
Genetic analysis of a recently detected urban population of *Lutzomyia evansi* (Diptera: Psychodidae) in Colombia

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Análisis genético de una población urbana de *Lutzomyia evansi* (Diptera: Psychodidae), recientemente detectada en Colombia

■ **RESUMEN.** *Lutzomyia evansi* (Núñez-Tovar) es el insecto transmisor del parásito *Leishmania infantum* en zonas rurales del norte de Colombia. Con el propósito de establecer el probable origen de una población urbana del vector, detectada en años recientes, se caracterizaron genéticamente ejemplares de *Lutzomyia evansi* de siete localidades geográficas del Caribe Colombiano. Los flebotomíneos fueron recolectados en ambientes rurales y urbanos de zonas endémicas y no endémicas de leishmaniasis visceral. Dentro del fragmento secuenciado de 315 pb correspondiente al extremo 3' del gen mitocondrial citocromo b, se encontraron nueve sitios polimórficos, nueve haplotipos nucleotídicos y un solo haplotipo aminoacídico. Las distancias genéticas pareadas entre los haplotipos, estimadas con el modelo de Kimura de dos parámetros, oscilaron entre 0,0032 y 0,0194. El análisis reveló la existencia de una baja variabilidad genética entre especímenes de localidades urbanas y rurales. Varios de los flebotomíneos recolectados en la zona urbana de la ciudad de Sincelejo, departamento de Sucre, donde en años recientes aparecieron casos autóctonos de leishmaniasis visceral, fueron genéticamente similares a los de El Contento, en el cercano departamento de Córdoba, foco rural de la enfermedad. Se discuten las implicaciones epidemiológicas de este hallazgo para la transmisión de *Leishmania infantum* en el Caribe Colombiano.

PALABRAS CLAVE. Flebotomíneos. Genética. *Lutzomyia evansi*. Leishmaniasis visceral. Colombia.

■ **ABSTRACT.** *Lutzomyia evansi* (Núñez-Tovar) is the vector of the parasite *Leishmania infantum* in rural zones of Northern Colombia. An attempt was made to determine the origin of a recently detected urban

population of *Lutzomyia evansi* by genetically characterizing specimens from seven geographically distinct localities in the Colombian Caribbean. Insect specimens were collected in rural and urban environments of areas endemic for visceral leishmaniasis or free of the disease. Nine polymorphic sites, nine nucleotide haplotypes and a single aminoacid haplotype were found within the 315 bp fragment sequenced, corresponding to the 3' end of the cytochrome b mitochondrial gene. Paired genetic distances between the haplotypes, estimated with the Kimura two-parameters model, varied from 0,0032-0,0194. Analysis revealed low genetic variability between specimens from urban and rural localities. Several of the sand flies collected in the city of Sincelejo (department of Sucre), where autochthonous visceral leishmaniasis cases have appeared in recent years, were genetically similar to those of a rural focus of the disease (El Contenido, on the neighboring department of Córdoba). The epidemiological implications of this finding for *Leishmania infantum* transmission in the Colombian Caribbean are discussed.

KEY WORDS. Sand flies. Genetics. *Lutzomyia evansi*. Visceral leishmaniasis. Colombia.

INTRODUCTION

Visceral leishmaniasis (VL), a re-emergent zoonosis in the Americas (Arias *et al.*, 1996; Feliciangeli *et al.*, 1999; Enserink, 2000), is caused by the trypanosomatid *Leishmania infantum* Nicolle. VL is the most severe of three clinical forms of leishmaniasis, generally producing death in children under five if left untreated. Formerly, VL was present only in rural areas but now occurs in urban and periurban habitats of Brazil, Venezuela and Colombia (Arias *et al.*, 1996; Aguilar *et al.*, 1998; Bejarano *et al.*, 2002; Cortés & Fernández, 2008). This may be attributed to both human migration and climatic or environmental changes that affect populations of vectors, some of which have adapted to anthropogenic environments (Walsh *et al.*, 1993).

In South and Central America *Lutzomyia longipalpis* (Lutz & Neiva), *L. cruzi* (Mangabeira) and *L. evansi* (Núñez-Tovar) are the known vectors of *Le. infantum* (Travi *et al.*, 1990; Ferro *et al.*, 1995; Santos *et al.*, 1998). The last of these transmits the parasite in Colombia and Venezuela (Travi *et al.*, 1990; Feliciangeli *et al.*, 1999). In 1999, *L. evansi* was detected in an urban area of the Colombian Caribbean (Bejarano *et al.*,

2001), the first cases of VL appearing two years later in the city of Sincelejo (Bejarano *et al.*, 2002). An entomological survey of dwellings in a neighborhood where the affected children lived revealed the presence of *L. evansi* (Bejarano *et al.*, 2002). *Lutzomyia longipalpis* has not been found, to date, in this neighborhood despite intensive entomological sampling.

This investigation was undertaken to determine the probable geographical origin of this recently detected urban population of the vector. Thus, specimens of *L. evansi* from rural and urban localities in Colombia were characterized using the mitochondrial cytochrome *b* gene. Mitochondria have matrilineal inheritance, which permits the geographical dispersion of females to be traced. Females have greater epidemiological importance than males through being directly involved in transmission of the parasite (Bejarano, 2001).

MATERIALS AND METHODS

Collection and identification of sand flies. The study was carried out in seven localities of the Caribbean coastal region of Colombia, each of which presents a distinct

Table I. Collection sites of *Lutzomyia evansi* in the Caribbean coast of Colombia and Genbank accession numbers of the DNA sequences obtained in this study.

Locality	Geographic coordinates	Environment	Epidemiologic status of leishmaniasis	Genbank accession nos.
Vereda El Contento (Córdoba)	9° 13' N, 75° 29' W	Rural	Endemic	EF457569, EF457570
Vereda Los Vidales (Córdoba)	9° 9' N, 75° 31' W	Rural	Endemic	EF457571, EF457572
Municipio de Sincelejo (Sucre)	9° 18' N, 75° 25' W	Urban	No endemic	EF457573, EF457574, EF457575
Vereda Loma de Piedra (Sucre)	9° 11' N, 75° 22' W	Rural	Endemic	EF457576, EF457577
Vereda Escobar Arriba (Sucre)	9° 20' N, 75° 21' W	Rural	Endemic	EF457578
Municipio de Santa Rosa de Lima (Bolívar)	10° 26' N, 75° 22' W	Rural	No endemic	EF457579, EF457580
Corregimiento de Isla Fuerte (Bolívar)	9° 23' N, 76° 23' W	Rural	No endemic	EF457581, EF457582

epidemiological profile for VL (Table I). Sand flies were collected in and around houses, using CDC light traps and Shannon traps respectively. The head, one wing and the genitalia of each specimen were removed to determine the species using the taxonomic key of Young & Duncan (1994), and Galati (2003).

DNA Extraction. DNA was extracted using the protocol of Collins *et al.* (1987) with the following modifications. Each specimen was macerated in 60 µl of lysis buffer (0,08 NaCl, 0,16 M saccharose, 0,06 M EDTA, 0,5% SDS, 0,1 M Tris-HCL at pH 7,5) and incubated at 65°C for 30 min, after which 14 µl of 8 M potassium acetate was added and the mixture incubated on ice for 30 min. It was then centrifuged at 12 000 × *g* for 10 min and the supernatant transferred to a new vial. The DNA was precipitated for 18 h with 200 µl of absolute ethanol at -20°C and centrifuged at 12 000 × *g* for 20 min. After decanting off the supernatant, the DNA was rinsed with 200 µl of 70% ethanol and then with 200 µl of absolute ethanol. Finally, it was left to dry at room temperature and re-suspended in 30 µl of ultrapure water.

DNA amplification. A segment of the mitochondrial DNA genome was amplified by PCR using the forward primer [5'-CA(T/C)ATTCAACC(A/T)GAATGATA-3'] and the reverse primer [5'-GGTA(C/T)(A/T)TTGCCTCGA(T/A)TTTCG(T/A)TATGA-3']

(Ready *et al.*, 1997). This region includes the 3' end of the cytochrome *b* gene. PCR amplifications were performed in a 50 µl reaction mix containing 1x PCR buffer (Promega Corporation, Madison, WI), 1,5mM MgCl₂, 0,2mM of deoxynucleoside triphosphate mix (Promega), 0,3µM of each primer, 1,5 U of *Taq* DNA polymerase (Promega) and 6 µl of DNA template. The cycling conditions included an initial 3-min denaturation step at 94°C, followed by 35 cycles of 1-min denaturation at 93°C, 1-min primer annealing at 50°C, and 1-min elongation at 72°C. Final extension was done at 72°C for 10 min, followed by a hold step at 4°C.

Nucleotide sequencing. PCR products separated by 1,5% agarose gel electrophoresis were visualized by ethidium bromide staining, purified by using Wizard® PCR Preps DNA Purification System Kit (Promega), and sequenced in both directions using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Sequences were determined on an ABI Prism® 377 automated DNA sequencer (Applied Biosystems) and assembled using the SeqMan program (DNASTAR, Inc., Madison, WI). The consensus nucleotide sequences were derived from 14 individuals of *L. evansi*. GenBank accession numbers for the sequence data are EF457569-EF457582 (Table I).

Genetic analysis. The gene that codes for the protein cytochrome *b* was selected for genetic analysis, since this presents the highest rate of substitution between the sequenced mitochondrial segments of *Lutzomyia* spp. (Bejarano, 2001). The segment analyzed is homologous with the region between positions 11 271 and 11 585 of the *Aedes albopictus* (Skuse) mitochondrial genome (Genbank accession no. AY072044). Nucleotide sequences were aligned with the Clustal W program running within the software package DAMBE 4.2.13 (Xia & Xie, 2001). Pairwise sequence alignments and protein translation were performed with MEGA version 3.1 (Kumar *et al.*, 2003). To determine the phylogenetic relationships of *L. evansi* populations, a neighbor-joining tree was constructed on the basis of the evolutionary distances calculated by the Kimura two-parameter model (Kimura, 1980) using MEGA software (Kumar *et al.*, 2003).

RESULTS

A total of nine nucleotide haplotypes of the cytochrome *b* gene was obtained from *L. evansi* specimens collected on the Caribbean coast of Colombia. These are the first nucleotide sequences reported for this sand fly vector species. The mean nucleotide composition of the cytochrome *b* gene was 9,9% guanine, 16,2% cytosine, 33,6% adenine and 40,3% thymine. Nine polymorphic and 306 conserved sites were found on this 315-bp segment of this gene (Table II). Most substitutions were transitions, five involving cytosine-thymine and three adenine-guanine. Only one corresponded to an adenine-thymine transversion. All the changes were silent, reflected in the presence of a single haplotype aminoacid among sand flies from the seven geographical locations analyzed.

Three of the nine nucleotide haplotypes observed appeared in the individuals of *L. evansi* from the city of Sincelejo. The number of nucleotide differences between haplotypes varied from 1-6 (0,32 and 1,94%), with an overall mean of 2,78 substitutions (0,89%). The first haplotype was shared between

recently urbanized sand flies (Sincelejo) and specimens from a rural focus of VL at El Contenido in the neighboring department of Cordoba. The second haplotype was observed in urban insects (Sincelejo) and the sand flies from Isla Fuerte, a small island 11 km off the Caribbean coast of Colombia. The third haplotype included individuals of all three localities (Sincelejo, Isla Fuerte and El Contenido). The remaining six haplotypes corresponded exclusively to sand flies collected in rural areas.

The paired genetic distances between each of the nine haplotypes, estimated using the Kimura two-parameter model, had low values of 0,0032-0,0194 (Table III). The highest value was recorded among haplotypes of rural locations that were VL foci, while the lowest ones appeared when haplotypes of rural and urban villages were paired. Two clusters were observed in the neighbor-joining tree (Fig. 1). The first cluster grouped haplotypes of urban, rural and island locations, while the second included those of three dissimilar rural villages from areas endemic or not for VL.

DISCUSSION

The present study represents the first analysis of intraspecific genetic variability of *Lutzomyia evansi* in Colombia, where previous genetic studies of sand flies have been focused on *L. longipalpis*, *L. shannoni* (Dyar), and other species of the *L. verrucarum* group (Cárdenas *et al.*, 2001; Uribe *et al.* 2001; Testa *et al.*, 2002; Pérez-Doria *et al.*, 2008). Analysis of the 3' end of this gene revealed low genetic variability among *L. evansi* isolates from the Colombian Caribbean. Genetic distance values were very low, similar to those observed with the same molecular marker in populations of *L. longipalpis* and *L. whitmani* (Antunes & Coutinho) (Ready *et al.*, 1997; Hodgkinson *et al.*, 2003). However, unlike these latter species, in which each nucleotide haplotype was present in a single population, several *L. evansi* haplotypes were shared by rural and urban individuals. Furthermore, on

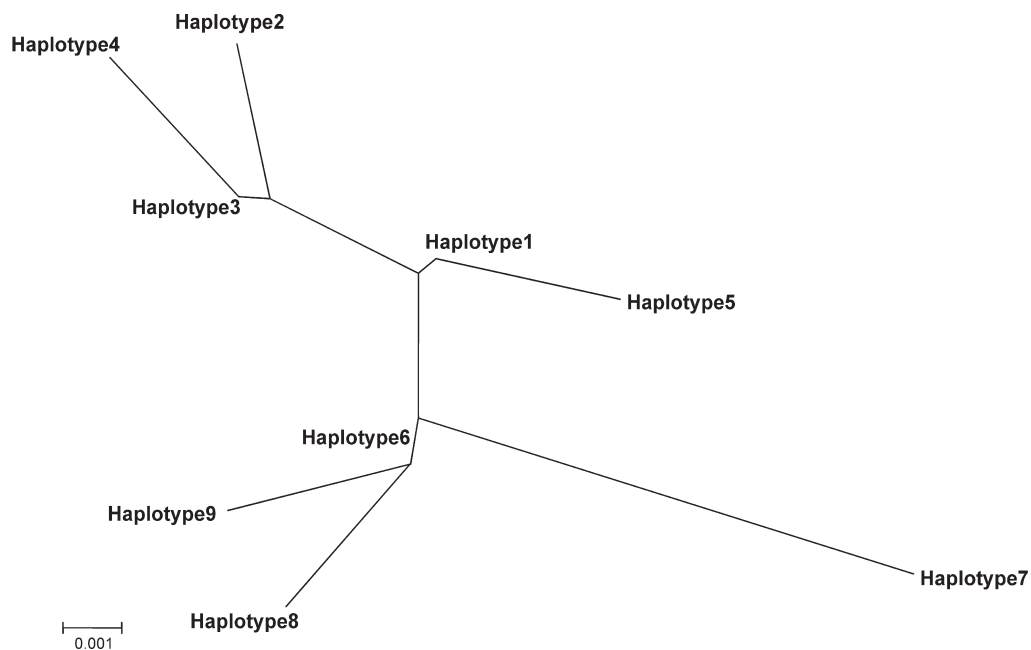


Fig. 1. Phylogenetic analysis of haplotypes of *Lutzomyia evansi* from Colombian localities based on a 315-bp fragment of the cytochrome *b* gene. Distances and groupings were determined by the Kimura's two-parameter and neighbor-joining method with the MEGA software. All haplotype numbers correspond to Table II.

Table II. Distribution of haplotypes of *Lutzomyia evansi* derived from the 3' end of the cytochrome *b* gene across Colombian localities. SI, Sincelejo; EC, El Contento; IF, Isla Fuerte; LV, Los Vidales; LP, Loma de Piedra; EA, Escobar Arriba; SR, Santa Rosa de Lima.

Haplotypes	Polymorphic Sites									Populations
	54	181	186	201	204	210	216	234	258	
1	C	T	A	T	A	A	C	G	C	SI, EC
2	C	T	A	T	A	G	C	G	T	SI, IF
3	C	T	A	T	A	G	C	G	C	SI, EC, IF
4	C	C	A	T	A	G	C	G	C	LV
5	C	T	G	T	A	A	C	G	C	LV
6	C	T	A	C	A	A	C	G	C	LP
7	C	T	A	C	T	A	C	A	T	LP
8	C	T	A	C	A	A	T	G	C	EA, SR
9	T	T	A	C	A	A	C	G	C	SR

the phylogenetic tree the mitochondrial haplotypes of urban sand flies did not appear on distinct clusters from those of rural insects, suggesting genetic homogeneity between the two groups.

The urban sand fly population of Sincelejo proved to be genetically related to the rural one of El Contento, an established VL focus since the 1980s (Vélez *et al.*, 1988).

Several of the sand flies of both geographical localities shared the same nucleotide haplotype, clearly showing a common genetic origin. This allows us to suggest that the urban population of *L. evansi* could have originated by dispersion from this rural focus of the disease, taking into account that these localities are approximately 13km apart. There are no apparent geographical barriers

Table III. Kimura's two-parameter genetic distance among nine nucleotide haplotypes of *Lutzomyia evansi* of Colombia. All haplotype numbers correspond to Table II.

	1	2	3	4	5	6	7	8
Haplotype 1								
Haplotype 2	0.0064							
Haplotype 3	0.0032	0.0032						
Haplotype 4	0.0064	0.0064	0.0032					
Haplotype 5	0.0032	0.0096	0.0064	0.0096				
Haplotype 6	0.0032	0.0096	0.0064	0.0096	0.0064			
Haplotype 7	0.0128	0.0128	0.0161	0.0194	0.0161	0.0096		
Haplotype 8	0.0064	0.0129	0.0096	0.0129	0.0096	0.0032	0.0128	
Haplotype 9	0.0064	0.0129	0.0096	0.0129	0.0096	0.0032	0.0128	0.0064

that would impede such dispersion. However, the previous existence of the vector in urban areas could also be possible, with the current population being descended from sand flies that survived the selection pressure of anti-malaria spraying campaigns carried out in the region during the 1980s.

It is difficult to determine whether vector dispersion contributed to that of the parasite, since the latter could also have arrived in infected mammal reservoirs. In recent years families displaced by politically motivated violence have arrived in Sincelejo from rural foci endemic for VL, bringing dogs with them that might be infected with *Le. infantum* and completing the elements required to establish a new microfocus of infection (Pérez-Doria *et al.*, 2006). Sporadic autochthonous cases of VL have appeared since 1999 (Bejarano *et al.*, 2002), when *L. evansi* was first detected in Sincelejo (Bejarano *et al.* 2001). This species was absent from all previous insect collections made in the city, which supports the hypothesis that it was a recent invader. Thus *Lutzomyia evansi* could be increasing the original limits of the *Le. infantum* transmission focus on the Caribbean coast of

Colombia, which would explain the presence of new VL cases in areas recently colonized by the vector (Bejarano *et al.*, 2002; Cortés & Fernández, 2008). This should be taken into account by public health entities when designing VL prevention and control measures in this country.

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