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Molecular Tracing with Mitochondrial ND5 of the Invasive Mosquito *Aedes* (Stegomyia) *albopictus* (Skuse) in Northern South America

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ABSTRACT

The widespread invasive *Aedes albopictus*, the Asian tiger mosquito, a vector of Dengue, Chikungunya and other arboviruses were discovered in Caracas (Venezuela) in 2009 and, separately in Colombia (Leticia 1998, Buenaventura 2001 and Cali 2007). The possible geographic origins were examined using mtDNA sequences NADH 5 (ND5). Sequences were aligned with those from GenBank. Venezuelan populations contained both unique (H14) and Asian-native haplotypes (H3), while the Colombian populations contain one unique (H15) and two common haplotypes (H1, H11) shared with the Brazilian, Hawaian, and Cameroon populations. Haplotype network analyses suggested: 1) Independent introduction into both countries; 2) two independent invasions into Colombia: from the Amazon River (H1) with evidence of founder effect or genetic bottleneck in Leticia, and, another via the Pacific port of Buenaventura from Hawaii (H11); 3) introduction to Venezuela directly from any Asian native range. Potential factors leading to limited genetic variation in mtND5 in the Columbian and Venezuelan populations were also discussed.

Keywords: *Aedes*, Dengue, Invasive Species, Mitochondrial DNA Gene, Nicotinamide Adenine Dinucleotide Dehydrogenase Subunit 5, Phylogeography.

1. Introduction

Invasive vector species are of great concern for human health and the natural environment, mainly due to global trade and travels [1,2]. *Aedes (Stegomyia) albopictus* (Skuse), commonly known as the Asian tiger mosquito, is currently the world's most invasive mosquito. It is of medical importance due to its aggressive daytime human-biting behavior and its ability to vector at least 21 viruses, including dengue, LaCrosse, yellow fever, Chikungunya, and West Nile [3,4,5]. Invasions into new areas of its potential range often begin through the transportation of eggs via the international trade in used tires. This species has desiccation-resistance eggs and its capacity to exploit anthropogenic container habitats such as discarded tires contributes to its rapid and successful colonization and widespread distribution. The distribution and invasion history of *Ae. albopictus* has been reviewed and updated frequently[5-8] and prospectively [4].

The widespread invasions during the past three decades are regarded as the "third wave" of human-aided dispersal of mosquito vectors of human disease, following the previous cosmopolitan or tropical spread of *Ae. aegypti* and the *Culex pipiens* Complex[2,9]. Mosquitoes spread by means of active adult flight and passive transportation of immature or adult stages via international trade and air travels. *Ae. aegypti* replaced *Ae. albopictus* in southeastern Asian cities in the first half of the 20th century [6]. Conversely, in the Americas, the introduction of *Ae. albopictus* was associated with a decline in the abundance of *Ae. aegypti* in the 1980´s [10]. The native range of *Ae. albopictus* is centered in the Oriental region and India [6] but extends west to the African island nations of Mauritius, the Seychelles, and Madagascar[2,5,6,11,12]. During the last century, *Ae. albopictus* spread to Hawaii, Guam, and other Pacific islands [2,5,6,11,12]. Independent introductions have been suggested for the U.S. and Brazil based on diapause responses [13,12] and also by mt DNA sequence analyses[15]. Following its establishment in the Americas, and spread in both the United States and Brazil, the Asian tiger mosquito invasion route has followed in Mexico, Central America [16] and in some Caribbean islands [5,17,18].

In South America, *Ae. albopictus* reached Colombia in Leticia in 1998, Buenaventura in 2001 and Cali in 2007[19-21], Paraguay [4] and northern Argentina in 1998 [22,23].

Recently, the presence of *Ae. albopictus* was reported for the first time in Venezuela in the general cemetery of Caracas [8], occupying the usual flower vases. Later, biting adults were collected in the Parque del Este (a zoo in the eastern part of Caracas), and also larvae and pupae were found in bamboo internodes and in fallen spathes palm and bromeliads [24].

Ae. albopictus has been implicated in sporadic dengue cases in Latin America[25]. The current intensification of intercontinental traffic might result in an increase in invasive species affecting human health as vectors of various pathogens [26].

With the objectives to identify the genetic origins and introduction paths for Venezuelan and Colombian *Ae. albopictus* populations, to compare with previous hypotheses of invasion pathways for Brazil and the US, and to assess the genetic variation of *Ae. albopictus* populations sampled in Venezuela and Colombia, we amplified and sequenced a 405-bp fragment of the mitochondrial gene Nicotinamide Adenine dinucleotide Dehydrogenase subunit 5 (ND5), and compared these sequences with the haplotype sequences obtained from GenBank.

Genes from the mitochondrial genome have been extensively used for insect phylogeography, phylogenetic, taxonomy and population genetics studies [27,28]. Furthermore, they are widely used to determine the sources of invasive *Aedes* populations based on phylogeography [15,29-32] or based on populations genetic [33-35].

2. Materials and Methods

A. Source of specimens. *Ae. albopictus* were collected from three locations in Caracas, Venezuela: Cementerio General del Sur (South General Cemetery, only 2 individuals), Parque del Este and Jardin Botanico de Caracas (botanical garden) (**Table 1)**, all at 950 m above sea level. The Colombian samples were collected in La Buitrera and at the campus of Universidad del Valle in Santiago de Cali, Valles del Cauca Department at 1,150 m, and in Buenaventura, the location of the second record for this species in Colombia located in a Port of the Pacific Ocean (**Table 1**). Adults were collected using human landing captures with mechanical aspirators and larvae and pupae larvae and pupae were collected from containers that contained water. The specimens were transferred to separate containers and transported to the insectary. Larvae were fed with fish food and reared to adults with fish food as previously described [36].

Table 1: Location and information of *Aedes albopictus* populations included in the present study.

Population	Collection site (n=number of sequences)	Geographic coordinates	Year of collection	Stage of collection
Venezuela	Jardin Botanico (n=30)	10°29'41,72'' N 66°53'17,67'' W	2010	Larvae and Adult
Venezuela	Cementerio General del Sur (n=2)	10°28'46.4'' N 66°55'09.2'' W	2009	Larvae
Venezuela	Parque del Este (n=30)	10°29'25,1'' N 66°50'18.5'' W	2010	Larvae and adult
Colombia	Buenaventura (n=36)	03°53' 07.7'' N 77° 01' 32.7'' W	2010	Adult
Colombia	Vereda La Buitrera (n=36)	03° 22' 19.9'' N 76° 34' 11.8'' W	2010	Adult
Colombia	Santiago de Cali (n=36)	03° 22' 24.22''N 76° 31' 49.51''W	2010	Adult

B. Genomic DNA Extraction and Polymerase Chain Reaction. Genomic DNA was extracted from two mosquito legs that were removed [37]. The remaining voucher specimens were pinned and stored in the collection of the Laboratorio de Biologia de Vectores, Instituto de Zoologia and Ecologia Tropical, Universidad Central de Venezuela.

Each pair of legs was ground in 25-30 µl of ice-cold TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with microfuge pellet pestle grinders (Kontes, Vineland, NJ), in a 1.5-ml Eppendorf tube. DNA was isolated using the method described by Arrivillaga *et al.*[38]. ND5 primers designed by Birungi and Munstermann [15] were used for polymerase chain reaction (PCR) amplifications. PCR was performed in 25-µl reaction volumes containing 3 µl of DNA template, PCR buffer containing 35 mM MgCl2, 100 mM Tris-HCl, 250 mM KCl, 1 mM each primer, 2 mM dNTP mix (Invitrogen), and 0.5 U of *Taq* polymerase (Invitrogen).

C. Sequencing and data analyzes PCR samples were purified and sequenced with forward and reverse ND5 primers by automated sequencing using an Applied Biosystems (Foster City, CA) ABI 377 automated sequencer or an ABI 3500 Genetic Analyzer and an ABI PRISM Dye terminator Cycle Sequencing Kit or BigDye Terminator v3.1 Cycle Sequencing Kit, following the manufacturer's instructions.

Contig (forward and reverse sequences) were assembled using Sequencher 4.2.2 (Gene Codes Corp, Ann Arbor, MI). Alignment of our sequences with those from GenBank [15,29,30] was performed using MacVector 7.2 (Accelrys, Madison, WI) software. The ND5 sequences generated from this study were aligned with those obtained from GenBank using the MacVector 7.2. These GenBank sequences (AY049968-AY049976, EU118294-EU118297, AJ971016-AJ971028 and JF309323) represent 14 different haplotypes that were reported previously [15, 29, 30]. Haplotypes were checked under a matrix exported to MacClade 4.08 [39]. We have not included analyses with ND5 sequences from GenBank with length much less than 409bp due to its yielded haplotype networks with lacking information and biases in the geographic origin interpretation.

To examine the level of nucleotide variation, the following statistics were computed using DnaSP[40]: Nucleotide diversity within populations was estimated according to Nei[41] equation 10.5. Genetic differentiation estimates were calculated using Hst

statistics (Hudson *et al.*[42], equation 2). Tajima´s D test for neutrality was calculated using the total number of segregating sites. To indicate cladistic relationships among our studied individuals/populations, we performed phylogenetic analyses using parsimony and maximum likelihood approaches using PAUPb10 [43]. The best substitution model and related parameters was selected by Modeltest[44]. The phylogenetic analyses were rooted by other *Aedes* species (*Ae. aegypti* from Venezuela) and also with the putative ancestral H3 *Ae. albopictus*, the Asian native haplotype. In addition, we generated a haplotype network using the statistical parsimony method based on Templeton *et al.*[45] and implemented in TCS[46] to visualize relationships. This approach identifies the number of haplotypes in the data and number of evolutionary steps taken from one haplotype to another. TCS has been shown to be useful in disentangling relationships at the intraspecific level[47]. We follow and keep the standard nomenclature of haplotypes after Usmani-Brown *et al.*[30].

3. Results

A. Mitochondrial Sequence Analysis. A total of 170 sequences were produced of which 62 were from the three locations in Caracas, Venezuela (30, 30 and 2 specimens, from Parque del Este, Jardin Botanico and Cementerio General del Sur, respectively), and 108 sequences were from the three locations in Colombia (36 sequences each in La Buitrera, Cali and Buenaventura). The C+G content was 23% for Venezuelan and Colombian sequences with evident A+T rich sequences. The sequences aligned had the following characteristics: 16 sites were polymorphic and six were parsimony-informative sites. Four substitutions were transversions, and the remaining were transitions. The 4 transversions were detected in H13 (**Table 3)**. Seven substitutions were synonymous (silent) and 9 were nonsynonymous.

Together with the GenBank ND5 *Ae. albopictus* sequences, a total of 16 variable sites were detected and these sites define 16 distinct haplotypes, two of which were not previously found (Table 2). These new haplotypes include H14 from Venezuelan and H15 from Colombian populations. The H3 native (from the geographical origin of *Ae. albopictus,* Asia) was the other and the most common haplotype found in Venezuela, while it was absent in Colombia. The local H15 was the most common haplotype in Colombia followed by H1 (shared with Brazil) and H11 (shared with Hawaii and Cameroon) haplotypes. H15 and H1 were found in the three Colombian locations, while H11 was found in Buenaventura only. The geographical locations and codes of all sequences and haplotypes are showed in **Table 2**.

Table 2: Geographic occurrence of total ND5 mt DNA haplotypes (405nt), with identification code in the publication reference. In **bold** the new haplotypes from this study.

Haplotype	Location (Country)	Code
H1	Anita Garibaldi, Praia de Fora, Jacarepagua, Manaus, Represa do Cigano, São Luís (Brasil); **La Buitrera, Buenaventura, Cali (Colombia)**	H1-Braz9968
H2	Anita Garibaldi and Praia de Fora, (Brasil)	H2-Braz9969
H3	Birungi & Munstermann : Jacksonville (USA), 3D Salvage (USA), AAA Salvage (USA), Oslo Mall (USA), Atlanta (USA), Malaysia Hanoi (Vietnam), Mousson *et al.* (2005) : Jacksonville (USA), Chiang Mai (Thailand), Seam Reap (Cambodia), Diego Suarez (Madagascar), MontSecret and Naintré (France), Nha Trang (Vietnam), Oahu (Hawaii) and La possession and la Providence (Réunion) Usmani-Brown *et al.* (2009): Rome (Italy). Zitko *et al.* 2011: East Adriatic. **Caracas (Venezuela)**	H3-Lous-9970
H4	Florida 3D Salvage and Jacksonvelle (USA)	H4-3DSalvageFla9971
H5	Florida 3D Salvage, USA	H5-3DSalvFla9972
H6	Oslo Mall, USA	H6-OMFla9973
H7	Florida AAA Salvage, USA	H7-AAASalvage9974
H8	Madagascar	H8-Madagascar9975
H9	Madagascar	H9-Madagascar9976
H10	Santchou, Mbalmaya and Douala, Cameroon (Usmani-Brown *et al.* 2009), Cameroon, Central Africa (Kamgang *et al.* 2011)	H10-Cameroon-8294
H11	Santchou, Mbalmaya and Douala, Cameroon Malama Ki and Nanawale, Hawaii, USA (Usmani-Brown *et al.* 2009), **Buenaventura, Colombia.** Cameroon, Central Africa (Kamgang *et al.* 2011)	H11-Cameroon8295
H12	Malama Ki and Nanawale, Hawaii, USA (Usmani-Brown *et al.* 2009)	H12-Hawaii8296
H13	Malama Ki and Nanawale, Hawaii, USA (Usmani-Brown *et al.* 2009)	H13-Hawaii8297
H14	**Caracas (Venezuela)**	H14-PE01-Venezuela
H15	**La Buitrera, Buenaventura, Cali (Colombia)**	H15-CO18
H16	Cameroon, Central Africa (Kamgang *et al.* 2011)	H16-Cameroon9323

B. Phylogenetic Relationships and Gene Network. The cladistics analysis with a heuristic search for only *albopictus* matrix yielded equally 36 parsimonious trees (L=20, CI= 0.85, RI=0.70, RC=0.595). The most parsimonious trees and consensus (strict and majority) tree showed a polytomy or unresolved relationships into the internal group of *Ae. albopictus* (both rooted with/without *Ae. aegypti*) sequences, revealing few changes and not resolved tree (polytomy) among all *Ae. albopictus* populations (trees do not show). The Maximum likelihood analysis also yields a polytomy under GTR+I+G model substitution. The haplotypes network generated by TCS illustrates the relationship between the 16 mtDNA haplotypes (**Fig. 1**). The statistical parsimony network distinguished two major groups originated from the native H3 haplotype and three non inter-connected haplotypes (H8, H9, H10, H16). The first group was characterized by populations from the unique haplotypes from the United States and Hawaii, unique H2 from Brazil and the shared haplotype H1 from Brazil and Colombia. The second group was represented by the private or local haplotypes from Venezuela and Colombia (H14 and H15) and the extraordinary haplotype H11 shared by Hawaii, Cameroon and Colombia by means of nt positions 184 and 268 (**Fig. 2**). The most frequent haplotype (H3) seemed to be widespread in Hawaii, Cameroon, Italy and Venezuela (**Table 2**). Nevertheless, recently 12 new ND5 haplotypes have been reported in Southeast Asia, with shorter 361 bp amplified [48] (all previous sequences have 409 or more bp) and the alignments that we done yielded many gaps (match between nt18-nt381) that produce unconnected haplotype networks or lacking geographic information networks when matrix without gaps was used (parsimony informative variable sites deleted). Additionaly, these sequences have not shared haplotypes with ours from South America and have no influence on the inferences of geographic origin. However, we have included all the variable sites found to date in order to show a comprehensive resume of the ND5 information (**Table 3)**.

Table 3: Haplotypes previously reported and new haplotypes (**Bold**) in this study.

	Variable sites																							
					+		1	1	1	2	2	2	** 2	2	2	3	3	3	3	3	3	3	3	+ 3
	1	6	6	8	**9**	9	3	8	9	0	1	3	6	7	9	1	2	7	7	7	8	9	9
Haplotypes	2	4	9	2	**2**	7	3	4	2	8	5	6	8	4	8	9	6	0	3	6	0	0	5
1	T	T	A	A	G	G	T	C	A	A	T	A	T	T	G	A	G	C	T	A	T	A	A	T
2	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3 (native)	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
5	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
7	C	.	.	G	.	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
9	.	.	.	.	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
11	.	.	.	.	.	.	.	T	.	.	.	.	**C**	.	.	.	.	.	.	.	.	.	.	.
12	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
13	.	.	.	.	.	.	.	T	.	.	.	.	G	.	.	.	.	.	.	G	A	G	T	G
14 (New)	.	.	.	.	**A***	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
15 (New)	.	.	.	.	**A***	.	.	T	.	.	.	.	**C**	.	.	.	.	.	.	.	.	.	.	.
16	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
17 ThLam5		.	T	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
18 ThLam1		.	.	G	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
19 Shan5		.	.	G	.	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
20 JNag3		.	.	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
21 Shan4		.	.	.	.	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
22 ThCh12		.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.		
23 Phu7		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
24 JTan15		.	.	.	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.		
25 ThCh7		.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.		
26 Phu6		.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.		
27 BGe1																		T	.	.	.			
29 BGe5																		.	C	.	.			

(*) nt position shared between Venezuelan and Colombian private haplotypes. (**) Hypervariable nt position. (+) new variable sites in ND5 (current study). Haplotypes H1-H16 showed in haplotypes network. H17-H29 haplotypes reported with shorter length sequences [48] and labels corresponding with Genbank (accession: JQ346948-959).

C. Population Differentiation. The newly sequenced geographical populations produced a global haplotype diversity H_T = 0.749. Haplotype diversity for Venezuela was 0.123 and for Colombia was 0.617. Pairwise comparisons also revealed no significant structuring between the Venezuelan and Colombian populations (Hst: 0.593 P: 0.4412). The nucleotide diversity in the total sample was 0.00358. The nucleotide diversity within population for Venezuela was 0.00030 and 0.00346 for Colombia, indicating overall low genetic variation.

Tajima´s D [49] value for Venezuela was -0.53969 (not significant, $P>0.10$) and 2.57600 (significant, $P<0.050$) for Colombia.

4. Discussion

A. Background and New Findings: Previous analyses with

albopictus/ND5 [15,29,30-32,34] reported low levels of ND5 genetic variation, which are consistent with our findings here. Including our new data, eight authors working with ND5 reach only 16 haplotypes in different countries from four continents sampled for *Ae. albopictus*: nine from Birungi and Munstermann [15]; then Usmani-Brown *et al.*[30] increased the haplotype diversity to thirteen and later Kamgang *et al.*[32] showed the Haplotype 16. Zitko *et al.*[50] found the haplotype 3 (H3) for East Adriatic, Raharimalala *et al.*[51] did not found any new haplotype from Madagascar and the new haplotypes (361 bp) from Asia[48], none of them are shared with any South American ones.

Here, we report 2 new haplotypes (H14, H15), one from Venezuela and one from Colombia representing changes on two respective variable sites, of which one of these sites is reported new (nt92). However the new haplotypes increases to reach 23, the total variable sites for ND5.

The Hawaiian populations revealed higher overall haplotype changes compared to the others and contain up to six changes in H13. US is the country with a higher local haplotype diversity (4 unique haplotypes), while Colombia had 3 haplotypes, one unique (H15) and two shared (H1, H11), while the Asian H3 is absent in Colombia. In contrast, one private haplotype occurs in Venezuela at a low frequency and the H3 Asian-native haplotype is commonly sampled.

Our data supports the previous reports from GenBank sequences, but also add a nt position (nt395) missed by Usmani-Brown *et al.*[30], that increased to 6 the variable sites for the H13 Hawaiian haplotype. This further supports the higher level of genetic diversity in the island locations [29,51].

Colombia and Venezuela are located at the northern continental border of South America and both countries are points of entrance for international trade from North America, Asia, Africa and Europe on both sides of the Panama Canal (Pacific and Atlantic Oceans).

B. Geographical origins and introduction pathway of the Colombian populations: *Aedes albopictus* was reported in 1998 from Colombia, and we suggest here that at least two introduction tracks for this invasive species occurred: the haplotypes shared with Hawaii-Cameroon (H11) and Brazil (H1), the network relationships of these haplotypes, but also the H15 private haplotype; their frequencies and the geographical locations support that hypothesis.

The strongest track is represented by the H1 haplotype, which occurs in the three Colombian locations sampled. Evidence suggests that H1 came from the Brazilian Amazon. First, Maia *et al.* [31] report H1 as the most frequent haplotype in Manaus, located in one of the most important fluvial ports on the Amazonas River. The city of Manaus is located in the fluvial way toward Leticia in the Colombian Amazonia where *Ae. albopictus* was first reported in Colombia[19]. Then, the important trade exchange between these cities is the most probable route of invasion of *Ae. albopictus* from Brazil to Colombia. Moreover, the higher frequency of H1 but also the absence of H3 in the Colombian samples suggest a founder effect from Brazilian H1 populations.

The H11 haplotype (lower frequency: 6%) is also shared by Hawaii and Cameroon populations. A single change from the Asian-native H3 in the nt268 suggests two hypotheses: 1) introduction from Hawaii or directly through the trade exchange from Africa (Cameroon), or 2) the variable nt268 position (there are other two changes in this nt position in the 16 haplotypes) can be an independent and highly probable change. The most robust hypothesis should be a direct introduction of H11 to Colombia from Hawaii by the Pacific port of Buenaventura, the location of the second report in Colombia where this haplotype was found [20].

The H15 private Colombian haplotype represents an interesting issue for discussion. This haplotype has two equally parsimonious origins (**Fig. 1**): one change from H11 (Hawaii-Cameroon share) and also one change from H14 (Venezuela). Both origin pathways can yield the haplotype with the highest frequency and widely distributed from Colombia (72%), whereas two changes are necessary to produce the H15 from the native H3, which has not been recorded in Colombia.

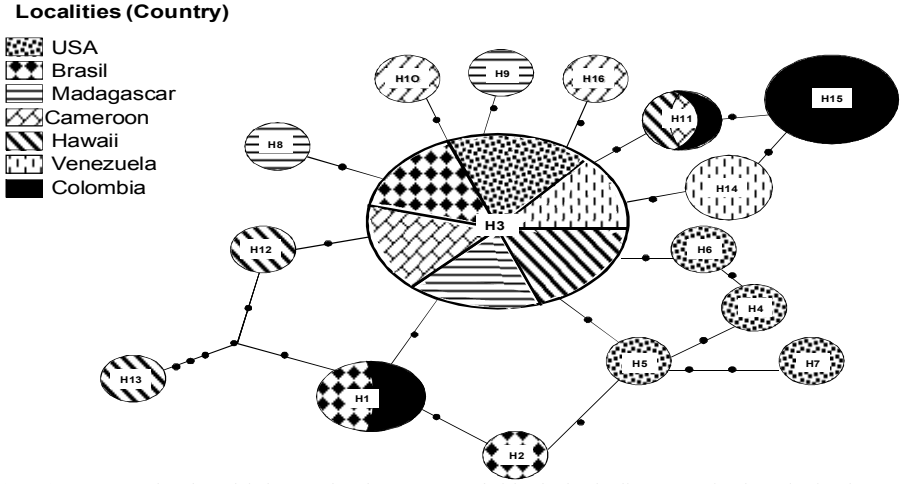

Fig 1: Haplotypes Network (TCS) among mitochondrial ND5 haplotypes. Each black dot indicates a single substitution. The numbers inside the circle indicate the Haplotype number (Tables 2 and 3). The size of the circle is proportional to the haplotype frequency only for the Venezuelan and Colombian populations.

Until H3 or H14 is detected in Colombia, the strongest hypothesis for the H15 origin is that it came from one change of the H11 introduced from Hawaii (supported by the highly probable nt268 change, which is shared with Cameroon), despite the frequent and strong trade exchange with Venezuela. However, a potential trade exchange with Cameroon should be demonstrated in order to establish a H11 link between Cameroon and Colombia.

The Tajima´s D negative values for Venezuela and Colombia are consistent with the neutral mutation hypothesis, the positive D value, which is significant for the Colombian population, suggests

a balancing selection as a consequence of multiple introductions [49]. Haplotype H1 from Colombia (introduced from Brazil and the most frequent haplotype in Manaus) affects the Tajima's D value. Excluding this H1, the Colombian populations yield a negative Tajima's D value, which is consistent with a purifying selection operating (or bottlenecks or founder effects). However, its inclusion made its value positive, suggesting that a balancing selection such as over-dominant selection is operating.

C. Geographical origins and introduction pathway of the Venezuelan populations.

The Venezuelan populations are highly represented by the H3 Asian-native haplotype (93.4%) and the local H14, though with a lower frequency (6.6%), both in the same location although Caracas is a large city.

The high frequency in the occurrence of H3 suggests the introduction through two equally probable ways: 1) A founder H3 population from Brazil or the US, or 2) directly from a native founder H3 Asian population based on the increasing trade exchange with this continent over the last 6-7 years. The negative Tajima's D value for Venezuelan populations supports a recent bottleneck or a recent expansion in the population. Also due the absence of shared haplotypes with Colombia, the hypothesis of introduction from this country is not supported and to date, there is no evidence of occurrence of *Ae. albopictus* in any location in Venezuela, close to Colombia.

D. Limited Variation in ND5 gene. Our results support the lower than expected genetic variation observed in all previous papers with populations using ND5 mtDNA; this involves four continents including the Hawaiian populations, which were introduced from Asia more than 100 years ago. Birungi & Munstermann and Mousson *et al.*[15,29] attributed this low genetic variation to: 1) small founder populations, 2) extensive insect control measures (to the reduction of effective population size and thus accelerate the drift effect), and/or 3) rapid range expansion of new mtDNA haplotypes via modern transportation means. However, Armbruster *et al.*[52] suggested the possible host mtDNA homogenization of *Ae. albopictus* as a consequence of a double infection by *Wolbachia*, based on studies with 18 widely distributed populations of *Ae. albopictus*. However, the phylogenetic structure of *Ae. albopictus* populations based on COI sequences[29,32] could suggest a different rate of evolution among these mtDNA genes. Also, our studies on *Culex* and *Anopheles* species using ND5[53] has found a wide variation of haplotype diversity among species and genera indicating that the genetic variation is strongly influenced by an effective selection against mildly deleterious mutations, which is consistent with a species-specific replication-dependent model in mt DNA[54].

Just two shared *Ae. albopictus* haplotypes had been found previously, the H7 (Perrotta) and H11 [30], and now, we have added another country to the H11 geographical distribution, which renders the analysis more complicated. We also found a new shared haplotype (H1) that represents a link to explain the Brazil-Colombia invasion of *Ae. albopictus*. The lack of extensive geographical field samples may be hiding the true routes of this invasive species. Also, the lack of confidence in the introduction date and the extremely low ND5 genetic variation made it difficult to estimate how long ago *Ae. albopictus* was introduced into the countries sampled.

Increased geographical sampling, including additional countries in the Americas (e.g. Argentina, Trinidad, Cuba, Dominican Republic, Mexico), and more Asian, European and African populations, as well as testing of other variable (nuclear and mitochondrial) molecular markers, will help to understand the ecological and anthropogenic invasion tracks of this important species and will improve the (genetics, phylogenetics, biological and ecological) methods to study other invasive species that affect human and animal health but also the biodiversity of local communities.

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6. Reference:

1. Moore CG. *Aedes albopictus* in the United States: current status and prospects for further spread. J Am Mosq Control Assoc 1999; 15: 221-227.
2. Lounibos LP. Invasions by insect vectors of human disease. Annu Rev Entomol 2002; 47:233-266.
3. Mitchell CJ. Geographic spread of *Aedes albopictus* and potential for involvement in arbovirus cycles in the Mediterranean Basin. J Vect Ecol 1995; 20:44–58.
4. Benedict MQ, Levine RS, Hawley WA, Lounibos LP. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector Borne Zoonotic Dis 2007; 7:76-85.
5. Gratz NG. Critical review of the vector status of *Aedes albopictus*. Med Vet Entomol 2004; 18:215-227.
6. Hawley WA. The biology of *Aedes albopictus*. J Am Mosq Control Assoc 1988; 4:1-39.
7. Enserink M. A mosquito goes global. Science 2008; 320:864-866.
8. Navarro JC, Zorrilla A, Moncada N. Primer registro de *Aedes albopictus* (Skuse) en Venezuela. Importancia como vector de Dengue y acciones a desarrollar. Bol Malariol Salud Amb 2009; 49: 161-166.
9. Calder L, Laird M. Mosquito travelers, arbovirus vectors and the used tire trade. Travel Med Internatl 1994; 12:3–12.
10. O'Meara GF, Evans LF, Gettman AD, Cuda JP. Spread of *Aedes albopictus* and decline of *Ae. aegypti* (Diptera: Culicidae) in Florida. J Med Entomol 1995; 32:554–562.
11. Mattingly PF. The sub-genus *Stegomyia* (Diptera: Culicidae) in the Ethiopian Region. II. Distribution of species confined to the East and South African sub-region. Bull Br Mus (Nat. Hist.) Entomol1958; 3: 1–65.
12. Craig GB. The diaspora of the Asian tiger mosquito. Biological pollution: the control and impact of invasive, exotic species, (B.N. McKnight Ed.). Indiana Acad Sci Indianapolis IN 1993; 261.
13. Hawley WA, Reiter P, Copeland RS, Pumpuni CB, Craig Jr GB. *Aedes albopictus* in North America probable introduction in tires from northern Asia. Science (Wash., D.C.) 1987; 236:1114-1116.
14. Forattini OP. Identificaçao de *Aedes* (*Stegomyia*) *albopictus* (Skuse) no Brasil. Rev Saúde Publ 1986; 20:244–245.
15. Birungi J, Munstermann LE. Genetic structure of *Aedes albopictus* (Diptera: Culicidae) populations based on mitochondrial ND5 sequences: evidence for an independent invasion into Brazil and the United States. Entomol Soc Am 2002; 95:126-132.
16. Ogata K, Lopez-Samayoa A. Discovery of *Aedes albopictus* in

Guatemala. J Am Mosq Control Assoc 1996; 12:503–506.
17. Chadee DD, Fat FH, Persad RC. First record of *Aedes albopictus* in Trinidad, West Indies. J Am Mosq Control Assoc 2003; 19:438-439.
18. Marquetti MC, Valdés V, Aguilera L. Tipificación de hábitats de *Aedes albopictus* en Cuba y su asociación con otras especies de culícidos, 1995-1998. Rev Cubana Med Trop 2000; 52:170-174.
19. Vélez ID, Quiñones ML, Suárez M, Olano V, Murcia LM, Correa E et al. Presencia de *Aedes albopictus* en Leticia Amazonas, Colombia. Biomedica 1998; 18:192–198.
20. Suárez M. *Aedes albopictus* (Skuse) (Diptera, Culicidae) en Buenaventura, Colombia. Inf. Quin Epidemiol. Nac 2001; 6:221–224.
21. Cuéllar-Jiménez ME, Velásquez-Escobar L, González-Obando R, Morales-Reichmann CA. Detección de *Aedes albopictus* (Skuse) (Diptera: Culicidae) en la ciudad de Cali, Valle del Cauca, Colombia. Biomédica 2003; 27:273-279.
22. Rossi GC, Pascual NT, Krsticevic FJ. First record of *Aedes albopictus* (Skuse) from Argentina. J Am Mosq Control Assoc 1999; 15:422.
23. Vezzani D, Carbajo AE. *Aedes aegypti, Aedes albopictus*, and dengue in Argentina: current knowledge and future directions. Mem Inst Oswaldo Cruz 2008; 103:66-74.
24. Zorrilla A, Quintero L, Ventura FD, Muñoz M, Moncada N, Navarro JC. Aspectos ecológicos de *Aedes albopictus* (Skuse, 1894) en Caracas, Venezuela. Bol Malariol San Amb 2011; 51:229-235.
25. Ibañez-Bernal S, Briceño B, Mutebi JP, Argot E, Rodriguez G. First record in America of *Aedes albopictus* naturally infected with dengue virus during the 1995 outbreak at Reynosa, Mexico. Med Vet Entomol 1997; 11:305–309.
26. Roderick GK. Geographic structure of insect populations: gene flow, phylogeography, and their uses. Ann Rev Ent 1996; 41:325–352.
27. Avise JC, Arnold RM, Ball RM, Bermingham E, Lamb T, Neigel JE, et al. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu Rev Ecol Syst 1987; 18:489-522.
28. Avise JC. Molecular markers, natural history and evolution. Chapman and Hall, New York, 1994.
29. Mousson L, Dauga C, Garerigues T, Schaffner F, Vazeille M, Failloux A. Phylogeography of *Aedes* (*Stegomyia*) *aegypti* (L.) and *Aedes* (*Stegomyia*) *albopictus* (Skuse) (Diptera: Culicidae) based on mitochondrial DNA variations. Genet Res 2005; 85:1-11.
30. Usmani-Brown S, Cohnstedt L, Munstermann LE. Population Genetics of *Aedes albopictus* (Diptera: Culicidae) Invading Populations, Using Mitochondrial nicotinamide Adenine Dinucleotide Dehydrogenase Subunit 5 Sequences. Ann Entomol Soc Am 2009; 102:144-150.
31. Maia RT, Scarpassa VM, Maciel–Litaiff LH, Tadei WP. Reduced levels of genetic variation in *Aedes albopictus* (Diptera: Culicidae) from Manaus, Amazonas State, Brazil, based on the analysis of the mitochondrial DNA *ND5* gen. Gen Mol Res 2009; 8:998-1007.
32. Kamgang B, Brengues C, Fontenille D, Njiokou F, Simard F, Paupy C. Genetic Structure of the Tiger Mosquito, *Aedes albopictus*, in Cameroon (Central Africa). PLoS ONE 2011; 6(5):e20257. doi:10.1371/journal.pone.0020257.
33. Urbanelli S, Bellini R, Carrieri M, Sallicandro P, Celli G. Population structure of *Aedes albopictus* (Skuse): the mosquito which is colonizing Mediterranean countries. Heredity 2000; 84:331–337.
34. Kambhampati S, Rai KS. Mitochondrial DNA variation within and among populations of the mosquito *Aedes albopictus.* Genome 1991; 34:288-292.
35. Kambhampati S, Black IV WC, Rai KS. Geographic origin of United States and Brazilian populations of *Aedes albopictus* inferred from allozyme analysis. Heredity 1991; 67:85-94.
36. Gerberg EJ. Manual for Mosquito rearing and experimental techniques. Published by the American Mosquito Control Association, inc. AMCA Bulletin No. 5, 1970.
37. Navarro JC, Hernandez C, Rangel-Diaz G, Guerrero E, Rangel Y, Arrivillaga J. Invalidación de autapomorfías putativas mediante análisis de filogenia molecular en *Anopheles* subgénero *Nyssorhynchus*. Bol Malariol Salud Amb 2009; 49:35-53.
38. Arrivillaga JC, Norris DE, Feliciangeli MD, Lanzaro GC. Phylogeography of the Neotropical sand fly *Lutzomyia longipalpis* inferred from mitochondrial DNA sequences. Infection, Genetics and Evolution 2002; 2:83-95.
39. Maddison DR, Maddison WP. MacClade version 4: Analysis of phylogeny and character evolution. Sinauer Associates, Sunderland, Massachusetts, 2000.
40. Rozas J, Rozas R. DnaSP version 3 an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 1999; 15:174-175.
41. Nei M. Molecular evolutionary genetics. Columbia University Press, New York, 1987.
42. Hudson RR, Boos DD, Kaplan NL. A statistical test for detecting geographic subdivision. Mol Biol Evol 1992; 9:138-151.
43. Swofford DL. PAUP a.0b10. Phylogenetic analysis using parsimony. Sinauer Associates, Inc. Sunderland, 2002, MA.
44. Posada D, Crandall KA. Modeltest: testing the model of DNA substitution. Bioinformatics 1998; 14:817-818.
45. Templeton AR, Crandall KA, Sing CF. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 1992; 132:619–633.
46. Clement M, Posada D, Crandall K. TCS: a computer program to estimate gene genealogies. Mol Ecol 2000; 9:1657-1660.
47. Woolley SM, Posada D, Crandall KA. A Comparison of Phylogenetic Network Methods Using Computer Simulation. PLoS ONE 2008; 3(4): e1913. doi:10.1371/journal.pone.0001913.
48. Porretta D, Mastrantonio V, Bellini R, Somboon P, Urbanelli S. Glacial History of a Modern Invader: Phylogeography and Species Distribution Modelling of the Asian Tiger Mosquito Aedes albopictus. PLoS ONE 2012; 7(9): e44515. doi:10.1371/journal.pone.0044515.
49. Tajima F. The effect of change in population size on DNA polymorphism. Genetics 1989; 123:597-601.
50. Žitko T, Kova A, Desdevises Y, Puizina J. Genetic variation in East-Adriatic populations of the Asian tiger mosquito, *Aedes albopictus* (Diptera: Culicidae), inferred from NADH5 and COI sequence variability. Eur J Entomol 2011; 108:501-508.
51. Raharimalala FN, Ravaomanarivo LH, Ravelonandro P, Rafarasoa LS, Zouache K, Tran-Van V *et al.* Biogeography of the two major arbovirus mosquito vectors, *Aedes aegypti* and *Aedes albopictus* (Diptera, Culicidae), in Madagascar. Parasites & Vectors 2012; 5:56.
52. Gillespie RG, Roderick GK. Arthropods on islands: colonization, speciation, and conservation. Annu Rev Entomol 2002; 47:595-632.
53. Armbruster P, Damsky WE, Giordano R, Birungi J, Munstermann LE, Conn JE. Infection of New- and Old-World *Aedes albopictus* (Diptera: Culicidae) by the intracellular parasite Wolbachia: implications for host mitochondrial DNA evolution. J Med Entomol 2003; 40:356-60.
54. Quintero L, Navarro JC. Filogenia molecular intraespecífica y variabilidad genética de *Culex quinquefasciatus* Say. Bol Malariol San Amb 2012; 52:45-65.
55. Weinreich DM. The rates of molecular evolution in rodent and primate mitochondrial DNA. J Mol Evol 2001; 52:40–50.