DNA barcode sequences used to identify *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) in La Tebaida (Quindío, Colombia)

"Código de barras" identifican *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) en La Tebaida (Quindío, Colombia)

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Abstract: *Aedes (Stegomyia) albopictus* has been identified for the first time in the municipality of La Tebaida (Quindio department of Colombia), an area with active transmission of dengue fever by *Ae. (Stegomyia) aegypti*. Specimens of *Ae. albopictus* were detected in a tire used as an ovitrap in a remnant of bamboo plantation in a rural area of the municipality of La Tebaida; *Ae. aegypti* presented co-occurrence and both species were molecularly typed using cytochrome oxidase I (DNA barcode region). The first DNA barcode sequences were recorded for 13 Colombian specimens of *Ae. albopictus*, identifying an asiatic origin (Singapore) and demonstrating the utility of this method for molecular identification. These sequences can be used to identify genetic flow with other populations in Colombia, in ecological studies, and in studies of vector incrimination in outbreaks of emerging and re-emerging arbovirus in Colombia.

Key words: Molecular identification. Cytochrome Oxidase I. Arboviruses. Medical entomology.

Resumen: Se identifica *Aedes* (*Stegomyia*) *albopictus* por primera vez en el municipio de La Tebaida (Quindío, Colombia) en un área con transmisión activa de dengue por *Ae.* (*Stegomyia*) *aegypti*. Los especímenes de *Ae*. *albopictus* fueron detectados en una llanta usada como ovitrampa en un remanente de plantas de guadua en área rural de La Tebaida; *Ae. aegypti* presentó co-ocurrencia y ambas especies fueron molecularmente tipificadas usando citocromo oxidasa I (región código de barras). Se registran las primeras secuencias código de barras para 13 especímenes de *Ae. albopictus*, identificando un origen asiático (Singapur) y evidenciando la utilidad de este método en la identificación molecular. En Colombia, estas secuencias pueden ser usadas para estudios en flujo de genes en otras poblaciones de mosquitos, ecología e incriminación vectorial en brotes epidémicos de arbovirus emergentes y re-emergentes.

Palabras clave: Identificación molecular. Citocromo Oxidasa I. Arbovirus. Entomología médica.

Introduction

Aedes albopictus (Skuse, 1894) is a prominent native mosquito originating from Southeast Asia with anthropophilic eating habits that is considered to be a vector-bridge between the enzootic cycle of West Nile virus and susceptible humans (Turell 2001; Sardelis 2002). Additionally, it is a vector of 22 arboviruses: Flavivirus (dengue, West Nile virus, yellow fever), Alphavirus (chikungunya, Eastern Equine Encephalitis virus), Orthobunyavirus (Tensaw virus, Potosi virus, Cachey Valley virus, LaCrosse virus), and nematodes [*Dirofilaria immitis* (Leidy, 1856), *Dirofilaria repens* Railliet & Henry, 1911] (Cancrini *et al*. 2003; Gratz 2004; Tilston *et al*. 2009). The range of geographic expansion of this species has increased to Europe, Africa, and America (Kraemer 2015) through the sale of used tires (Reiter 1998) and bamboo (Demeulemeester *et al*. 2014) and the carriage of adults in commercial aircraft (Gratz 2000). In Colombia, it was first recorded in Leticia-Amazonas in a collection using human bait (Vélez *et al*. 1998) and was subsequently identified in Buenaventura (Suárez 2001), Cali (Cuellar *et al*. 2007), Barrancabermeja (Gutiérrez *et al*. 2010), and Medellin (Rúa *et al*. 2011). The presence of this species in geographically distinct locations is probably the result of land transport, human passive dispersal, and the failure of entomological surveillance programs associated with dengue in Colombia (Rúa-Uribe *et al*. 2012). The proven vector competence of *Ae. albopictus* (Gratz, 2004); the circulation of Flavivirus, Alphavirus and Orthobunyavirus in Colombia (Groot 1964; Rivas *et al*. 1995; Groot *et al*. 1996; Mattar *et al*. 2005; Hoyos *et al*. 2012; Muñoz and Navarro 2012); the recent introduction of the Chikungunya virus in 22 departments of the country (Mattar and González 2015); and favorable ecological conditions for the establishment of the insect populations in both rural and urban areas could imply a long-term vectorial role and serious epidemiological implications for human health. During dengue epidemiological surveillance activities, *Ae. albopictus* was identified in the municipality of La Tebaida (Quindio, Colombia) and the first sequences cytochrome oxidase I (DNA barcode) were recorded to identify possible phylogeographic origins and permit the tasks of entomological surveillance and vector incrimination.

Materials and methods

Following information from the vector control program of the secretary of health of the Quindio department about the identification of *Ae. albopictus* in a larvae trap in the municipality of La Tebaida, an entomological surveillance outing was coordinated in February 2015 in the Sector Las Brisas - Vereda La Palmita, of the rural jurisdiction of the municipality of La Tebaida (4°25'50.61"N, 75°51'37.24"W), Quindio department in order to collect immature mosquitoes. Sampling was done following Belkin *et al*. (1969). Immatures

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were collected in both artificial and natural breeding places associated with human domicile using a plastic pipette. Samples were placed in containers previously encoded for transport to the Center for Biomedical Research (CIBM) of the University of Quindio for observation until adulthood and later identification considering external morphological characteristics and dichotomous keys (Lane 1953; Forattini 2002; Rueda 2004; González and Carrejo 2007). Legs from specimens identified as *Ae. albopictus* were removed and DNA extracted utilizing the potassium acetate (AcK) method modified by Rosero *et al*. (2010). DNA extracts were used to amplify the DNA fragment barcode (barcode) of ~ 700 nt of mitochondrial gene cytochrome oxidase I with the MTNF / MTNR (Hebert *et al*. 2003; Kumar *et al*. 2007) oligonucleotides. Each PCR mixture contained 1x NH_4SO_4 buffer, 1 mM each dNTP, 5 mM $MgCl_2$, 0.5 µM primers, 0.4 U of Taq polymerase (Bioline, Maryland), 4 uL DNA, and a final volume of 50 µL with molecular water (Gibco BRL). Amplification parameters in the multigene thermocycler (Labnet, New Jersey) included: one cycle of 94 °C for 10 min; followed by 35 cycles of 95 °C for 60 s, 50 °C for 60 s and 72 °C for 60 s, respectively; final extension at 72 °C for 5 min; and 4 °C for preservation. PCR products were visualized on agarose gel (1%) with GELSTAR® stain (Lonza, Rockland) diluted 1/50 and Dark Reader (Image, Alexandria). Positive PCR products were sequenced using the amplification primers (Macrogen - Seoul, Korea). These sequences were edited manually in the Bioeditv7.2.0 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.htm) and consensus in fasta format were aligned in ClustalW (Larkin *et al*. 2007). Genetic distances were estimated in MEGAv6.0 (Tamura *et al*. 2013) using the Kimura 2-parameter model (K2P) (Kimura 1980) and molecular operational taxonomic units (MOTU's) were identified according to genetic distances calculated and clusters within a dendrogram inferred by the Neighbor-Joining (NJ) algorithm (Saitou and Nei 1987) (K2P model bootstrapp = 1.000 replications) (Felsenstein 1985). *Aedes aegypti* (Linnaeus, 1972) and *Culex quinquefasciatus* (Say, 1823) were used as outgroups, because are good references for inter-species genetic distances. Genetic diversity parameters were estimated by polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity, and Tajima neutrality tests using DNAspv5.0 software (Librado and Rozas 2009) for the molecularly characterized *Ae. albopictus* population. A phylogenetic network was estimated using the algorithm "NeighborNet" in package software SplitsTree4 (Huson y Bryant 2006) and COI - sequences published by Zhong *et al*. (2013) for determined the geographic origin with 66 haplotypes registered in China, Taiwan, Japan, Singapore, Italia a several locations of USA.

Results

A total of 55 *Aedes* immature in stages, from L_1 to L_4, including five pupae, were found in a tire used as ovitrap, in a remnant of bamboo (*Guadua angustifolia*) (Kunt) (Fig. 1). All individuals emerged being 28 individuals identified as *Ae. albopictus* (8♂, 20♀) and 27 as *Ae. aegypti* (9♂, 18♀). From these, 9 male and 5 female *Ae. albopictus*, 3 *Ae. aegypti* and 2 *Cx. quinquefasciatus* were characterized for the COI gene - barcode DNA fragment (GenBank accession numbers = KP877569-KP877572). The sequences obtained have a length of 737 nt for *Ae. albopictus*, and correspond to positions 1658 to 2393 of the mitochondrial gene cytochrome oxidase I (reference sequence in GenBank AY072044.1 of COI - *Ae. albopictus*) and 540 nt belong to the barcode region (positions 1658-2198) (Hebert *et al*. 2003). No insertion-deletion events were evident in the sequences analyzed, nor was the presence of stop codons, characteristic of nuclear copies of mitochondrial genes (NUMT's) (Black IV and Bernhardt 2009). Five haplotypes were identified, recording

Figure 1. Tire-ovitrap in the guadua remanent (rural area from municipality of La Tebaida, Quindío Department) where were found *Ae. albopictus* and *Ae. aegypti*.

Table 1. Polymorphic sites between close COI-haplotypes of Singapore and *Ae. albopictus* sequences from La Tebaida (Quindío).

	Polimorphic sites
Haplotypes	226666
	243699
	1739903
H34	CTCTAGA
H33	——
H27	——
'La_Tebaida__M4'	TG.A.TT
'La_Tebaida__F5'	TG.A.TT
'La_Tebaida__F6'	T..A.TT
'La_Tebaida__F8'	TGTA.TT
'La_Tebaida__F9'	TGTA.TT
'La_Tebaida__F10'	TG.A.TT
'La_Tebaida_M12'	TG.A.TT
'La_Tebaida_M14'	TG.A.TT
'La_Tebaida_M15'	TG.AGTT
La	T..A.TT
'La_Tebaida_M18'	TG.AGTT
'La_Tebaida_M19'	TG...TT
'La_Tebaida_M20'	TG...TT

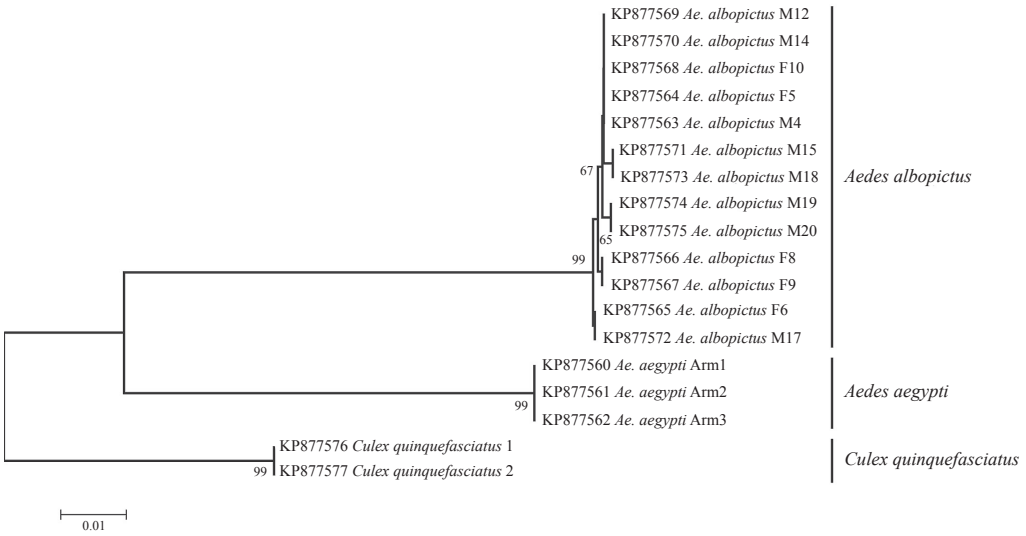

Figure 2. Neighbor-joining dendrogram estimated with sequences of cytochrome oxidase I obtained of *Aedes albopictus* adults, *Ae. aegypti* and *Cx. quinquefasciatus* (Kimura-2-parameter, bootstrap = 1000 replicates). The branch values indicate the bootstrapp clusters in same MOTU (values > 50). The final alignment was of 692 nucleotides and GenBank numbers accessions are in front of every specimen typing.

a high haplotype diversity (Hd = 0.795) and four polymorphic sites at positions 249 (guanine-thymine), 265 (cytosine-thymine), 661 (adenine-thymine) and 691 (adenine-guanine). The Tajima test was not statistically significant (D = 0.55880), nucleotide diversity was low (0.00146), and intra-species genetic distances under Kimura-2 model parameters was low (0.001), indicating the presence of conspecific individuals of the same species. Inter-species genetic distances for *Ae. albopictus* - *Ae. aegypti* (0.137) and *Ae. albopictus* - *Cx. quinquefasciatus* (0.122) were correspondent to recorded estimates for species differentiation in mosquitoes (Cywinska *et al*. 2006; Kumar *et al*. 2006) and were correspondent with species-specific MOTU's in the Neighbor-joining dendrogram (Fig.2). The phylogenetic network evidenced a close relationship with haplotypes 27, 33 y 34 (*sensu* Zhong *et al*. 2013) from Singapore (Fig. 3). Polymorphic sites between *Ae. albopictus* - La Tebaida and Singapore haplotypes were eight and corresponded mainly to transitions (Table 1).

Discussion

Differentiation and taxonomic identification of Culicidae is a priority in vector incrimination and disease control (Besansky *et al*. 2003), however, the high morphological similarity in diagnostic features among vector and non-vector species, species complexes, and cryptic diversity prevents

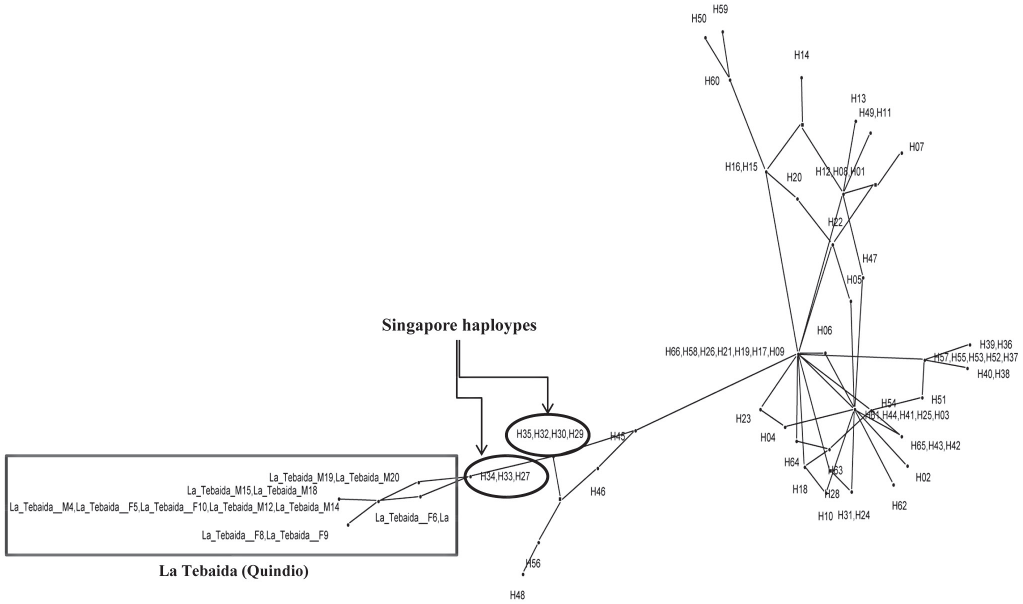

Figure 3. Phylogenetic Network estimated with haplotypes registered by Zhong *et al*. (2013) and sequences characterized for *Ae. albopictus* from La Tebaida (Quindío). The haplotypes enclosed in red belonging to Singapore.

biodiversity and epidemiological studies (Cywinska *et al*. 2006). Significant efforts in the characterization of molecular markers in order to resolve these taxonomic problems and provide for the rapid recognition of vectors have been made. As was shown our results, cytochrome oxidase I - barcode fragment (Hebert *et al*. 2003) is highly reliable for the identification of a wide range of mosquitoes, the split of species complexes (Cywinska *et al*. 2006; Kumar *et al*. 2007), and confirmation of invasive mosquitoes (Golding *et al*. 2012). In our case, the sequences of COI-barcode reported belonging to *Ae. albopictus* allowed differentiate to species with which it shares habitats and geographical areas in Colombia, thus this tool can help clarify important ecological questions about the occupation and/or segregation of habitats in rural and urban areas (Olano and Tinker 1993; Silva *et al*. 2006; Valentini *et al*. 2008), identification of immatures stages (Dhananjeyan *et al*. 2010), prediction of niche (Medley 2010), ecological competition (Murrell and Juliano 2008), taxonomic confirmation (Oter *et al*. 2013) and arboviruses transmission (Cook *et al*. 2005). Another relevant interest about DNA barcode methodology to level-species is that marker should be indicating population aspects about structure, gene flow and phylogeography (Hajibabaei *et al*. 2007), in a context for invasive species as *Ae. albopictus* in Colombia. The phylogeographic origin of the La Tebaida population related to Singapore specimens, similar results find Zhong *et al*. (2013) for specimens collected from 2001 in Los Angeles - California, this information is complementary to Asiatic route for introduction of *Ae. albopictus* in Colombia, where Navarro *et al*. (2013) related Colombian haplotypes with Hawaii populations using ND5, suggesting as most probably hypothesis the introduction from Hawaii or directly through the trade exchange from Africa through the Pacific port of Buenaventura, location with presence of haplotypes related to Asian populations. Interestingly, there is a significant molecular differentiation between haplotypes La Tebaida and Singapore, in this sense, multiple introductions and adaptation to Colombian ecosystems may involve new variability in COI, reflecting population evolution. The COI - DNA barcode characterization for populations of *Ae. albopictus* in Medellin, Buenaventura, Leticia and Barrancabermeja could help to identify phylogeographic origins, colonization and dispersion routes taking account the limited information by other mitochondrial regions (ND5, COI, CytB) and increasing the geographical sampling in American countries with reported *Ae. albopictus* (Argentina, Cuba, Mexico) and others African/ Asiatic populations (Birungi *et al*. 2002; Mousson *et al*. 2005; Navarro *et al*. 2013). By the way, this is the major advantage of COI-DNA barcode: connectivity and common language of DNA sequences for different research groups working in locations inside geographic range of target - species insect (Hoyos *et al*. 2012), allowing typing more sequences of different sites and taking advantage of the high genetic variability of COI for studies in flow and structure populations (Cook *et al*. 2005)

The emergence and re-emergence of pathogenic microorganisms depends on the convergence of ecological and evolutionary factors that allow the disease in susceptible human hosts (Hoyos *et al*. 2012); the recent introduction of chikungunya and its epidemic outbreak in Colombia, the presence of arboviruses of epidemiological importance, and the geographical records of *Ae. albopictus* are indicative of risk for the emergence of new pathogens and consequent outbreaks in human populations. On this regard, the vectorial role of *Ae. albopictus* in outbreaks of dengue/chikungunya and the role it plays in communities of competent vectors on an ecological level in the occupation of natural and artificial habitats is important. Molecular characterization of *Ae. albopictus* with COI - DNA barcode region should contribute to knowledge about the flow and genetic structure of Colombian populations of this species and identify the phylogeographic origin of recent populations detected in Medellin, for identify possible points of entry into Colombia and to increase entomological surveillance in these locations for the purpose of intercepting new sources of foreign mosquito introduction (Oter *et al*. 2013; Demeulemeester *et al*. 2014).

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