

Molecular Evidence Confirms the Taxonomic Separation of *Lutzomyia tihuiensis* from *Lutzomyia pia* (Diptera: Psychodidae) and the Usefulness of Pleural Pigmentation Patterns in Species Identification

ALVEIRO PÉREZ-DORIA,¹ EDUAR ELÍAS BEJARANO,^{1,2} DIANA SIERRA,³ AND IVÁN DARÍO VÉLEZ⁴

J. Med. Entomol. 45(4): 653–659 (2008)

ABSTRACT The phlebotomine sand flies *Lutzomyia pia* (Fairchild & Hertig 1961) and *Lutzomyia tihuiensis* Le Pont, Torrez-Espejo & Dujardin 1997 (Diptera: Psychodidae) belong to the *pia* series of the *Lu. verrucarum* species group, which includes several species that bite humans in Andean foci of leishmaniasis. The females of these two species exhibit isometry and isomorphism in anatomical structures of the head and terminalia commonly used in taxonomic identification of sand flies. They can only be differentiated based on subtle differences in the pigmentation of the pleura. In *Lu. tihuiensis*, this is restricted to the basal portions of the katopimeron and katopisternum, whereas in *Lu. pia* both structures are totally pigmented. Taking into account the subtle morphological differences between these species, the objective of the current study was to evaluate the specific taxonomic status of *Lu. tihuiensis* with respect to *Lu. pia*. A 475-bp portion of the mitochondrial genome was sequenced, composed of the 3' end of the cytochrome *b* gene, intergenic spacer 1, the transfer RNA gene for serine, intergenic spacer 2, and the 3' end of the gene NAD dehydrogenase 1. Genetic analysis confirms that *Lu. tihuiensis* and *Lu. pia* constitute two distinct species and this is supported by four strong lines of evidence, i.e., the paired genetic distances, size differences and amino acid composition of the cytochrome *b* protein, presence and absence of intergenic spacer one and divergence observed in the sequence of the transfer RNA gene for serine. It also confirms the validity of the pleural pigmentation pattern as a species diagnostic character and the importance of performing a detailed examination of this character during morphological determination of phlebotomine sand flies in the series *pia*.

KEY WORDS sand flies, *Lutzomyia verrucarum* group, *Lutzomyia pia*, *Lutzomyia tihuiensis*, cytochrome *b*

The series *pia* Galati, 1995, of the *Lutzomyia verrucarum* group Theodor, 1965, consists of seven phlebotomine species (Diptera: Psychodidae) whose females exhibit sac-like, striated spermathecae with a smooth apical ring with almost straight borders. The males have five spines arranged on the distal half of the style (Bejarano et al. 2004). These species are *Lutzomyia pia* (Fairchild & Hertig 1961); *Lutzomyia reclusa* Fernandez & Rogers, 1991; *Lutzomyia suapiensis* Le Pont, Torrez-Espejo & Dujardin 1997; *Lutzomyia tihuiensis* Le Pont, Torrez-Espejo & Dujardin 1997; *Lutzomyia tocaniensis* Le Pont, Torrez-Espejo & Dujardin, 1997; *Lutzomyia limafalcaoae* (Wolff & Galati 2002); and

Lutzomyia emberai Bejarano, Duque & Velez, 2004. In the taxonomic scheme proposed by Galati (2003a), these species are grouped within the series *pia* of the subgenus *Pifanomyia* Ortiz & Scorza, 1963 in the genus *Pintomyia* Costa Lima, 1932.

The geographic distribution of the series *pia* is restricted to Colombia, Venezuela, Peru, and Bolivia, except for *Lu. pia* whose range extends northwards to Panama and Costa Rica. Throughout these countries, some species of the series are often collected biting humans (Zeledón et al. 1985, Alexander et al. 1992, Le Pont et al. 1997, Cáceres et al. 2001, Bejarano et al. 2006). Most important of these is *Lu. pia*, which is an opportunistic human-biter that is also susceptible to infection by *Leishmania braziliensis* (Vianna 1911) Matta, 1916, the etiological agent of cutaneous and mucocutaneous leishmaniases (Warburg et al. 1991).

To date, four species of the series *pia* have been recorded from Colombia: *Lu. pia*, *Lu. limafalcaoae*, *Lu. emberai*, and *Lu. tihuiensis*. The last of these species was recorded from specimens collected in the Valle de Aburrá (Bejarano et al. 2006), department of Antio-

¹ Grupo de Investigaciones Biomédicas, Universidad de Sucre, Cra. 14 No. 16-B-32, Apartado Aéreo 406, Sincelejo, Colombia.

² Corresponding author: e-mail, eduardelias@yahoo.com.

³ Department of Microbiology, Immunology and Pathology, Colorado State University, Central receiving 200 W. Lake Street Campus Delivery 1619, CO 80523.

⁴ Programa de Estudio y Control de Enfermedades Tropicales, Universidad de Antioquia, Calle 62 No. 52-59, Apartado Aéreo 1226, Medellín, Colombia.

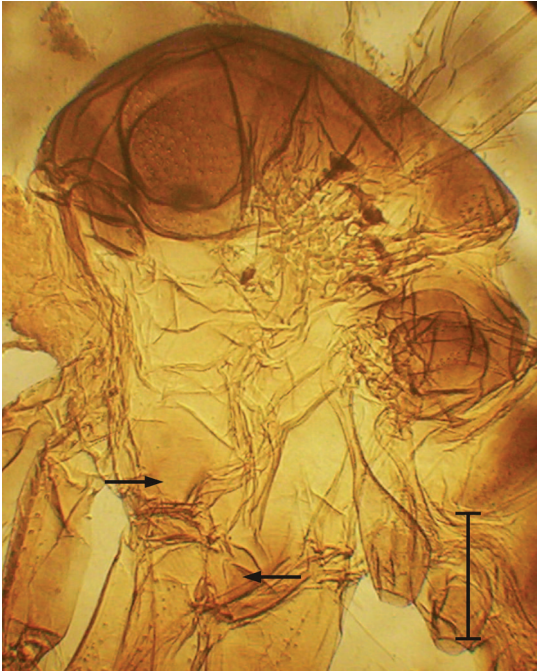


Fig. 1. Thorax of the sand fly *Lu. tihuilienis* with the base of the katapisternum (left arrow) and katepimeron (right arrow) partially pigmented. Scale line = 200 μm .

quia, where it had originally been identified as *Lu. pia* (Agudelo et al. 2002), due to its close morphological similarity. It is important to emphasize that the females of *Lu. pia* and *Lu. tihuilienis* exhibit isometry and isomorphism in anatomical structures of the head and terminalia, used frequently as diagnostic characters for phlebotomine species. Their identification is only possible by the subtle differences in the pigmentation of the pleura, which in *Lu. tihuilienis* is restricted to the basal portion of the katepimeron and katapisternum, whereas in *Lu. pia* both structures are totally pigmented (Fig. 1).

To determine whether the morphological differences between *Lu. pia* and *Lu. tihuilienis* have a genetic basis, genetic variation between these taxa was estimated by molecular characterization of a portion of the mitochondrial genome. The mitochondrial genes most used to date in studies of the genus *Lutzomyia* are cytochrome *b*, cytochrome oxidase I, NAD dehydrogenase 4, and the small ribosomal subunit (Ready et al. 1997; Suguri et al. 1997; Uribe et al. 1998; 2001; Ishikawa et al. 1999; Bejarano 2001; Rojas 2001; Arrivillaga et al. 2002; Testa et al. 2002; Beati et al. 2004). These molecular markers have proved to be useful for analysis of both interspecific and intraspecific genetic variability, including separation of morphologically similar species. The genomic region selected for the current study extends from the 3' end of the cytochrome *b* gene to the 3' end of NAD dehydrogenase subunit one gene. Nucleotide sequence analysis confirmed the specific status of *Lu.*

tihuilienis and the validity of pleural pigmentation as a diagnostic character for species of the series *pia*.

Materials and Methods

Collection of Phlebotomines. Specimens of *Lu. pia* were collected in the municipality of Marsella (04° 55' N, 05° 58' W), in the Colombian department of Risaralda, using two CDC light traps operated from 1800 to 0600 hours and a Shannon trap operated from 1800 to 2200 hours. Specimens of *Lu. tihuilienis* were collected in a secondary forest in Vereda El Vallano, municipality of Envigado (06° 08' N, 75° 34' W) in the neighboring department of Antioquia. The latter species could only be captured by human-landing catches made from 1800 to 2200 h. Specimens were transported dry to the PECET Laboratory of the Universidad de Antioquia and stored at -4°C .

Taxonomic Determination. Sand flies were examined under the stereomicroscope and dissected by separating the head and the final segments of the abdomen, which include the female genitalia. These structures were cleared in lactophenol (1:1 lactic acid: phenol) for 24 h, and then they were mounted on slides in Hoyer's medium. Species determination was performed using the keys and descriptions of Young and Duncan (1994), Wolff and Galati (2002), Galati (2003b), and Bejarano et al. (2004), based principally on the length of the labro-epipharynx and second palpomere, ratio of the lengths of the common and individual sperm ducts, and the pleural pigmentation pattern.

Extraction, Amplification and Sequencing of DNA. Total DNA was extracted from the thorax and proximal segments of the abdomen by using the protocol described by Collins et al. (1987) with the following modifications: maceration in 60 μl of lysis buffer (0.08 NaCl, 0.16 M saccharose, 0.06 M EDTA, 0.5% SDS, and 0.1 M Tris-HCl, pH 7.5), precipitation with 200 μl of absolute ethanol, and resuspension in 30 μl of sterile water. Amplification was performed by polymerase chain reaction (PCR), by 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)TTCC(T/A)TATGA-3' (Ready et al. 1997). These primers amplify a region composed of the 3' end of the cytochrome *b* gene (Cyt *b*), intergenic spacer 1 (IG1), transfer RNA gene for serine (UCN) (tRNA^{Ser}), intergenic spacer 2 (IG2), and the 3' end of the NAD dehydrogenase 1 gene (NAD1). PCR was carried out in 50 μl of reaction mix containing PCR 1 \times buffer (Promega, Madison, WI), 1.5 mM MgCl₂, 0.2 mM of a mixture of deoxyribonucleotides (Promega), 0.3 μM of each primer, 1.5 U of *Taq* DNA polymerase (Promega), and 6 μl of DNA solution.

A three-stage thermic amplification profile was used consisting of 1) denaturation at 94°C for 3 min; 2) 35 cycles of denaturation for 1 min at 90°C, annealing for 1 min at 50°C and extension at 72°C for 1 min; and 3) a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis in 1.5% agarose gel. They were stained with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide

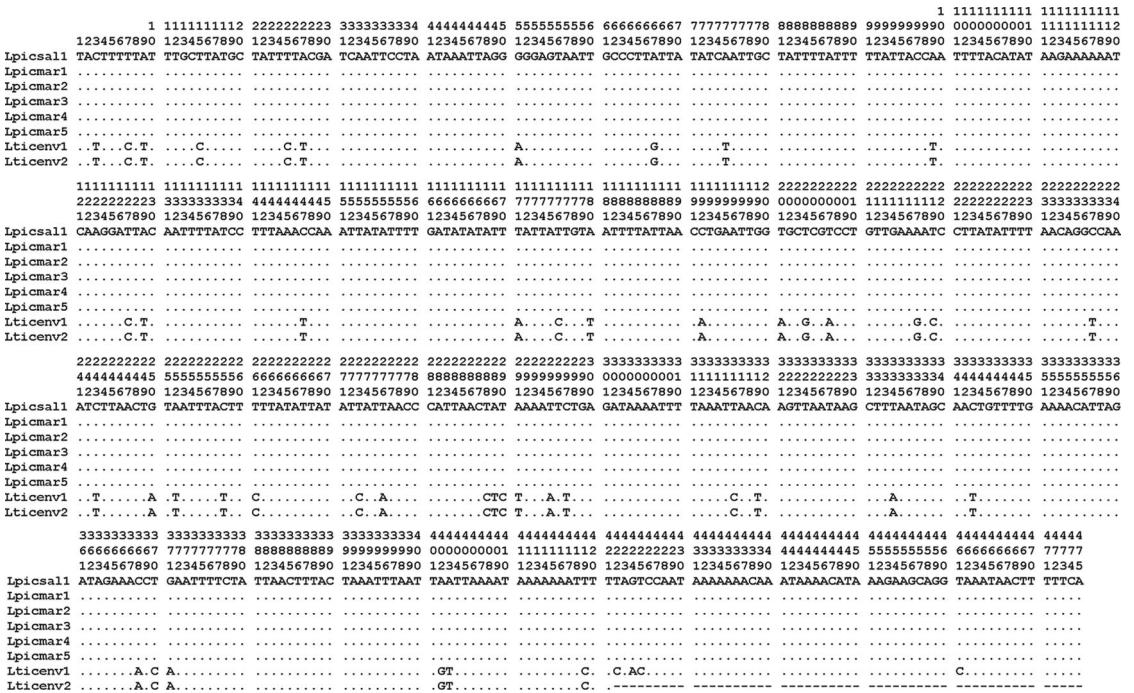


Fig. 2. Multiple alignment of the nucleotide sequences of the 3' end of the cytochrome *b* gene, intergenic spacer 1, the transfer RNA gene for serine (UCN), intergenic spacer 2, and the 3' end of the gene NAD dehydrogenase derived from adult females of *Lu. tihuiensis* (isolates Lticenv1 and Lticenv2) and *Lu. pia* (isolates Lpicsall, Lpicmar1, Lpicmar2, Lpicmar3, Lpicmar4, and Lpicmar5). Dots denote homology, dashes denote missing data, and letters denote the respective nucleotide.

and visualized in a UV light transilluminator. They were then purified using a Wizard PCR Preps DNA Purification System kit (Promega). Nucleotide sequences of the purification products were determined on an ABI Prism 3730xl electrophoresis capillary automatic sequencer (Applied Biosystems, Foster City, CA) using fluorescent chain terminators (Big Dye Terminator Cycle Sequencing Ready Reaction kit, Applied Biosystems).

Sequence Analysis. Nucleotide sequences were deposited into GenBank with accession numbers EF033642–EF033648. Using the nucleotide-nucleotide search option of the BLAST program available on-line (Altschul et al. 1990) the position of the new sequences was determined with respect to the mitochondrial genome of *Anopheles gambiae* Giles (Beard et al. 1993). Multiple alignments of the sequences were carried out using the Clustal W program (Thompson et al. 1994) integrated with DAMBE 4.2.13 (Xia and Xie 2001). Nucleotide sequences were translated to amino acids on the MEGA 3.1 program (Kumar et al. 2004), which also was used to determine the polymorphic sites and paired genetic distances (p) between the two species. The distances between *Lu. pia* and *Lu. tihuiensis* were then compared with a reference standard constituted by intra- and interspecific genetic distances for *Lu. verrucarum* group species (Bejarano 2001, Rojas 2001). A *Lu. pia* homologous sequence (GenBank accession no. AF403486) also was included in the analysis, from a specimen

collected in Salamina (5° 24' N, 75° 28' W) in the Colombian department of Caldas (Rojas 2001). The secondary structure of the tRNA^{Ser} was inferred using the tRNAscan-SE 1.21 program (Lowe and Eddy 1997), and secondary structure diagrams were manually edited.

Results

Fourteen females belonging to the genus *Lutzomyia* were identified during the study (11 *Lu. pia* and three *Lu. tihuiensis*), from which seven 475-bp nucleotide sequences were obtained of the genes Cyt *b*, IG1, tRNA^{Ser}, IG2, and NAD1. The first and last nucleotides of these sequences are homologous to no. 11232 and 12575, respectively, of the Cyt *b* and NAD1 gene of the *An. gambiae* mitochondrial genome (Genbank accession no. NC 002084). A single nucleotide haplotype was found to be shared by five *Lu. pia* specimens from Marsella, Risaralda, which was identical to isolate Lpicsall (accession no. AF403486) from Salamina, Caldas, deposited in GenBank by Rojas (2001). Thus, a single haplotype for the two *Lu. tihuiensis* specimens sequenced during the study was obtained, the first known for this species. The nucleotide alignments of all the sequences analyzed are shown in Fig. 2.

Each haplotype is 475 bp, except for a single *Lu. tihuiensis* 421-bp sequence. The haplotypes isolated are conformed by a partial sequence of the 3' end of the Cyt *b* gene, which in the alignment (Fig. 2) ex-

	1	1111111112	2222222223	3333333334	4444444445	5555555556
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
Lticenv1	FLFAYAILRS	IPNKLGGVIA	LVMSIAILFL	LPILHMSKNQ	GLQFYPLNQI	MFWYMLITVI
Lticenv2
Lpicmar1I.....	F.I..
Lpicmar2I.....	F.I..
Lpicmar3I.....	F.I..
Lpicmar4I.....	F.I..
LpicsallI.....	F.I..
				1	111111	
	6666666667	7777777778	8888888889	9999999990	000000	
	1234567890	1234567890	1234567890	1234567890	1234567890	
Lticenv1	LLTWIGARPV	EDPYILTQQI	LTIIYFLYYI	MNPLTSKIWD	KILNQ*	
Lticenv2	
Lpicmar1N.....	.V.....	I...M.F..	*-	
Lpicmar2N.....	.V.....	I...M.F..	*-	
Lpicmar3N.....	.V.....	I...M.F..	*-	
Lpicmar4N.....	.V.....	I...M.F..	*-	
LpicsallN.....	.V.....	I...M.F..	*-	

Fig. 3. Multiple alignment of the partial amino acid sequence of the cytochrome *b* protein derived from adult females of *Lu. tihuiensis* and *Lu. pia*. Dots indicate homology, asterisks indicate stop signals, dashes indicate insertion-deletion events, and letters indicate the respective amino acid. All isolate names correspond to those in Fig. 2.

tends from nucleotides 1 to 318 in *Lu. pia* and from 1 to 321 in *Lu. tihuiensis*. This demonstrates an important difference between *Lu. pia* and *Lu. tihuiensis* with respect to the size of the Cyt *b* protein, conferring on the latter species the ability to code for an additional amino acid on the carboxyl-terminal end (Fig. 3). This is due to the substitution of the first nucleotide of the stop codon, situated at position 316, which corresponds to thymine in *Lu. pia* and cytosine in *Lu. tihuiensis*. In the latter species, this substitution changes the TAA stop codon (UAA for messenger RNA) to the CAA codon that codes for glutamine.

A noncoding DNA sequence was located on IG1 that is only present in *Lu. pia*, apparently conformed by two nucleotides (cytosine and adenine) at positions 319 and 320 of the multiple alignment (Fig. 2). It is important to emphasize that *Lu. tihuiensis* lacks IG1, another important difference between the two species.

The tRNA^{Ser} gene lies between positions 321 and 387 of the alignment (Fig. 2), consisting of 67 nucleotides in both species. In *Lu. pia* the nucleotides of this gene are for exclusive coding, whereas in *Lu. tihuiensis* the gene shares its primer nucleotide with the third position of the Cyt *b* stop codon. This suggests that there is an overlap between the Cyt *b* and tRNA^{Ser} genes in *Lu. tihuiensis*. Furthermore, five divergent sites were found in the nucleotide constitution of the gene tRNA^{Ser} gene, at positions 334, 343, 368, 370, and 371 of the alignment. When the secondary structures (Fig. 4) inferred for this molecule were compared, it was observed that the anticodon arm contains a non-Watson-Crick U-U base pair in *Lu. tihuiensis*, and a non-Watson-Crick C-U base pair in *Lu. pia*. All this demonstrates the existence of type-specific tRNA^{Ser} molecules in *Lu. tihuiensis* and *Lu. pia*.

Adjacent to this gene is IG2, a noncoding DNA sequence that extends from positions 388–403 of the alignment (Fig. 2). IG2 has a constant length of 16 nucleotides in the two species, although it exhibits some differences in the nucleotide constitution. Finally, a partial sequence of the 3' end of the NAD1

gene is presented between positions 404 and 475 of the mitochondrial fragment (Fig. 2), which was not included in the analysis because of its size and the lack of information for one of the *Lu. tihuiensis* specimens.

Multiple alignment revealed the presence of 38 nucleotide substitutions between *Lu. pia* and *Lu. tihuiensis* at the 3' end of the Cyt *b* gene. When the amino acid sequence coded by this gene was inferred, the most important changes were found to occur at the first position of the codon, provoking nine nonsynonymous substitutions on the amino acid sequence (Fig. 3). The matrix of paired genetic distances (*p*) was calculated from a 318-bp fragment of Cyt *b*, homologous with the mitochondrial region sequenced in other species of the *Lu. verrucarum* group (Bejarano 2001, Rojas 2001). The results provide a value of 0.1164 between *Lu. tihuiensis* and *Lu. pia*.

Discussion

The phlebotomine sand flies *Lu. pia* and *Lu. tihuiensis* belong to the *pia* series of the *Lu. verrucarum* species group, which includes several species that bite humans in Andean foci of leishmaniasis. Although both species are anthropophilic, small differences in behavior have been observed in Colombia. Warburg et al. (1991) found that *L. pia* in Valle del Cauca did not attempt to bite immediately after settling, whereas Zuleta (2002) observed that in Valle de Aburrá *L. tihuiensis* began to bite as soon as it landed on human skin (Bejarano et al. 2004). These subtle bionomic differences could favor the vectorial role of *Lu. tihuiensis* over *Lu. pia*, and subsequently make it necessary to distinguish them.

The results of the current study confirm *Lu. pia* and *Lu. tihuiensis* as being two distinct species, demonstrating the validity of the pleural pigmentation pattern as a species diagnostic character. The specific status of *Lu. tihuiensis* is supported by four strong lines of evidence, including 1) paired genetic distances, 2) differences in size and amino acid compo-

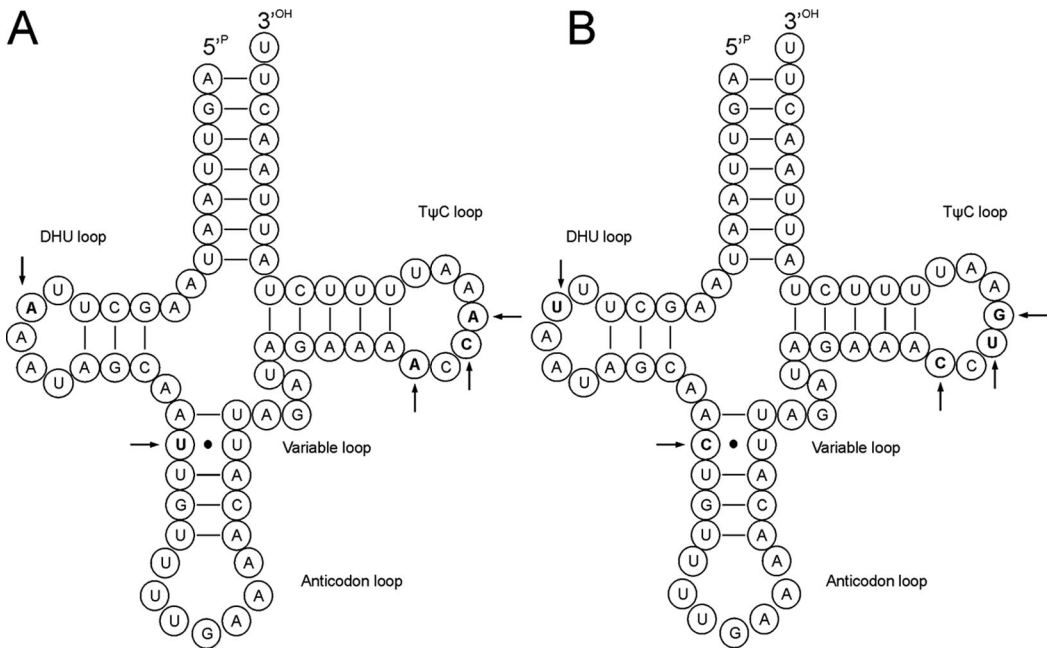


Fig. 4. Predicted secondary structure for the mitochondrial serine transfer RNA (UCN) from the sand flies (A) *Lu. tihuihensis* and (B) *Lu. pia*. Dashes and dots indicate Watson–Crick base pairing and non-Watson–Crick base pairing, respectively. Polymorphic sites are marked with bold letters and arrows.

sition of the protein cytochrome *b*, 3) absence of IG1, and 4) divergence exhibited by the sequence of the tRNA^{Ser} gene.

The paired genetic distance between *Lu. pia* and *Lu. tihuihensis* (0.1164) lies within the range of interspecific distances for the *Lu. verrucarum* group species, e.g., that between *Lutzomyia evansi* (Nunez-Tovar, 1924) and *Lutzomyia ovallesi* (Ortiz 1952) (0.1195–0.1384). It greatly exceeds the genetic distances between other morphologically similar species, such as *Lutzomyia spinicrassa* Morales, Osorno, Osorno & Munoz, 1969, and *Lutzomyia longiflososa* Osorno, Morales, Osorno & Munoz, 1970 (0.0629–0.0660) (Rojas 2001), and it is much higher than the intraspecific distances seen for this group. For example, the values between *Lu. evansi* populations from Colombia and Venezuela vary from 0.0252 to 0.0377 (Bejarano 2001).

The second line of evidence to support the specific status of *Lu. tihuihensis* derives from the amino acid sequence inferred for the Cyt *b* gene. The protein product of this gene in *Lu. tihuihensis* has an additional amino acid at the 3' end. This type of difference does not occur within a single species but it has been found between distinct species, e.g., *Lutzomyia columbiana* (Ristorcelli & Van Ty, 1941) and *Lu. ovallesi* (Rojas 2001). The latter presents a glutamine at the carboxyl-terminal end of the Cyt *b* gene, resulting in a larger protein product. Such differences in the size of this protein may reflect an important evolutionary divergence between species (Bejarano 2001). In addition, nine divergent amino acid sites were observed between *Lu. pia* and *Lu. tihuihensis*, explaining the differences between protein products coded by the Cyt

b gene of these species. These results contrast with the 38 substitutions found at the nucleotide level, denoting a high rate of neutral mutation at the third position of the different codons, as expected for genomic coding regions (Futuyma 1998).

The presence and absence of IG1 in *Lu. pia* and *Lu. tihuihensis*, respectively, is another important difference between the two species. The IG1 is a noncoding DNA sequence, which ranges in size from 0 to 10 bp within the genus *Lutzomyia* (Bejarano 2001, Rojas 2001). Some studies on comparative genomics have shown that length variations of intergenic spacers are one of the causes of differences in size of the mitochondrial genome of insects. Moreover, it is well known that a drastic mitochondrial genome size reduction has occurred through the evolutionary history.

The fourth line of evidence corresponds to the five divergent sites presented by the nucleotide sequence of the tRNA^{Ser} gene and differences in the configuration of the secondary structure inferred from this molecule, including the non-Watson–Crick base pairs of the anticodon arm. Although this gene tends to be highly conserved in *Lutzomyia*, apparently exhibiting only interspecific differences (Rojas 2001, Vivero et al. 2007), five polymorphic sites were found in the DHU and TψC loops, and the anticodon stem. Given that the mutational dynamics and evolutionary rate of the tRNA^{Ser} gene are low in phlebotomines, these differences may reflect a marked evolutionary divergence between the species.

The genetic evidence derived from the current study supports the separation of *Lu. tihuihensis* and *Lu. pia*,

confirming the validity of the pleural pigmentation pattern as a diagnostic character that should be examined carefully during taxonomic determination of these species. Finally, it confirms the usefulness of mitochondrial genes as molecular tools in differentiating morphologically similar species of the genus *Lutzomyia*.

Acknowledgments

We thank Boris René Zuleta for help with fieldwork in Antioquia. This study was financed by the Fundación para la Promoción de la Investigación y la Tecnología of the Banco de la República, Colombia (code 1606).

References Cited

- Agudelo, L. A., J. Uribe, D. Sierra, F. Ruiz, and I. D. Vélez. 2002. Presence of American cutaneous leishmaniasis vectors surrounding the city of Medellín, Colombia. *Mem. Inst. Oswaldo Cruz* 97: 641–642.
- Alexander, B., C. Ferro, D. G. Young, A. Morales, and R. B. Tesh. 1992. Ecology of phlebotomine sand flies (Diptera: Psychodidae) in a focus of *Leishmania (Vianina) braziliensis* in northeastern Colombia. *Mem. Inst. Oswaldo Cruz* 87: 387–395.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215: 403–410.
- Arrivillaga, J. C., D. E. Norris, M. D. Feliciangeli, and G. C. Lanzaro. 2002. Phylogeography of the Neotropical sand fly *Lutzomyia longipalpis* inferred from mitochondrial DNA sequences. *Infect. Genet. Evol.* 2: 83–95.
- Beard, C. B., D. M. Hamm, and F. H. Collins. 1993. The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. *Insect Mol. Biol.* 2: 103–124.
- Beati, L., A. G. Cáceres, J. A. Lee, and L. E. Munstermann. 2004. Systematic relationships among *Lutzomyia* sand flies (Diptera: Psychodidae) of Peru and Colombia based on the analysis of 12S and 28S ribosomal DNA sequences. *Int. J. Parasitol.* 34: 225–234.
- Bejarano, E. E. 2001. Variabilidad genética y especiación en *Lutzomyia (verrucarum) evansi* (Nuñez-Tovar, 1924), vector de leishmaniosis visceral americana. M. Sc. thesis, Universidad de Antioquia, Medellín, Colombia.
- Bejarano, E. E., P. Duque, and I. D. Vélez. 2004. Taxonomy and distribution of the series *pia* of the *Lutzomyia verrucarum* group (Diptera: Psychodidae), with a description of *Lutzomyia emberai* n. sp. *J. Med. Entomol.* 41: 833–841.
- Bejarano, E. E., D. Sierra, A. Pérez-Doria, and I. D. Vélez. 2006. Primer hallazgo de *Lutzomyia tihuilienis* (Diptera: Psychodidae) en el valle de Aburrá, Colombia. *Biomédica* 26 (Suppl. 1): 228–231.
- Cáceres, A., L. Quate, E.A.B. Galati, and H. Bath. 2001. Flebotomios (Diptera: Psychodidae) de San Pedro, distrito de Kosñipata, Paucartambo-Cusco y nuevos reportes para el Perú. *Rev. Med. Exp.* 18: 24–26.
- Collins, F. H., M. A. Mendez, M. O. Rasmussen, P. C. Mehafeey, N. J. Besansky, and V. Finnerty. 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *Am. J. Trop. Med. Hyg.* 37: 37–41.
- Futuyma, D. J. 1998. *Evolutionary biology*, 3rd ed. Sinauer Inc., Sunderland, MA.
- Galati, E.A.B. 2003a. Classificação de Phlebotominae, pp. 23–51. In E. F. Rangel and R. Lainson [eds.], *Flebotomíneos do Brasil*. Editora Fiocruz, Rio de Janeiro, Brazil.
- Galati, E.A.B. 2003b. Morfologia, terminología de adultos e identificação dos táxons da América, pp. 53–175. In E. F. Rangel and R. Lainson [eds.], *Flebotomíneos do Brasil*. Editora Fiocruz, Rio de Janeiro, Brazil.
- Ishikawa, E. A., P. D. Ready, A. A. De Souza, J. C. Day, E. F. Rangel, C. R. Davies, and J. J. Shaw. 1999. A mitochondrial DNA phylogeny indicates close relationships between populations of *Lutzomyia whitmani* (Diptera: Psychodidae, Phlebotominae) from the rain-forest regions of Amazonia and northeast Brazil. *Mem. Inst. Oswaldo Cruz* 94: 339–345.
- Kumar, S., K. Tamura, and M. Nei. 2004. MEGA 3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief. Bioinform.* 5: 150–163.
- Le Pont, F., M. J. Torrez-Espejo, and J. P. Dujardin. 1997. Phlébotomes de Bolivie: description de quatre nouvelles espèces de *Lutzomyia* (Diptera: Psychodidae). *Ann. Soc. Entomol. Fr.* 33: 55–64.
- Lowe, T. M., and S. R. Eddy. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* 25: 955–964.
- Ready, P. D., J. C. Day, A. A. De Souza, E. F. Rangel, and C. R. Davies. 1997. Mitochondrial DNA characterization of populations of *Lutzomyia whitmani* (Diptera Psychodidae) incriminated in the peri-domestic and silvatic transmission of *Leishmania* species in Brazil. *Bull. Entomol. Res.* 87: 187–195.
- Rojas, W. 2001. Relaciones filogenéticas en *Lutzomyia* spp. del grupo *verrucarum*. M.S. thesis, Universidad de Antioquia, Medellín, Colombia.
- Suguri, S., M. Harada, M. Marumo, A. Hosokawa, R. A. Sud, E.A.L. Gómez, and Y. Hashiguchi. 1997. A preliminary study on nucleotide sequence variations of *Lutzomyia* spp. in the cytochrome c oxidase subunit I gene, pp. 25–27. In Y. Hashiguchi [ed.], *Studies on New World leishmaniasis and its transmission, with particular reference to Ecuador*. Kyowa Printing Co, Kochi, Japan.
- Testa, J. M., J. Montoya-Lerma, H. Cadena, M. Oviedo, and P. D. Ready. 2002. Molecular identification of vectors of *Leishmania* in Colombia: mitochondrial introgression in the *Lutzomyia townsendi* series. *Acta Trop.* 84: 205–218.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.
- Uribe, S., C. Porter, and I. D. Vélez. 1998. Amplificación y obtención de secuencias de rRNA mitocondrial en *Lutzomyia* spp. (Diptera: Psychodidae) vectores de leishmaniosis. *Rev. Colomb. Entomol.* 24: 109–115.
- Uribe, S., T. Lehmann, E. D. Rowton, I. D. Vélez, and C. H. Porter. 2001. Speciation and population structure in the morphospecies *Lutzomyia longipalpis* (Lutz & Neiva) as derived from the mitochondrial ND4 gene. *Mol. Phylogenet. Evol.* 18: 456–461.
- Vivero, R. J., M. A. Contreras-Gutiérrez, and E. E. Bejarano. 2007. Análisis de la estructura primaria y secundaria del ARN de transferencia mitocondrial para Serina en siete especies de *Lutzomyia*. *Biomédica* 27: 429–438.
- Warburg, A., J. Montoya-Lerma, C. Jaramillo, A. L. Cruz-Ruiz, and K. Ostrovska. 1991. Leishmaniasis vector potential of *Lutzomyia* spp. in Colombian coffee plantations. *Med. Vet. Entomol.* 5: 9–16.
- Wolff, M., and E.A.B. Galati. 2002. Description of *Pintomyia limafalcaoae* and *Pintomyia antioquiensis*, two new spe-

- cies of phlebotomine sand fly (Diptera, Psychodidae) from the Colombian Andes. *Mem. Inst. Oswaldo Cruz* 97: 317–324.
- Xia, X., and Z. Xie. 2001. DAMBE: software package for data analysis in molecular biology and evolution. *J. Hered.* 92: 371–373.
- Young, D. G., and M. A. Duncan. 1994. Guide to the identification and geographic distribution of *Lutzomyia* sand flies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). *Mem. Am. Entomol. Inst.* 54: 1–881.
- Zeledón, R., J. Murillo, and H. Gutierrez. 1985. Flebótomos antropófilos y leishmaniasis cutánea en Costa Rica. *Bol. Of. Sanit. Panam.* 99: 163–72.

Received 8 February 2007; accepted 9 January 2008.
