Tropical Biomedicine 35(1): 100–110 (2018)

Molecular identification and genetic diversity of *Lutzomyia gomezi* (Diptera: Psychodidae) using DNA-barcodes in Cordoba, Colombia

Carrero-Sarmiento, D.[1] and Hoyos-López, R.[2*]
[1]Universidad de Pamplona, Laboratorio de Entomología, Grupo de Ecología y Biogeografía, Pamplona, Colombia
[2]Universidad del Sinú, Grupo de Investigación en Resistencia Bacteriana y Enfermedades Tropicales, 230000, Montería, Colombia
[*]Corresponding author e-mail: rhoyoslopez@gmail.com
Received 18 July 2017; received in revised form 21 November; accepted 22 November 2017

Abstract. *Lutzomyia gomezi*, a suspected vector of cutaneous leishmaniasis in Colombia is recorded for natural infection of *Leishmania* parasites, its anthrophilic behaviour and significant abundance supports its vectorial role. The difficulties associated with taxonomic identification due to lack of males require the use of molecular markers (DNA barcodes), which allows us to distinguish the species. In this study, the cytochrome oxidase I fragment was proposed as DNA barcode to identify specimens collected from Cordoba, Colombia (Planeta Rica: Arenoso/Centro Alegre, Sahagún: Santiago Abajo/San Andresito) by using protocols for DNA extraction, PCR, and sequencing. These sequences allowed for testing the genetic diversity, genetic distance, population structure and gene flow. A phylogenetic analysis was performed with sequences registered in Genbank for this and related species such as *Lutzomyia lichyi*, *Lutzomyia longipalpis* and *Lutzomyia bifoliata*. In total, 24 PCR products were sequenced, resulting in an alignment of 677 nt in length, and 9 haplotypes were identified for *L. gomezi*. Values for polymorphic sites, haplotype and nucleotide diversity were high for specimens belonging to Sahagún and Planeta Rica. The genetic distances (TN93) and localities studied were significant (0,011–0,024), F_{ST} evidenced with mild and significant structure (0,10–0,52) and limited genetic flow (Nm=0,45–24,5). The phylogenetic analysis showns three lineages with significant distances (0,026-0,48) and sympatric between haplotypes from different zones; however, the limited sampling size and the absence of specimens belonging to other Colombian geographic areas implied more lineages. The DNA barcode methodology can answer questions about phylogeography, vector competence and genetic structuration between populations using a common marker for the scientific community.

INTRODUCTION

Lutzomyia gomezi (Nitzulescu, 1931) is a species belonging to *Lutzomyia* subgenus, that is morphologically characterized in males with no upturned paramere, a coxite tuft of 20 or fewer setae, and individual sperm ducts relatively thin in females (Young & Duncan, 1994). It has a wide geographic distribution and is found in Central America, including Mexico, Panama, Trinidad & Tobago, Guatemala (Valderrama *et al*., 2014). In South America, it has been recorded from Colombia, Venezuela, Ecuador, Peru, French Guiana and Brazil (Galati, 2009). Its habitats are mainly associated with humid and dark places and are close to animal burrows in tropical rainforest but exist in low abundance (Christensen *et al*., 1983). Due to fragmentation of forest and changes in the availability of native habitats has caused changes its adaptive behavior to forest gaps; the exploitation of peridomestic animals as blood source, biting activity, and colonization of pastures and mango crops (Porter & Young, 1986; Travi *et al*., 2002; Duque *et al*., 2004; Hoyos *et al*., 2012; Paternina *et al*.,

2016). *L. gomezi* is recognized by its anthropophilic and endophilic behavior, and is a suspected vector for cutaneous leishmaniasis in several endemic regions in Colombia (Travi *et al.*, 1988; Sandoval *et al.*, 1998).

Strong evidence on natural infection reported with parasites supports the hypothesis that it is likely to be a vector of cutaneous leishmaniasis. It has been found to be naturally infected with non-identified trypomastigotes in Panama (Jhonson *et al.*, 1963), Ecuador (Calvopina *et al.*, 2004) and Colombia (Morales *et al.*, 1981; Young *et al.*, 1987); and was found to be infected with *Leishmania panamensis* from Boyaca – Colombia (Santamaría *et al.*, 2006). Other records for *L. gomezi* of *Leishmania braziliensis* in Venezuela (Feliciangeli *et al.*, 1994; Rodriguez *et al.*, 1999), *Leishmania naiffi* in Panama (Azpurua *et al.*, 2010), and experimental infection assays prove a high competence for *Le. Panamensis* (Walters *et al.*, 1989; Jaramillo *et al.*, 1994). In Caribbean Colombian, the regions of Sucre and Cordoba have a high prevalence for human cutaneous leishmaniasis, and species related to this clinical form, such as *Le. braziliensis*, *Le. panamensis* and *Leishmania guyanensis* have been isolated and identified in patients (Martinez *et al.*, 2010).

L. gomezi has been associated with peri-urban and rural areas in Colombia. The active zones for leishmaniasis are in Bucaramanga – Santander (Sandoval *et al.*, 1998), Bahia Solano – Choco (Duque *et al.*, 2004), Otanche and Pauna – Boyaca (Santamaría *et al.*, 2006), San Carlos (Parra & Echavarria, 2005) and Santa Fe de Antioquia (Hoyos *et al.*, 2013). In Caribbean region, it has been reported in the urban areas of Carmen de Bolivar – Sucre (Cortez & Fernandez, 2008) and Sincelejo (Bejarano *et al.*, 2002, Lambraño *et al.*, 2012). The Cordoba populations of *L. gomezi* was identified to have a mixed focus for leishmaniasis in San Andres de Sotavento (Travi *et al.*, 2002), in rural areas the active transmission of cutaneous leishmaniasis occur in Tierralta and Valencia (Vivero *et al.*, 2017), and recently, near the delta of Sinú river in San Bernardo del Viento (Toro-Cantillo *et al.*, 2017).

DNA barcodes have been proposed as a tool for rapid identification on the biodiversity in groups with medical importance and complex taxonomy (Hebert *et al.*, 2003; Besansky *et al.*, 2003). This method requires amplification of the cytochrome oxidase I fragment and sequencing obtained from library references, which is used to identify queries characterized to any species collected from different ecosystems, or in our case, active zones for leishmaniasis (Azpurua *et al.*, 2010; Contreras *et al.*, 2014; Pinto *et al.*, 2015). Several advances have been recorded in Colombia; the molecular typification of *Lutzomyia longipalpis* complex (Hoyos *et al.*, 2012), DNA barcodes for 36 sand fly species collected from different geographic locations from Colombia (Contreras *et al.*, 2014); DNA barcodes for 19 sand fly species of mixed leishmaniasis collected from Sucre, Colombia (Sincelejo, Colosó and Ovejas) (Romero *et al.*, 2016), and the identification of the immatures stages of sand flies in endemic zones for cutaneous and visceral leishmaniasis (Vivero *et al.*, 2017). The recent application to immature stages opens the possibility for research in micro-habitats, breeding sites and ecology for vector species, allowing future measures of control more adjusted to the ecology of the disease. However, it is necessary but requires considerable effort to gain this information, as less than 20% of the *Lutzomyia* diversity has been identified for America (Romero *et al.*, 2016); even for Colombia, the percentage is very low and focused in species with incrimination of vectors that are naturally infected with *Leishmania* spp., or species that are abundant in active zones for leishmaniasis transmission. There are five aspects to consider in future DNA barcode research for sand flies: 1. Increase the samples of a species, but with individuals belonging to different geographic locations. 2. Test the genetic diversity between localities within species to clear topics of cryptic diversity. 3. Delimitation in the barcode-gap for

evolutionary close species. 4. Possible uses in answering evolutionary question, as exemplified in *L. longipalpis* (Hoyos *et al.*, 2012). 5. Metagenomic studies vector-parasite, as exemplified in mosquitoes (Talaga *et al.*, 2017).

In our study, we present DNA barcode sequences of *L. gomezi* taken from two locations in Cordoba (Colombia). DNA barcode methodology testing are used to determine the parameters of genetic diversity and evaluate the potential use in genetic populations of sand fly species.

MATERIALS AND METHODS

Specimens of *Lutzomyia gomezi* in this study were collected using CDC light traps (180 hours of sampling for each locality) and Shannon traps (12 hours of sampling for each locality) between August – November 2016, in rural areas of Planeta Rica (Centro Alegre, 8°10'36.39" N, 75°37°54.63" W; Arenoso, 8°13'6.15" N, 75°44'15.79" W) and Sahagún (San Andresito, 8°47'52.31" N, 75°25'44.84" W; Santiago Abajo 8°35'15.67" N, 75°23'33.70" W) (Cordoba, Colombia). Thirty adults, stored in nitrogen liquid, were fragmented for morphological identification (head and last abdominals segments) using pictorial keys (Young & Duncan, 1994; Galati, 2009). The thorax, legs and partial abdomen were used for DNA extraction protocols (Hoyos *et al.*, 2012). Amplification of the DNA barcode fragment was achieved using universal primers LCO-1490/HCO-2198 (Folmer *et al.*, 1994; Hebert *et al.*, 2003).

Each volume of PCR-mix contained 3µl Buffer [1X], 2.4µl of DNTP [2 mM], 1.8µl of MgCl2 [1.5 mM], 0.9µl each primer [0.3 uM], 0,4 U of taq polymerase (Bioline, Maryland) and 2µl of DNA template and was made up to a total volume of 30µl using water for molecular biology quality. The PCR thermocycler parameters included: a single cycle at 95°C for 5 min, followed by 34 cycles of 95°C for 30 s, 54°C for 1 min and 72°C for 1 min, respectively terminating with a 72°C for 10 min of final extension step and 4°C hold. PCR products were visualized on 1% agarose gels, containing GELSTAR® (Lonza, Rockland) diluted 1/50 using a Dark Reader lector (IMGEN, Alexandria). PCR products were sequenced using same primers LCO/HCO (Folmer *et al.*, 1994) in the Macrogen sequencing service (Seoul, Korea).

Sequences obtained were manually edited in Bioeditv7.2.0 software (Hall, 1999) and consensus in FASTA format and aligned in ClustalW (Larkin *et al.*, 2007). Genetic distances were estimated in MEGAv6.0 (Tamura *et al.*, 2013) using the Tamura-Nei model (TN93) (Romero *et al.*, 2016), and dendrogram inferred by the Neighbor-Joining (NJ) algorithm (Saitou & Nei, 1987) using bootstrap as confidence measure (Felsenstein, 1985). *Lutzomyia bifoliata* (KC921233.1), *Lutzomyia lichyi* (KC921259.1) and *Lutzomyia longipalpis* (GU909505.1) were used as the outgroup, because they are representative for *Lutzomyia* subgenus. Genetic diversity parameters were estimated by polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity, Tajima neutrality tests, genic flow and index population structure using DNAspv5.0 software (Librado & Rozas, 2009). A phylogenetic tree was built using maximum likelihood inference in Phyml 3.0 software (Guindon & Gascuel, 2003) (bootstrapp = 1000 replicates).

RESULTS

Thirty adults were identified as *L. gomezi*, and 24 sequences were obtained from four locations in Cordoba (Colombia) (Genbank accession numbers: KY860853-77), and they all have a length of 677 nt. No insertions/deletion sites were evident in the sequences analyzed, and the presence of stop codons, characteristic of nuclear copies of mitochondrial genes (NUMT's), was not detected (Leite, 2012).

Nine haplotypes in total were evidenced, and Santiago Abajo – Sahagún have more diversity haplotype, but polymorphic sites and nucleotide diversity was significant (0,01256) in Arenoso – Planeta Rica. San Andresito – Sahagún had a low values (0,00882) for haplotypes, polymorphic sites

and diversity haplotype. The Tajima test was not significant for any sampled population, and intra-population genetic distances using Tamura – Nei model (TN93) were low for Arenoso – Planeta Rica, but different values were found for Centro Alegre – Planeta Rica and localities from Sahagún (Table 1). 636 sites were unchanged and 41 were polymorphic positions (18 parsimony informative sites, 26 single mutations) (Table 2). The F_{ST} values show moderate and significant differentiation between San Andresito – Arenoso/Centro Alegre/Santiago Abajo; in fact, the gene flow of these populations is slight (0,45 – 1,619) or negligible (Table 3). The Neighbor-joining

Table 1. Parameters of genetic diversity for different localities where was collected *L. gomezi* (Cordoba, Colombia)

Locality (Cordoba, Colombia)	n[a]	H[b]	S[c]	Hd[d]	Đ[e]	Tajima test	GD[f]
Planeta Rica – Arenoso	5	3	22	0.700	0.01300	-1.2390 (N.s, P>0.10)	0.0–0.014
Planeta Rica – Centro Alegre	5	3	18	0.700	0.01064	-1.2275 (N.s, P>0.10)	0.0–0.011
Sahagún – San Andresito	5	2	15	0.400	0.00882	-1.2185 (N.s, P>0.10)	0.0–0.013
Sahagún – Santiago Abajo	9	6	19	0.833	0.01256	0.91119 (N.s, P>0.10)	0.001–0.009
Total	24	9	41	0833	0.01415	–	–

[a]Sequences characterized. [b]Haplotypes. [c]Polymorphism sites. [d]Haplotype diversity. [e]Nucleotide diversity. [f]Intra-specific genetic distances (Tamura-Nei Model). N.s = Not statistical significance.

Table 2. Polymorphic sites between DNA – barcode haplotypes of *L. gomezi* from Cordoba (Colombia)

	Polymorphic sites
	1111222334455555555555555555566666666
Haplotypes	2345611792993859022233333333444444923333346
	0289036947362987767812356789013479890123714
Lgomezpra1	CAGCTCCTAGCCAATACTTGTATTCTGCTGTTTTCCCGGAGGTA
Lgomezpra2	.....GC....CT
Lgomezpra3	.....GC....CT
Lgomezpra4	.............TAAGAGGAGGAAAGAAGACT.GC....CT
Lgomezpra5	.....GC....CT
Lgomezprac1	TG.TCTTCGATTGT.....TGC...T..
Lgomezprac2	.....GC....CT
Lgomezprac4	.....GC....CT
Lgomezprac5	.....GC....CT
Lgomezssab1	.............C.....GC....CT
Lgomezssab2	.............C.....GC....CT
Lgomezssab3	T.ATCTTCGATT.T.....TGCT....T
Lgomezssab4	.............C.....GC....CT
Lgomezssab5	.............C.....GC....CT
Lgomezssab6	T..TCTTCGATTGT.....TGC.....T
Lgomezssab7	.....GC....CT
Lgomezssab8	.G.....GC....CT
Lgomezssab9	.............C.....GC....CT
Lgomezssan1	T..TCTTCGATTGT.....TGCTGA..T
Lgomezssan2	.............C.....GC....CT
Lgomezssan3	T..TCTTCGATTGT.....TGC.....T
Lgomezssan4	T..TCTTCGATTGT.....TGC.....T
Lgomezssan5	T..TCTTCGATTGT.....TGC.....T

Table 3. Genetic Diversity of *Lutzomyia gomezi* sampled from Cordoba department. Index of subpopulation structure (F_{ST}), gene flow (N_m) and genetic distances between populations of *L. gomezi* from Cordoba (Colombia)

	Arenoso	Centro Alegre	Santiago Abajo	San Andresito
1. Arenoso	–	*0.012*	*0.015*	*0.024*
2. Centro Alegre	**0.0204** (24.5)	–	*0.011*	*0.016*
3. Santiago abajo	**0.1193** (3.69)	**0.1039** (4.31)	–	*0.015*
4. San Andresito	**0.5219** (0.45)	**0.3678** (0.85)	**0.2359** (1.619)	–

In bold values corresponding to F_{ST} and within brackets N_m. Values in italics corresponded to genetic distances.

dendrogram and phylogenetic tree inferred by maximum likelihood with *L. gomezi* sequences reported to Contreras *et al.* (2014) and Romero *et al.* (2016), exhibited a similar pattern, with high diversification and partition in three lineages without geographic segregation. The maximum likelihood phylogenetic tree showns the following lineages (Figure 1): 1. Haplotype of Victoria, Caldas. 2. Haplotypes from Santiago Abajo/ San Andresito – Sahagún, Centro Alegre/ Arenoso – Planeta Rica, Colosó/Sincelejo – Sucre and Victoria – Caldas. 3. Haplotypes from Colosó – Sucre, Santiago abajo/San Andresito – Sahagún, Bahia Solano – Choco, Riosucio – Caldas and Ulloa – Valle del Cauca. Genetic distance values between lineages were high (2,6 – 4,8%) (Table 4).

DISCUSSION

DNA barcodes can be useful markers to indicate population aspects related to structure, gene flow and phylogeography (Hajibabaei *et al.*, 2007). Our results confirm a high genetic polymorphism for *L. gomezi* in a micro-geographic area from Cordoba, where we identified 44 polymorphic sites, and agree with the 40 identified and reported by Contreras *et al.* (2014) for *L. gomezi*. In fact, this species has high values for intra-specific divergence (6.0%) from many different localities (Choco, Antioquia, Valle del Cauca, Caldas), which explained the high values for genetic distances in comparison with our results (2.4% – TN93 model). Similar divergences were reported by Romero *et al.* (2016) for two close localities where *L. gomezi* specimens were collected (2.4% for Kimura-2-parameters and 2.5% – TN93).

An inter-specific limit of 3.0% for insects was proposed by Hebert *et al.* (2003), but values proposed by Contreras *et al.* (2014) and Romero *et al.* (2016) were different and range between 6.0% – 2.9% respectively, demonstrating a possible evolutionary process for phlebotomine sand fly species tested. For *L. gomezi*, the first report as a possible "cryptic species" was registered by Azpurua *et al.* (2010) in Barro Colorado (Panama), which found 11 polymorphic sites for a barcode sequence of 549 nt. However, intra-specific divergences or values for genetic diversity were not reported.

The dendrogram of Neighbor-Joining and phylogenetic tree inference obtained using maximum likelihood were similar, splitting into three lineages grouping sequences belonging to five Colombian departments. However, specimens were not included for the regions of central Colombia (Boyaca, Cundinamarca), Santander, Amazonas and others Caribbean areas. In this sense, *L. gomezi* has a wide distribution reported in another 22 Colombian departments (Bejarano & Estrada, 2016), indicating the likely existence of more lineages and genetic diversity if the sampling size is increased.

L. longipalpis is a recognized as a "species complex" with a geographic distribution similar to *L. gomezi*, has a significant values for genetic distances (under Kimura-2-parameters model) in a range of 5.0–6.0% for Colombian populations, and indicated three molecular clusters (Hoyos *et al.*, 2012). Contreras *et al.* (2014) concluded the possible presence of two or

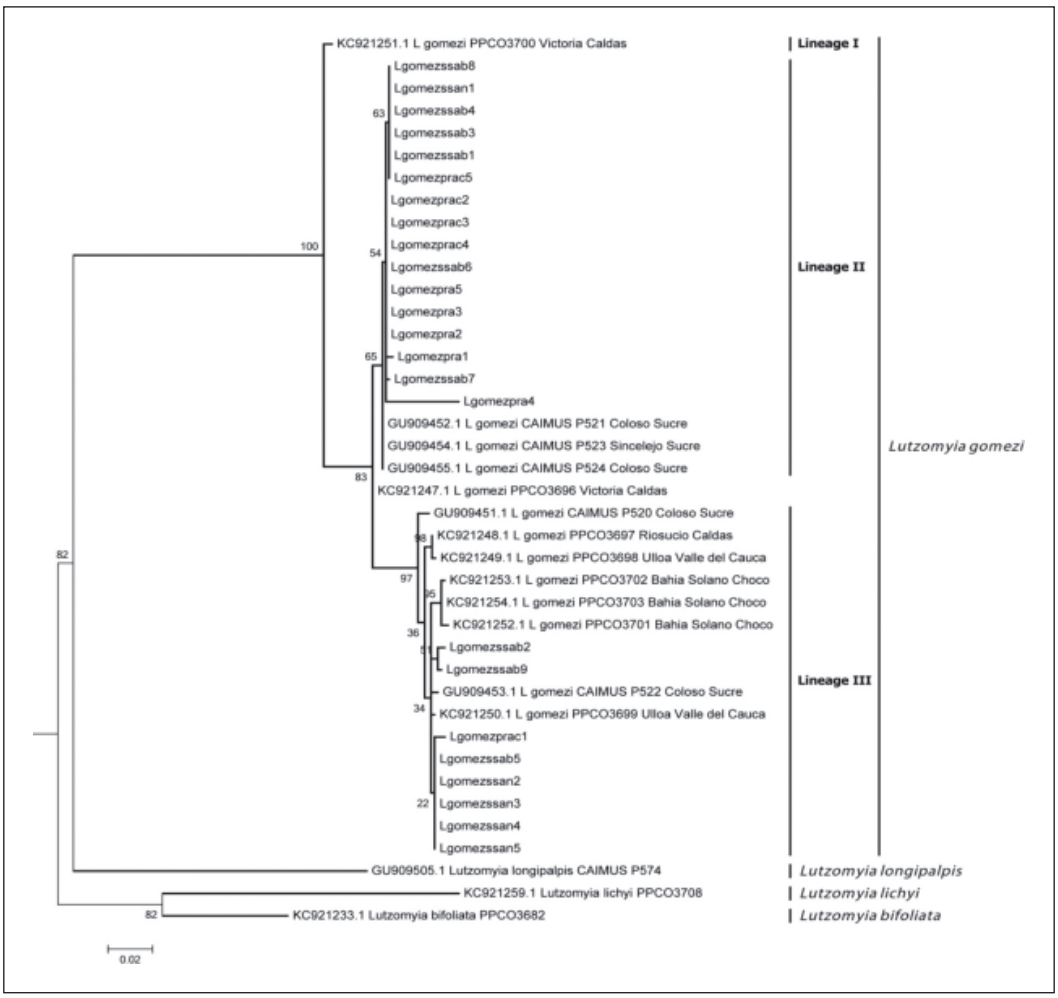

Figure 1. A phylogenetic tree estimated by the maximum likelihood analysis of 37 DNA Barcode sequences of *Lutzomyia gomezi* under the TN93+I+G model of nucleotide substitution. Three outgroups were included in the analysis (*L. longipalpis*, *L. bifoliata* and *L. lichyi*).

Table 4. Genetic distances (TN93 model) between lineages identified in phylogenetic analysis populations of *L. gomezi* registered from Colombia

	1	2	3	4	5
1. Lineage 2					
2. Lineage 3	0.026				
3. Lineage 1	0.033	0.048			
4. *L. lichyi*	0.253	0.246	0.229		
5. *L. bifoliata*	0.192	0.183	0.169	0.151	
6. *L. longipalpis*	0.209	0.204	0.209	0.198	0.186

three cryptic species for *L. gomezi* according to its extensive geographic distribution in America. Similar results were obtained by Souza-Pinto *et al.* (2015), where this species achieve the maximum upper limit intra-specific divergence by testing specimens collected in different localities from Brazil. Valderrama *et al.* (2014), used sequences of cytochrome b/start NADH1 and nuclear gene α-1 for testing phylogeographic hypotheses and genetic structures in the Panama isthmus and, from the 38 localities where *L. gomezi* was collected, found a high genetic variability, mild population structure, the presence of three clusters well-differentiate, and evidence of gene flow in close sites (7-8 kms). Our studied localities are separate by 72.2 kms explaining the values of F_{ST} estimated for Sahagún and Planeta Rica, but mild structure were founded for localities within these municipalities (Santiago abajo vs San Andresito – Arenoso vs Centro Alegre), indicating different micro-evolutionary trajectories.

This considerable polymorphism can be associated with survival and adaptability because the natural ecosystems and habitats for this species are lost by anthropogenic disturbance; in this sense, this species is tagged as "semi-domestic" due to its capacity to colonize pastures, farms, peri-urban areas (Porter & Young, 1986; Bejarano *et al.*, 2002; Lambraño *et al.*, 2012; Hoyos *et al.*, 2013, 2016), increasing in abundance from fragment forest to human houses (Duque *et al.*, 2004). Otherwise, different micro-evolutionary process (random drift, selective pressure, habitat fragmentation and geographic barriers) within populations of *L. gomezi* implied differences in competence or capacity as vector for *Leishmania* parasites, as observed for *L. longipalpis* (Rocha *et al.*, 2011). For this reason, it is relevant to estimate the real genetic divergences for *L. gomezi* as species complex, and test the phylogeographic hypothesis in its geographic range of distribution. In this way, DNA barcodes have a considerable advantage as molecular marker: the connectivity and common language of DNA sequences for different research groups work in different zones inside a range of distribution for an evaluated species (Zamora *et al.*, 2015). The easy amplification and automatic sequencing allow a very high number of DNA barcodes for representative individuals belonging to different sites and the advantage of high genetic variability for studies in genetic flow and structure populations (Hajibabaei *et al.*, 2007; Hoyos *et al.*, 2015).

The molecular identification of *L. gomezi* is a goal for studies that are focused on the ecology of transmission in zones that are endemic for cutaneous leishmaniasis. Rapid identification in human houses due to taxonomical differentiation is a main aim in zones with presence of another similar species as *Lutzomyia sherlocki*, whose male is morphologically similar to characteristics proposed for taxonomic identification. These include the width in ejaculatory ducts in comparison with basal setae tuft in gonocoxite, a slightly curved up paramere, the number of setae tufts in the gonocoxite, and the individual spermatic conducts are greater in width in *L. sherlocki* compared to *L. gomezi* (Martins *et al.*, 1972; Llanos *et al.*, 1975). However, all these morphological characteristics require a specialized knowledge in phlebotomine sand fly taxonomy, making research difficult in zones active for *Leishmania* transmission. The molecular characterization of *L. sherlocki* for testing the use of DNA barcodes in geographic areas with simpatry to *L. gomezi* as Amazonian Brazil (Young & Duncan, 1994; Galati, 2009).

DNA barcodes can contribute to research related to blood-meal sources (Human index blood), natural infection with *Leishmania* spp., identification to immature stages and metagenomics approaches; however, it is necessary to increase the barcode sequences in a wide geographic range for Colombia and other countries for assigning taxonomic status to unknown specimens with a reference library and delimitation of species within *L. gomezi* species complex.

Acknowledgments. To research program in health and tropical infections "Contract 754-2013" between Cordoba department government and Universidad del Sinú-"Elias Bechara Zainum" and Colciencias –

Convocatory 528 (Scholarship to Richard Hoyos-López). To biologist Fernando Flórez Arrieta and Rafael Bolaños by his assistance in entomological collections and taxonomic identification of specimens used in this study. To Angie Toro-Cantillo by molecular protocols used in this study.

REFERENCES

Azpurua, J., de la Cruz, D., Valderrama, A. & Windsor, D. (2010). *Lutzomyia* Sand Fly Diversity and Rates of Infection by *Wolbachia* and an Exotic *Leishmania* Species on Barro Colorado Island, Panama. *PLoS Neglected Tropical Diseases* **4**: e627.

Bejarano, E., Uribe, S., Rojas, W. & Vélez, I.D. (2002). Phlebotomine sand flies (Diptera: Psychocidae) associated with the appearance of urban leishmaniasis in the city of Sincelejo, Colombia. *Memórias do Instituto Oswaldo Cruz* **97**: 645-647.

Bejarano, E. & Estrada, L. (2016). Family Psychodidae. *Zootaxa* **4122**: 187-238.

Besansky, N., Severson, D. & Ferdig, M. (2003). DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. *Trends in Parasitology* **19**: 545-546.

Calvopina, M., Armijos, R. & Hashiguchi, Y. (2004). Epidemiology of leishmaniasis in Ecuador: current status of knowledge – A review, *Memória do Instituto Oswaldo Cruz* **99**: 663-672.

Christensen, H.A., Herrer, A. & Telford, S.R. (1983). Enzootic cutaneous leishmaniasis in eastern Panama. II. Entomological investigations. *Annals of Tropical Medicine and Parasitology* **66**: 55-66.

Contreras-Gutierrez, M., Vivero, R., Vélez, I.D., Porter, C.H. & Uribe, S. (2014). DNA barcoding for the identification of sand fly species (Diptera, Psychodidae, Phlebotominae) in Colombia. *PLoS One* **15**: e85496.

Cortés, L.A. & Fernández, J.J. (2008). Especies de *Lutzomyia* en un foco urbano de leishmaniasis visceral y cutaìnea en El Carmen de Bolìvar, Bolìvar, Colombia. *Biomédica* **28**: 433-440.

Duque, P., Vélez, I., Morales, M. & Sierra, D. (2004). Sand flies fauna involved in the transmission of cutaneous leishmaniasis in Afro-Colombian and Amerindian communities of Chocó, Pacific Coast of Colombia. *Neotropical Entomology* **33**: 263-264.

Feliciangeli, M., Rodríguez, N., Bravo, A., Arias, F. & Guzman, B. (1994). Vectors of cutaneous leishmaniasis in North-Central Venezuela. *Medical and Veterinary Entomology* **8**: 317-324.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.

Folmer, O., Blac, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294-299.

Galati, E. (2009). Bioecologia e Identificacäo de Phlebotominae: Classificacäo, Morfologia e Terminologia e Identificacäo de Adultos. *Vol I-II*. Universidade de Säo Paulo, Säo Paulo.

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696-704.

Hajibabaei, M., Singer, G., Hebert, P. & Hickey, D. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics* **23**: 167-172.

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Hebert, P.D., Cywinska, A., Ball, S.L. & De Waard, J.R. (2003). Biological identifications through DNA barcodes. *Proceedings Biological Sciences* **270**: 313-321.

Hoyos, R., Velez, I. & Uribe, S. (2012). Typification of Colombian specimens of *Lutzomyia longipalpis* (Diptera: Psychodidae) by "Barcoding". *Revista Colombiana de Entomología* **38**: 134-140.

Hoyos, R., Vivero, R., Contreras, M. & Uribe, S. (2013). Phlebotomines sandflies (Diptera: Psychodidae) on a rural area from Santa Fe de Antioquia, Colombia. *Revista Colombiana de Entomologia* **39**: 51-55.

Hoyos-López, R., Roman, S., Castaño, J.C. & Gallego-Gómez, J.C. (2015). DNA barcode for typing of immature mosquitoes from Armenia and Circasia (Quindío, Colombia). *Revista Colombiana de Entomologia* **41**: 218-227.

Hoyos-López, R., Bolaños, R., Contreras-Gutierrez, M. & Carrero-Sarmiento, D. (2016). Phlebotomine sandflies (Diptera: Psychodidae) in a sub-Andean forest from the Norte de Santander, Colombia. *Journal of Vector Borne Diseases* **53(1)**: 70-76.

Jaramillo, C., Travi, B. & Montoya, J. (1994). Vector competence of some Neotropical sandflies for the *Leishmania* (*Viannia*) *braziliensis* complex. *Memória do Instituto Oswaldo Cruz* **8**: 1-7.

Johnson, P., McConnell, E. & Hertig, M. (1963). Natural infections of leptomonad flagellates in Panamian phlebotomus sandflies. *Experimental Parasitology* **14**: 107-122.

Lambraño, L., Manjarrez, G. & Bejarano, E. (2012). Temporal variation of Lutzomyia sand flies (Diptera: Psychodidae) in the urban area of Sincelejo (Colombia). *Salud Uninorte*, **28**: 191-200.

Larkin, M., Blackshields, G., Brown, NP., Chen-Na, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A. & Lopez, R. (2007). Clustal W and CLUSTALX version 2.0. *Bioinformatics* **23**: 2947-2948.

Leite, L. (2012). Mitochondrial pseudogenes in insect DNA barcoding: differing points of view on the same issue. *Biota Neotropica* **12**: 301-308.

Librado, P. & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451-1452.

Llanos, B.Z., Martins, A.V. & Da Silva, J. (1975). Estudos sobre os flebotomíneos do Peru (Diptera, Psychodidae, Phlebotominae) 1. Departamento de Cuzco; 2. Descriçao das fèmeas de *Lutzomyia campbelli* e *Lutzomyia sherlocki* e redescriçao do macho e descriçao de fêmea de *Lutzomyia octavioi*. *Revista Brasileira De Biologia* **35**: 655-664.

Martínez, L., Rebollo, J., Luna, A., Cochero, S. & Bejarano, E. (2010). Molecular identification of the parasites causing cutaneous leishmaniasis on the Caribbean coast of Colombia. *Parasitology Research* **106**: 647-652.

Martins, A.V., Silva, E. & Falçao, A.L. (1971). *Lutzomyia sherlocki* sp. n., do Estado de Mato Grosso, Brasil (Diptera, Psychodidae). *Revista Brasileira De Biologia* **31**: 415-418.

Morales, A., Corredor, A., Cáceres, E., Ibagos, A. & Rodríguez, C. (1981). Aislamiento de tres cepas de *Leishmania* a partir de *Lutzomyia trapidoi* en Colombia. *Biomédica* **4**: 37-41.

Nitzulescu, V. (1931). Sur un Phlebotome nouveau du Venezuela. P. gomezi n. sp. *Annales de Parasitologie Humaine et Comparee* **9**: 247-255.

Parra, G.J. & Echavarría, J.C. (2005). Experiencias en el control de un foco de leishmaniasis cutanéa en San Carlos, Antioquia. *CES Medicina* **19**: 31-36.

Paternina, L., Verbel-Vergara, D., Romero-Ricardo, L., Perez-Doria, A., Paternina-Gómez, M., Martínez, L. & Bejarano, E.E. (2016). Evidence for anthropophily in five species of phlebotomine sand flies (Diptera: Psychodidae) from northern Colombia, revealed by molecular identification of bloodmeals. *Acta Tropica* **153**: 86-92.

Porter, C.H. & Young, D. (1986). A new species of phlebotomine sandfly (Diptera: Psychodidae) from Guatemala. *Journal of Medical Entomology* **23**: 236-243.

Rocha, L., Falqueto, A., Dos Santos, C., Grimaldi, G. & Cupolillo, E. (2011). Possible implication of the genetic composition of the *Lutzomyia longipalpis* (Diptera: Psychodidae) populations in the epidemiology of the visceral leishmaniasis. *Journal of Medical Entomology* **48**: 1016-1022.

Rodríguez, N., Aguilar, C., Barrios, M. & Barker, D. (1999). Detection of *Leishmania braziliensis* in naturally infected sandflies by the polymerase chain reaction. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **93**: 47-49.

Romero-Ricardo, L., Lastre-Meza, N., Perez-Doria, A. & Bejarano, E. (2016). DNA barcoding to identify species of phlebotomine sand fly (Diptera: Psychodidae) in the mixed leishmaniasis focus of the Colombian Caribbean. *Acta Tropica* **159**: 125-131.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406-425.

Sandoval, C., Angulo, V., Gutiérrez, R., Muñoz, G. & Ferro, C. (1998). Especies de *Lutzomyia* (Diptera: Psychodidae) posibles vectores de leishmaniasis en la ciudad de Bucaramanga, Santander, Colombia. *Biomédica* **18**: 161-168.

Santamaría, E., Ponce, N., Zipa, Y. & Ferro, C. (2006). Presencia en el peridomicilio de vectores infectados con *Leishmania* (*Viannia*) *panamensis* en dos focos endémicos en el occidente de Boyacá, piedemonte del valle del Magdalena medio, Colombia. *Biomédica*, **26**: 82-94.

Souza-Pinto, I., Das Chagas, B.D., Rodrigues, A., Ferreira, A.L., Rezende, H.R., Bruno, R.V. & Brazil, R.P. (2015). DNA barcoding of neotropical sand flies (Diptera, Psychodidae, Phlebotominae): Species identification and discovery within Brazil. *PLoS One* **10**: e0140636.

Talaga, S., Leroy, C., Guidez, A., Dusfour, I., Girod, R., Dejean, A. & Murienne, J. (2017). DNA reference libraries of French Guianese mosquitoes for barcoding and metabarcoding. *PLos One* **12**: e0176993.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725-2729.

Toro-Cantillo, A., Atencia-Pineda, M. & Hoyos-López, R. (2017). Phlebotomine sand flies (Diptera: Psychodidae) collected in rural area from San Bernardo del Viento (Córdoba, Colombia). *Revista MVZ Córdoba* **22**(Supl-1**)**: 6044-6049.

Travi, B., Montoya, J., Solarte, Y., Lozano, L. & Jaramillo, C. (1988). Leishmaniasis in Colombia. I. Studies on the phlebotomine fauna associated with endemic foci in the Pacific Coast region. *The American Journal of Tropical Medicine and Hygiene* **39**: 261-266.

Travi, B., Adler, G., Lozano, L., Cadena, H. & Montoya-Lerma, J. (2002). Impact of habitat degradation on phlebotominae (Diptera: Psychodidae) of tropical dry forests in northern Colombia. *Journal of Medical Entomology* **39**: 451-456.

Valderrama, A., Tavares, M. & Andrade, J. (2014). Phylogeography of the *Lutzomyia gomezi* (Diptera: Phlebotominae) on the Panama Isthmus. *Parasites & Vectors* **7**: 1-11.

Vivero, R., Bejarano, E., Estrada, L., Florez, F., Ortega-Gómez, E., Aparicio, Y., Torres-Gutierrez, C., Uribe-Soto, S. & Muskus-López, C. (2017). DNA barcode for identification of immature stages of sand flies (Diptera: Psychodidae) collected from natural breeding sites. *Zootaxa* **4277**: 228-236.

Vivero, R., Quintero, L., Peña, H.C., Alvar-Beltran, J., Tovar, C., Atencia, C.M., Vélez, I.D. (2017). Composition and distribution of medically important phlebotomines (Diptera: Psychodidae) in the municipalities of Tierralta and Valencia (Córdoba, Colombia). *Journal of Vector Borne Diseases* **54**: 87-95.

Walters, L., Chaplin, G., Modi, G. & Tesh, R. (1989). Ultrastructural biology of Leishmania (Viannia) panamensis (=*Leishmania braziliensis panamensis*) in Lutzomyia gomezi (Diptera: Psychodidae): a natural host-parasite association. *The American Journal of Tropical Medicine and Hygiene* **40**: 19-39.

Young, D., Morales, A., Kreutzer, D., Alexander, B., Corredor, A. & Tesh, R. (1987). Isolation of *Leishmania braziliensis* (Kinetoplastida: Trypanosomatidae) from cryopreserved Colombian sandflies (Diptera: Psychodidae). *Journal of Medical Entomology* **24**: 587-589.

Young, D. & Duncan, M. (1994). Guide to the identification and geographic distribution of *Lutzomyia* sand flies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). *Memoirs of the American Entomological Institute* **54**: 1-881.

Zamora-Delgado, J., Castaño, J.C. & Hoyos-López, R. (2015). DNA barcode sequences used to identify *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) in La Tebaida (Quindío, Colombia). *Revista Colombiana de Entomologia* **41**: 212-217.