America. Numbers to the left side of each node indicate posterior probabilities (PP). Numbers to right are estimates of mean divergence times (Mya) from the Beast calibrating with a rate of 0.0115 substitutions/site/Mya (albF = *An. albitarsis* F; albI = *An. albitarsis* I; jan = *janconnae*; alb = *An. albitarsis* s.s.; ory = *An. oryzalimnetes*; albJ = *An. albitarsis* J; mar = *marajoara*; dea = *An. deaneorum*; albH = *An. albitarsis* H; albG = *An. albitarsis* G).

(sister species) corresponds with the geographic and ecological proximity detected previously (Foley et al. 2014).

In summary, Ruiz-Lopez et al. (2012) identified *An. albitarsis* H as a new lineage and, based on genetic approaches (i.e., network analysis, significant genetic differentiation), we confirm *An. albitarsis* H as a new species within the Albitarsis Group. The novel lineage detected, named here as *An. albitarsis* J, was separated from *An. albitarsis* H by 8 mutational steps (Fig. 2). Morphologically, *An. albitarsis* J is similar to the primary malaria vector *An. marajoara*. It was collected in Itaituba, Pará state, Brazil, in sympatry with *An. albitarsis* G and *An. oryzalimnetes*.

The F_{ST} was not significant for any comparison of *An. albitarsis* J with the other nine Albitarsis Group taxa; however, the network analysis supported *An. albitarsis* J as a distinct cluster (Fig. 2). The small sample size was a limitation; further detailed morphological, genetic, ecological studies and a large sample size are necessary before concluding if this lineage represents a new species within the Albitarsis Group.

A significant correlation between genetic and geographical distances was observed in *An. albitarsis* G and *An. oryzalimnetes*. A possible explanation for this, is the large distribution of both species; *An. albitarsis* G is present in Brazilian Amazon, while *An. oryzalimnetes* is widely distributed in Brazil, mainly in the central region (Fig. 1). Analysis of the ecological niche for *An. oryzalimnetes* revealed that its climatic and environmental profiles are very distinct from the other species of the Albitarsis Group (Foley et al. 2014). In addition, ecological and environmental determinants (e.g., mountains, rivers, climatic changes, ecoregions, demographic history) may also influence the genetic divergence of the Albitarsis Group, e.g., Amazon River appears to be important in the speciation of this group (Foley et al. 2014).

Lehr et al. (2005) using the *COI* gene sequence data and Wilkerson et al. (2005) using the *COI*, ND4, ITS2, and D2 data of *An. albitarsis*, *An. albitarsis* B (= *An. oryzalimnetes*), *An. deaneorum* and *An. marajoara* found similar results; however, Lehr et al. (2005) also detected paraphily of *An. deaneorum* and *An. marajoara*, suggestive of potential introgression or perhaps a recent speciation event. Lehr et al. (2005) hypothesized that *An. marajoara* may comprise two or more phylogenetic species. Four "*An. marajoara*" outliers in their Bayesian topology were later confirmed as *An. albitarsis* F in Ruiz-Lopez et al. (2012).

The undescribed species—*An. albitarsis* F, *An. albitarsis* G, *An. albitarsis* H, *An. albitarsis* I, and *An. albitarsis* J—are morphologically very similar to *An. marajoara*. Based on the *white* gene, ITS2, and *COI* sequences, *An. albitarsis* F (Brochero et al. 2007, Ruiz-Lopez et al. 2012) and *An. albitarsis* I (Gutierrez et al. 2010, Ruiz-Lopez et al. 2012) from Colombia were identified as new taxa. McKeon et al. (2010) analyzed *COI* gene sequences of *An. marajoara* from 14 populations in the Brazilian Amazon and detected a new species, which Ruiz-Lopez et al. (2012) later also found, and called *An. albitarsis* G. Ruiz-Lopez et al. (2012) discussed the distributions and taxonomic positions of the above species in relation to the pertinent literature for *An. marajoara* and concluded that *An. albitarsis domesticus*—the current synonym of *An. marajoara*—(Rios et al. 1984) did not refer to any of the newly recognized species (*An. albitarsis* F, *An. albitarsis* G, *An. albitarsis* I) /lineage (*An. albitarsis* H).

The only species of the Albitarsis Group that can be recognized morphologically is *An. deaneorum* (only in the larval stage based on seta 3-C) (Rosa-Freitas 1989), and its distinction from some species (*An. albitarsis* s.s., *An. oryzalimnetes*, *An. marajoara*) in the Albitarsis Group was supported by early allozyme loci, mtDNA and

hybridization studies (Rosa-Freitas et al. 1990, Narang et al. 1993, Lima et al. 2004). The phylogenetic analysis of the mtDNA *COI* barcode gene further supported the separation of all species of the Albitarsis Group (Ruiz-Lopez et al. 2012). The current network analysis clearly showed distinct clusters of *An. marajoara* and *An. deaneorum*, corroborating the findings of Ruiz-Lopez et al. (2012).

The network analysis of the *COI* barcode region sequences further supports the finding of Ruiz-Lopez et al. (2012), confirming the status of the eight previously proposed species, establishes *An. albitarsis* H as a new species in the Albitarsis Group and identifies a novel lineage named *An. albitarsis* J. Based on our results, the Albitarsis Group includes: *An. albitarsis* s.s., *An. oryzalimnetes*, *An. marajoara*, *An. deaneorum*, *An. janconnae*, *An. albitarsis* F, *An. albitarsis* G, *An. albitarsis* H, *An. albitarsis* I and likely a novel lineage, *An. albitarsis* J. The *COI* barcode region is an important marker for species delimitation (Hebert et al. 2003, Cywinska et al. 2006, Kumar et al. 2007, Dai et al. 2012) in the Albitarsis Group and the parsimony network approach was shown to be a useful tool for species delineation in this highly diverse group, comprising essentially isomorphic taxa.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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