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Systematic relationships among *Lutzomyia* sand flies (Diptera: Psychodidae) of Peru and Colombia based on the analysis of 12S and 28S ribosomal DNA sequences

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Abstract

Lutzomyia spp. are New World phlebotomine sand flies, many of which are involved in the transmission of human diseases, such as leishmaniases and bartonellosis. The systematic classification of the approximately 400 species in the genus has been based on morphological characters, but the relationships within the genus are still very much in question. We have inferred phylogenies of 32 species of phlebotomine sand flies belonging to seven sub-genera and two species groups, by using fragments of the mitochondrial small subunit (12SrRNA) and of the nuclear large subunit (28SrRNA) ribosomal gene sequences. The subgenus *Helcocyrtomyia* and the *Verrucarum* species group, prominent representatives of the Peruvian sand fly fauna, were represented by 11 and 7 species, respectively. Although based on a limited number of taxa, the resulting phylogenies, based on 837 characters, provide an initial phylogenetic backbone for the progressive reconstruction of infrageneric relationships within *Lutzomyia.*

Keywords: *Lutzomyia*; Phylogeny; *Helcocyrthomyia*; *Verrucarum* species group; Ribosomal RNA; Sand flies

1. Introduction

The genus *Lutzomyia* (Diptera, Psychodidae, Phlebotominae) encompasses approximately 400 sand fly species (Young and Duncan, 1994) which are distributed throughout the Neotropical and the southern Nearctic regions. At least 33 species have been incriminated as vectors of human illnesses, such as leishmaniasis and bartonellosis (Townsend, 1913; Grimaldi and Tesh, 1993; Anderson and Neuman, 1997). *Leishmania* spp. and *Bartonella bacilliformis* have proved to be genetically, biologically, and ecologically very diverse (Bañuls et al., 1999; Birtles et al., 2002), and their diversity is likely to determine the nature of vector–pathogen relationships (Pimenta et al., 1994). However, to better understand the nature of these relationships, more accurate tools must be developed for the identification and the classification of sand flies. Despite the medical importance of the Phlebotominae, their overall morphological systematics remains controversial (Fairchild, 1955; Theodor, 1965; Lewis et al., 1977; Martins et al., 1978; Lane, 1986; Artemiev, 1991; Williams, 1993; Young and Duncan, 1994; Galati, 1995; Galati et al., 1995a). In New World taxa, the interpretation of morphological characters is frequently hampered by the occurrence of intraspecific polymorphism and of cryptic species (Dujardin et al., 1996; Lampo et al., 1999; Mukhopadhyay et al., 2001), and by the absence of distinctive morphological characters for the females of many species (Feliciangeli et al., 1992; Young and Duncan, 1994). Therefore, additional informative characters are needed for a more detailed taxonomy as well as for reassessment of the systematic relationships within the genus *Lutzomyia.*

Presently, two main discordant classifications of *Lutzomyia* sand flies coexist. Young and Duncan (1994)

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reviewed the genus *Lutzomyia* and subdivided it into 15 unranked subgenera and 11 species groups. The *Verrucarum* group was further subdivided into series, but this is the only example of a fully hierarchical organization proposed within the genus. Shortly thereafter, Galati (1995) attempted to resolve the evolutionary relationships among New World sand flies by a cladistic analysis of 88 quantitative and qualitative morphological characters. The resultant classification was predicated by that of Artemiev (1991) and has come to be favoured among South American taxonomists. The cladograms inferred from this analysis proposed the first substantial hypotheses on the intrageneric evolutionary relationships among *Lutzomyia* sand flies (Fig. 1A and B). Galati (1995) subdivided Phlebotominae into two tribes, (1) Hertigini, which includes the genera *Warileya*, *Hertigia*, and *Chinius* and (2) Phlebotomini, which was subdivided into six subtribes—Phlebotomina (*Phlebotomus*), Australophlebotomina, Brumptomyiina, Sergentomyiina, Lutzomyina (*Lutzomyia*) and Psychodopygina. Psychodopygina included several subgenera (sensu Young and Duncan, 1994), which were elevated to the generic rank (*Psathyromyia*, *Viannamyia*, *Nyssomyia*, *Trichophoromyia*, *Psychodopygus*). Similarly, the organisation within Lutzomyina was radically modified. Some species groups and subgenera were elevated to the generic rank, and the *Verrucarum* group was included into genus *Pintomyia*, subgenus *Pifanomyia.*

Molecular data sets have been introduced only recently as tools for the study of sand fly relationships. Ribosomal gene sequences (18SrDNA and the D2 domain of 28SrDNA) were used to reevaluate higher-level relationships within the family Phlebotominae (Depaquit et al., 1998; Aransay et al., 2000) and within genera *Phlebotomus* and *Sergentomyia* (Aransay et al., 2000). The more rapidly evolving mitochondrial genes (cytochrome b and ND4) and nuclear ITS2 and elongation factor-α gene sequences were used to resolve intraspecific and subgeneric relationships, respectively (Ishikawa et al., 1999; Depaquit et al., 2000; Uribe Soto et al., 2001; Testa et al., 2002). Analyses specifically aimed at inferring the overall *Lutzomyia* phylogeny were based on the *cacophony* IVS6 gene and the *period* gene sequences of eight *Lutzomyia* taxa in the subgenera *Nyssomyia* and *Lutzomyia*, and the *Migonei* group. The resulting *Lutzomyia* clusters were not fully resolved. Nevertheless, they indicated monophyly for subgenus *Nyssomyia*, and paraphyly for subgenus *Lutzomyia* and the *Migonei* group. The *Nyssomyia* lineage appeared to be basal when using a *Phlebotomus* species as an outgroup (Lins et al., 2002; Mazzoni et al., 2002).

We sequenced fragments of the 12SrRNA gene sequences of Peruvian and Colombian *Lutzomyia* species to build a molecular database to be used for sand fly identification. In addition, the 12SrDNA and 28SrDNA sequences from the same flies were examined for suitability as tools for reconstructing *Lutzomyia* phylogenies at the subgenus and species levels. The limitations of conserved genes in resolving subgeneric, and specific relationships have been well established (Depaquit et al., 1998; Aransay et al., 2000). The 12SrRNA and a relatively rapidly evolving fragment of the 28SrRNA gene have proved to be promising tools for tracing the history of more recent evolutionary events (Hillis and Dixon, 1991). The phylogenies based on our rDNA sequences were then used to test the two models of *Lutzomyia* systematics outlined by Young and Duncan (1994) and Galati (1995) (Tables 1 and 2). For clarity, the nomenclature proposed by Young and Duncan (1994) will be used throughout this article.

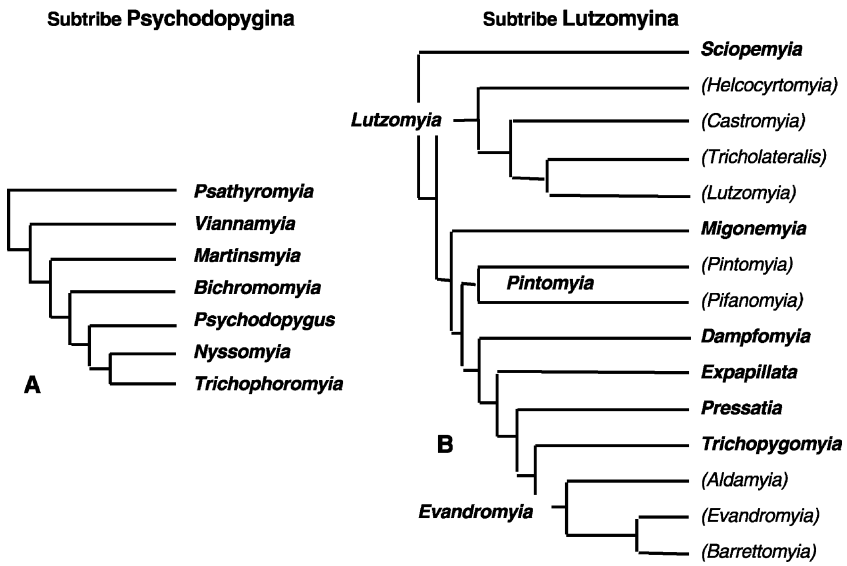

Fig. 1. Simplified version of the cladogram representing relationships among Psychodopygina and Lutzomyina taxa (sensu Galati, 1995).

Table 1
List of *Lutzomyia* specimens analysed in this study, of authors, collection sites, and GenBank sequence accession numbers (with the exception of the three specimens from Colombia, all other sand flies were collected in Peru)

Species	Authors	Collected in	12S	28S
L. ayacuchensis	Cáceres and Galati (1988)	Ayacucho, Lucanas, Sancos	AY352660	AY349483
L. battistini 1	Hertig (1943)	Huancavelica, Tayacaja	AY352661	AY349487
L. battistini 2	Hertig (1943)	Huancavelica, Acomayo	AY352662	
L. caballeroi	Blancas, Cáceres and Galati (1989)	Ancash, Huarmey, Fortaleza	AY352663	AY349501
L. castanea 1	Galati and Cáceres (1994)	Amazonas, Utcubamba	AY352664	
L. castanea 2	Galati and Cáceres (1994)	Amazonas, Utcubamba	AY352665	AY349502
L. cortelezzi	Brèthes (1923)	San Martín, San Martín, Morales, Sombrillas	AY352666	AY349490
L. fisheri	Pinto (1926)	Puno, Sandia	AY352667	AY349489
L. flaviscutellata	Mangabeira (1942)	San Martín, San Martín, Morales, Herta Floresta, Moramilla	AY352668	AY349506
L. geniculata	Mangabeira (1941)	San Martín, San Martín, La Banda de Shilcayo, Ahuafhiyacu	AY352669	AY349505
L. gonzaloi	Ogusuku et al. (1997)	Huánuco, Huamalies	AY352670	
L. guderiani 1	Torréz-Espejo, Cáceres and Le Pont (1995)	Puno, Sandia	AY352671	AY349504
L. guderiani 2	Torréz-Espejo, Cáceres and Le Pont (1995)	Puno, Sandia	AY352672	
L. guderiani 3	Torréz-Espejo, Cáceres and Le Pont (1995)	Puno, Sandia	AY352673	
L. longipalpis	Lutz and neiva (1912)	Colombia-INS (Callejon)	AY352674	AY349492
L. maranonensis 1	Galati, Cáceres and Le Pont (1995)	Amazonas, Utcubamba	AY352675	
L. maranonensis 2	Galati, Cáceres and Le Pont (1995)	Cajamarca, San Ignacio	AY352676	AY349497
L. maranonensis 3	Galati, Cáceres and Le Pont, 1995	Cajamarca, San Ignacio	AY352677	
L. migonei 1	França (1920)	Cusco, La Convención	AY352678	
L. migonei 2	França (1920)	Cusco, La Convención	AY352679	AY349499
L. munaypata 1	Ogusuku, Chevarría, Porras and Pérez (1999)	Huancavelica, Tayacaja, Cochabamba, Sueruro	Ay352680	
L. munaypata 2	Ogusuku, Chevarría, Porras and Pérez (1999)	Cusco, Calca		AY349488
L. nevesi 1	Damasceno and Arouck (1956)	San Martin, San Martin, Boca Toma del Cumbaza	AY352681	
L. nevesi 2	Damasceno and Arouck (1956)	San Martin, San Martin, Tarapoto, Tarapotillo	AY352682	AY349496
L. noguchi	Shannon (1929)	Lima, Huarochiri, Surco	AY352683	AY349483
L. nuneztovari 1	Ortiz (1954)	Huancavelica, Tacayaia, Cochabamba, Sueruro	AY352684	AY349493
L. nuneztovari 2	Ortiz (1954)	Puno, Sandia	AY352685	
L. nuneztovari 3	Ortiz (1954)	Cusco, Calca	AY352686	
L. ovallesi	Ortiz (1952)	Colombia-INS	AY352687	AY349498
L. peruensis 1	Shannon (1929)	La Libertad, Santiago de Chuco, Mollepata	AY352688	AY349484
L. peruensis 2	Shannon (1929)	La Libertad, Santiago de Chuco, Mollepata	AY352689	
L. peruensis 3	Shannon (1929)	Cusco, Calca	AY352690	
L. pescei 1	Hertig (1943)	Cusco, Anta	AY352691	
L. pescei 2	Hertig (1943)	Huancavelica, Acomayo	AY352692	
L. pescei 3	Hertig (1943)	Huancavelica, Acobamba, Rosario, Auquimbra	AY352693	AY349491
L. quillabamba	Ogusuku, Chevarría, Porras and Pérez (1999)	Cusco, La Convención	AY352694	
L. robusta 1	Galati, Cáceres and Le Pont (1995b)	Cajamarca, San Ignacio	AY352695	AY349494
L. robusta 2	Galati, Cáceres and Le Pont (1995b)	Cajamarca, San Ignacio	AY352696	
L. scorzai 1	Ortiz (1965)	Puno, Sandia	AY352697	AY349503
L. serrana 1	Damasceno and Arouck (1949)	Huanuco, Huanuco, Monzon	AY352699	
L. serrana 2	Damasceno and Arouck (1949)	Colombia-INS	AY352700	AY349495
L. sherlocki 1	Martins, Silva and Falcão (1971)	San Martín, San Martín, Morales, Villa Antonio		
L. sherlocki 2	Martins, Silva and Falcão (1971)	San Martín, San Martín, Morales, Villa Antonio	AY352698	AY349486
L. tejadai 1	Galati and Cáceres (1990)	Huanuco, Huanuco, Chichao	AY352701	
L. tejadai 2	Galati and Cáceres (1990)	Huanuco, Huanuco, Chichao	AY352702	
L. tuberculata	Mangabeira (1941)	Puno, Sandia, Pacaisuizo	AY352703	AY340509
L. verrucarum 1	Townsend (1913)	Lima, Huarochiri, Surco	AY352704	AY349500
L. verrucarum 2	Townsend (1913)	La Libertad, Santiago de Chuco, Mollepata	AY352705	
L. yuilli	Young and Porter (1972)	San Martín, San Martín, La Banda de Shilcayo, Ahuafhiyacu	AY352706	AY349507
L. yuilli yuilli	Young and Porter (1972)	San Martín, San Martín, La Banda de Shilcayo, Ahuafhiyacu	AY352707	AY349508
Trichophoromyia sp.	Undescribed species	San Martin, San Martin, Boca Toma del Cumbaza	AY352708	AY349510

Table 2
Classification of the *Lutzomyia* species considered in our study according to Young and Duncan (1994)

Family	Psychodidae	
Sub-family	Phlebotominae	
Genus	*Lutzomyia*	
Subgenera	*Lutzomyia*	*L. longipalpis*
		L. battistini
		L. sherlocki
	Pintomyia	*L. fisheri*
	Viannamyia	*L. tuberculata*
	Nyssomyia	*L. yuilli yuilli*
		L. yuilli
		L. flaviscutellata
	Trichophoromyia	*L. (Trichophoromyia)*spp.
	Psychodopygus	*L. geniculata*
	Helcocyrtomyia	*L. peruensis*
		L. ayacuchensis
		L. noguchii
		L. tejadai
		L. pescei
		L. scorzai
		L. caballeroi
		L. castanea[a]
		L. munaypata[a]
		L. quillabamba[a]
		L. guderiani[a]
		L. gonzaloi[a]
Species groups	*Migonei*	*L. migonei*
		L. cortelezzii
	Verrucarum	
Series	*serrana*	*L. robusta*[a]
		L. serrana
Series	*verrucarum*	*L. nevesi*
		L. maranonensis[a]
		L. ovallesi
		L. nuneztovari
		L. verrucarum

Non-represented subgenera: *Sciopemyia*, *Coromyia*, *Dampfomyia*, *Pressatia*, *Evandromyia*, *Psathyromyia*, *Trichopygomyia*, and *Micropygomyia*. Non-represented species groups: *Saulensis*, *Baityi*, *Rupicola*, *Aragaoi*, *Lanei*, *Delpozoi*, *Dreisbachi*, *Pilosa*, and *Oswaldoi*.

[a] Species described after 1994 and which were not included in Young and Duncan (1994) original classification.

2. Materials and methods

2.1. Sand flies

Lutzomyia specimens (Table 1) were collected in Peru with light-traps developed by the US Centers for Disease Control. Specimens of *Lutzomyia longipalpis*, *Lutzomyia ovallesi* and *Lutzomyia serrana* were from laboratory reared colonies and were provided by C. Ferro (Instituto Nacional de Salud, Bogotà, Colombia). Each sand fly was preserved in 70% alcohol until subjected to DNA extraction. Voucher specimens corresponding to each species were kept at the collection of the Division de Entomología, Instituto Nacional de Salud and Instituto de Medicina Tropical 'Daniel A. Carrión' Universidad Nacional Mayor de San Marcos in Lima, Peru. The two alternative classifications of the species in our sample (Young and Duncan, 1994; Galati and Cáceres, 1994; Galati, 1995; Galati et al., 1995a) are presented in Tables 1 and 2.

2.2. DNA extraction, PCR amplification and sequencing

Each specimen was blotted dry with filter paper and DNA was extracted by using the DNeasy Tissue kit (Qiagen, Valencia, California) using the protocol modification of Beati and Keirans (2001). A ≈ 360 bp fragment of the 12SrDNA sequence, corresponding to part of domain II and domain III of the ribosomal small-subunit RNA gene sequence (Hickson et al., 1996), was amplified by primers T1B (5′-aaa cta gga tta gat acc ct-3′) and T2A (5′-aat gag agc gac ggg cga tgt-3′) (Beati and Keirans, 2001). These primers have successfully amplified DNA from mites (Beati and Keirans, 2001) and fleas (unpublished data). PCR conditions were 5 cycles of 94 °C denaturation (20 s), 52 °C annealing (30 s), and 70 °C elongation (25 s), followed by 30 cycles of 94 °C denaturation (20 s), 54 °C annealing (30 s), and 72 °C elongation (25 s), with an intial denaturation of 94 °C (5 min) and a final extension of 72 °C (5 min). The 28SrDNA sequences (about 710 bp) were amplified by primers 28V (5′-aag gta gcc aaa tgc ctc gtc atc-3′) and 28 X (5′-ggc tct tcc tat cat tgt gaa gca gaa ttc ac-3′) (Hillis and Dixon, 1991) and correspond to part of the IV and V domains of the 28SrRNA sequence (Larsen, 1992). PCR conditions were 35 cycles of 94 °C denaturation (20 s), 60 °C annealing (25 s), and 72 °C elongation (50 s), with an initial denaturation of 94 °C (5 min) and a final extension of 72°C (5 min). Each PCR reaction (25 μl) contained 2.5 μl of template DNA, 5 μl of Taq enhancer (5 ×), 2.5 μl of Taq buffer (10 ×), 1.25 μl of each primer (10 pm/μl), 200μM each of dNTPs, 1.25 Ml of $MgCl_2$ (25 mM) and 0.1 μl of Taq polymerase (5 U/μl) (Eppendorf Scientific, Wesbury, NY). Amplified products were purified with a QIAquick PCR Purification kit (Qiagen) and sequenced at the Keck Facilty (Yale University). The 12SrDNA sequences were obtained from several specimens of a single species in order to evaluate intraspecific variability and to test the reliability of the morphological identification, whereas 28SrDNA sequences were produced for one representative of each species (Table 1).

2.3. Sequence analysis

Sequences of the two strands of each PCR sample were assembled with Seqman software (Lasergene, DNASTAR, Madison, WI) and manually aligned by using MacClade (Maddison and Maddison, 2000) according to the recognised secondary structure of the ribosomal gene sequences (Larsen, 1992; Hadjiolov et al., 1984; Hickson et al., 1996). Pairwise distance values were calculated by using PAUP* (v 4.0 beta 8) (Swofford, 2000). Phylogenetic reconstructions were obtained by maximum parsimony and maximum likelihood method implemented in PAUP* and by Bayesian

analysis using MrBAYES (v2.01) (Huelsenbeck, 2000; Huelsenbeck and Ronquist, 2001). Maximum parsimony heuristic searches for optimum trees were performed by swapping branches using the tree bissection reconnection (TBR) algorithm, with all substitutions given equal weight and gaps treated as missing characters. Branch support was assessed by 500 bootstrap replicates. Maximum likelihood searches were performed after the best nucleotide substitution model was selected by Modeltest (v 3.06) (Posada and Crandall, 1998), and by using the maximum parsimony tree with the best likelihood score as starting tree. Bayesian Markov Monte Carlo analyses were performed by running simultaneously four chains for 500 000 replicates and by using the nucleotide substitution model previously selected by Modeltest. Trees were sampled every 100 iterations. Topologies that had been saved before the likelihood values stabilised were discarded from the final sample. A 50% majority-rule consensus tree of the remaining sampled trees was performed in PAUP, and posterior probability values recorded for each branch. The topologies of the trees were compared and their congruence was tested to establish if the two data sets were compatible for combination in a total evidence analysis. Congruencies were evaluated by the partition-homogeneity test (100 replicates with MAXTREE set to 100, significance threshold value $P > 0.05$) as implemented in PAUP (Bull et al., 1993).

3. Results

3.1. 12SrDNA sequences

The length of the 12SrDNA fragments varied from 358 to 363 bp and the resulting alignment was of 362 bp after gaps created by single sequences were excised. The GenBank accession numbers of the sequences are listed in Table 1. The alignment is available upon request from the corresponding author (LB). Intraspecific variability occurred within several *Lutzomyia* taxa, with distance values usually varying between 0 and 0.4%, except between *Lutzomyia nuneztovari* (0.8%) and *Lutzomyia battistini* (1.9%). At the subspecies level, the distance between *Lutzomyia yuilli yuilli* and *Lutzomyia yuilli* was 1.9%. The distances between specimens of *L. serrana* and *Lutzomyia robusta* varied between 0.3% (*L. robusta* and the Peruvian *L. serrana*) and 0.6% (*L. robusta* and the Colombian *L. serrana*). The sequence of *Lutzomyia quillabamba* differed from that of *Lutzomyia tejadai* by 2.5%. Distance values between other recognised species varied from 4 to 13%. A preliminary phylogenetic analysis of all samples verified that sequences obtained from all specimens of a single species were clustered in monophyletic clades. This was true for all but the *L. serrana–robusta* cluster. Furthermore, the sequences of two female specimens, tentatively identified as *Lutzomyia nevesi*, differed by 5% from the sequences of clearly identified male specimens of the same species. The two samples were therefore eliminated from our dataset. One representative sequence from each recognised species was retained for the pylogenetic analyses (Table 1). The maximum parsimony heuristic search recovered three most parsimonious trees (length = 339; CI = 0.507; RI = 0.553; 75 parsimony-informative characters), all included in a single island, which indicates that all best trees shared the same basic topology. The strict consensus tree of the three topologies is presented in Fig. 2A. The reconstruction was not well resolved and the sole deduction possible is that all taxa belonging to the Psychodopygina and Lutzomyina (sensu Galati) are clustered in monophyletic groups. Modeltest selected TVM + I + G as the model for best fitting the 12S sequences (Nst = 6; base = 0.42 − 0.04 − 0.10; Rmat = 0.57 − 2.52 − 0.52 − 0.0001 − 2.52; Rates = gamma; Shape = 0.42; Pinvar = 0.37). The maximum likelihood search was interrupted after 20 000 rearrangments, and the tree with the best likelihood score was saved for comparison. The Bayesian analysis produced a tree with a topology perfectly identical to the maximum likelihood reconstruction; therefore, only the Bayesian analysis tree is presented in Fig. 3A. The resolution in this tree was slightly better than that of the maximum parsimony cladogram (12 supported nodes in the Bayesian tree, versus five in the MP tree). The separation between Lutzomyina and Psychodopygina lineages was confirmed. Some additional clusters appeared to be monophyletic within the subgenus *Helcocyrtomyia*, although they did not support separating this subgenus into the subdivisions (series) *osornoi*, *sanguinaria* and *peruensis* (Table 3) as proposed by Galati and Cáceres (1994). The remaining monophyletic group was a cluster of taxa that were included in the subgenus *Lutzomyia* and the *Verrucarum* group. Neither of the series proposed by Young and Duncan (1994) and Galati et al. (1995a) within the *Verrucarum* group (Galati's genus *Pintomyia*) appeared to be monophyletic (Tables 2 and 3).

3.2. 28SrDNA sequences

Sequences of approximately 710 bp were obtained from the same samples as used for the 12SrDNA phylogenetic analysis, with the exception of three species (Table 1). The GenBank accession numbers of the sequences are listed in Table 1. After alignment, the conserved and uninformative fragments at the beginning and at the end of the alignment were eliminated, and the gap positions created by single sequences were excised. The alignment is available upon request from the corresponding author (LB). The final data set included 475 characters, of which 100 were parsimony informative. Intraspecific distances between taxa varied from 1.1% (between *Lutzomyia castanea* and *Lutzomyia caballeroi* or *Lutzomyia scorzai*) to 12.9% (between *L. yuilli* and *Lutzomyia sherlocki*). However, the distance between *L. serrana* and *L. robusta* was only 0.2%. By maximum parsimony, PAUP found the single most parsimonious tree

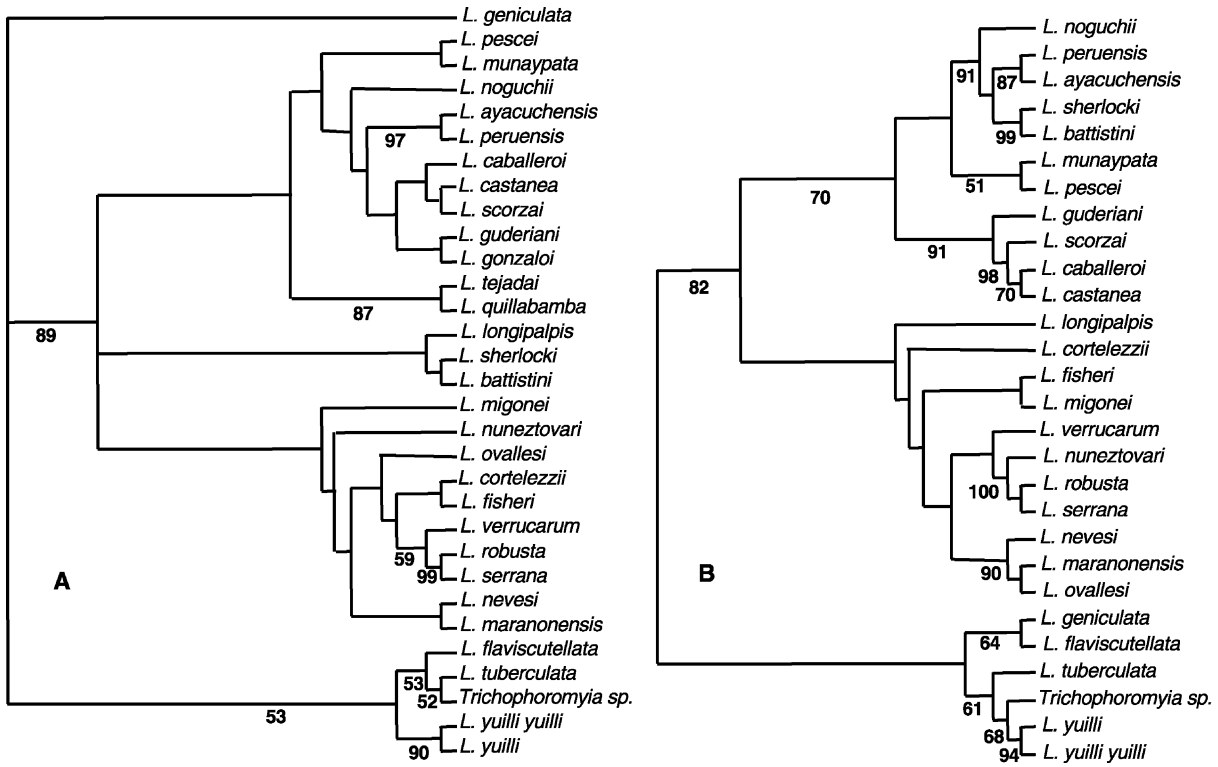

Fig. 2. Maximum parsimony trees for the considered sand fly taxa (*Lutzomyia*) obtained by analysing 12SrDNA (A) and 28SrDNA (B) data sets. Bootstrap values are shown below branches (500 replicates).

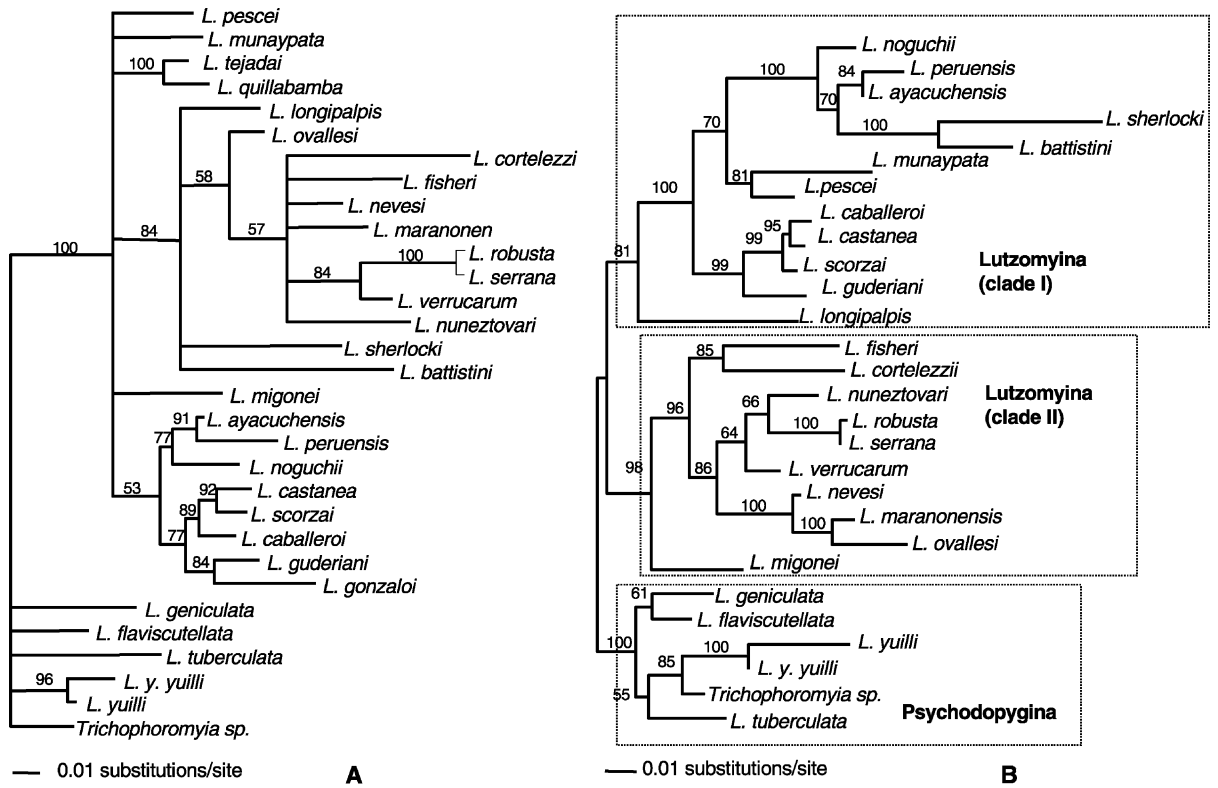

Fig. 3. Phylogenetic trees for the coonsidered taxa (*Lutzomyia*) obtained by Bayesian analysis of the 12SrDNA (A) and the 28rDNA (B) data sets. Posterior probabilities are shown above branches.

Table 3
Classification of the sampled sand fly taxa, genera *Viannamyia*, *Psychodopygus*, *Nyssomyia*, *Bichromomyia*, *Trichophoromyia*, *Lutzomyia*, *Pintomyia*, *Migonemyia*, and *Evandromyia*, according to Galati (1995), Galati and Cáceres, 1994 and Galati et al. (1995a)

Family	Psychodidae		
Sub-family	Phlebotominae		
Tribe	Phlebotomini		
Subtribe	Psychodopygina		
Genus	*Viannamyia*		*V. tuberculata*
Genus	*Psychodopygus*		*P. geniculatus*
Genus	*Nyssomyia*		*N. yuilli*
			N. yuilli yuilli
	Bichromomyia		*B. flaviscultellata*
Genus	*Trichophoromyia*		*Trichophoromyia. sp.*
Subtribe	Lutzomyina		
Genus	*Lutzomyia*		
Subgenus	*Lutzomyia*		*L. longipalpis*
			L. battistini
Subgenus	*Helcocyrtomyia*	series *peruensis*	*L. peruensis*
			L. ayacuchensis
			L. noguchii
			L. tejadai
			L. pescei
		series *osornoi*	*L. caballeroi*
			L. castanea
			L. munaypata
			L. quillabamba
		series *sanguinaria*	*L. gonzaloi*
			L. scorzai
			L. guderiani
	Tricholateralis		*L. sherlocki*
Genus	*Pintomyia*		
Subgenus	*Pintomyia*		*P. fisheri*
Subgenus	*Pifanomyia*	series *serrana*	*P. robusta*
			P. serrana
		series *evansi*	*P. nevesi*
			P. maranonensis
			P. ovallesi
			P. nuneztovari
		series *verrucarum*	*P. verrucarum*
Genus	*Migonemyia*		
Subgenus	*Migonemyia*		*M. migonei*
Genus	*Evandromyia*		
Subgenus	*Barrettomyia*		*E. cortelezzii*

(length = 371; CI = 0.523; RI = 0.649) shown in Fig. 2B. Although the inferred tree was not fully resolved, several of its branches were supported by >70% bootstrap values. These included the lineage leading to the Psychodopygina (Galati, 1995), the cluster containing all *Helcocyrtomyia* taxa, and the species pair *L. sherlocki* and *Lutzomyia battistini* (both in subgenus *Lutzomyia* according to Young and Duncan (1994)). The other member of subgenus *Lutzomyia* examined, *L. longipalpis*, was not included in the same clade. Within *Helcocyrtomyia*, once again the well supported subdivision of the lineages did not correspond to the organisation of Galati and Cáceres (1994) series. Modeltest suggested the use of a HKY + G model for maximum likelihood and Bayesian analyses (Base = 0.26–0.19–0.22; Nst = 2; Tratio = 1.92; Rates = gamma; Shape = 0.14; Pinvar = 0). The maximum likelihood search was interrupted after 20 000 rearrangements and the tree with the best likelihood score was saved for further comparisons. Its overall topology did not differ from the Bayesian tree shown in Fig. 3B. Generally, the branch support by Bayesian analysis was higher than by maximum parsimony analysis and showed support for the subdivision of Psychodopygina and Lutzomyina (sensu Galati). Two sister lineages appeared to be well resolved within *Lutzomyina* (clade I and clade II in Fig. 3B). Clade I included all *Helcocyrtomyia* and *Lutzomyia* taxa, whereas clade II included the *Migonei* and the *Verrucarum* groups. *Helcocyrtomyia* and *Lutzomyia* were both paraphyletic, and the composition of the supported *Helcocyrtomyia* clades did not correspond to the subdivision of the subgenus into series. The structure of clade II closely resembled the structure of Galati's cladogram (Fig. 1A). The subdivision of the *Verrucarum* group into series (Table 3), however, was not supported.

3.3. The combined data set

The 12SrDNA and the 28SrDNA sequences were combined in a single data set. The partition homogeneity test rejected the null hypothesis of data set homogeneity ($P = 0.01$), and therefore the two data sets were not combined.

4. Discussion

The first aim was to provide tools for the molecular identification of *Lutzomyia* sand flies. This was achieved by matching nucleic acid sequences to clearly recognised species. However, the morphological identification of *Lutzomyia* in many cases was difficult, and verification was necessary so that all specimens identified as a given species had identical or closely related sequences. A preliminary phylogenetic analysis of all 12SrDNA sequences confirmed that sequences obtained from identical species were clustered into monophyletic clades; this strongly indicated the absence of cryptic species in the samples. However, two species, *L. serrana* and *L. robusta*, were comingled in a single clade and distance values between them were as low as those observed within recognised species. Distance values between their 28SrDNA sequences were also smaller than those observed between other recognised species. This strongly suggested that these two taxa, recently differentiated by a variance analysis of five morphological traits (Galati et al., 1995b), constitute a single species. By collecting samples of both species from more diverse geographical areas the genetic structure of populations of these two taxa can be better determined. The specific status of other recently described species (Galati et al., 1994; Torres-Espejo et al., 1995; Ogusuku et al., 1997, 1999) was not questioned, however.

The second aim was to assess the systematic relationships among the available taxa based on the phylogenetic analysis of fragments of 12SrDNA and 28SrDNA sequences and to verify whether these reconstructions corroborated previously published classifications based on morphometric characters.

In general, overall resolution was better in the Bayesian analyses than in maximum parsimony analyses. In the 28S data set, gaps frequently occurred in the variable and most informative segments of the sequences. Because of this, maximum parsimony, with gaps considered as missing characters, may not be the best tool for analysing such sequences. Maximum likelihood and Bayesian analyses produced identical topologies. This is not particularly surprising since both analyses were based on the same model of nucleotide substituions selected by Modeltest.

Although 12SrDNA sequences were informative at the intraspecific level for the subtribes *Psychodopygina* and *Lutzomyina* (sensu Galati), they did not provide well-resolved topologies within genera and subgenera (Fig. 2A). Bayesian analysis of the same sequences did not improve the overall quality of the phylogenetic analysis (Fig. 3A). Because interspecific mitochondrial introgression events cannot be excluded (Testa et al., 2002), the use of mitochondrial gene sequences for inferring sand fly phylogenies may be questionable. However, the analysis by all methods (maximum parsimony, maximum likelihood and Bayesian analysis) of both the 12SrDNA and 28SrDNA datasets supported the clustering of subgenera *Viannamyia*, *Nyssomyia, Trichophoromyia*, and *Psychodopygus*. Therefore, the establishment of the two clades Lutzomyina and Psychodopygina (Galati, 1995), appears to be fully justified.

The best resolution within Psychodopygina was provided by Bayesian analysis of the 28S dataset (Fig. 3B). Although the branching order of its basal lineages was not resolved, the reconstruction supported Galati's (1995) consideration of *Trichophoromyia* and *Nyssomyia* as the most recently evolved sister clades in the subtribe (Fig. 1 and Table 3). It also corroborated Galati's placement of *Lutzomyia flaviscutellata* in genus *Bichromomyia* in contrast with its position in the subgenus *Nyssomyia* by Young and Duncan (1994). Furthermore, the 28SrDNA data indicated that *Trichophoromyia* is more closely related to *Nyssomyia* taxa than to *L. flaviscutellata*.

For the *Lutzomyina* genera (sensu Galati, 1995) Bayesian and maximum likelihood analysis of the 28SrDNA provided good resolution among the remaining taxa, which were subdivided into two sister clades (clade I and clade II in Fig. 3B).

Clade I contained all *Helcocyrtomyia* taxa and all *Lutzomyia* taxa (Table 2) which, according to Galati (1995) classification, all belong to genus *Lutzomyia* (Table 3). In the Galati classification, *L. sherlocki* had been separated from the *L. battistini–L. longipalpis* lineage and shifted to the subgenus *Tricholateralis* of genus *Lutzomyia*. The 28SrDNA data did not corroborate this distinction and indicated that *L. longipalpis* is more distantly related from *L. sherlocki* and *L. battistini*, than these are to each other. However, the length of the branches leading to *L. battistini* and *L. sherlocki* indicated that this association may be the result of long-branch attraction. An analysis of other, less rapidly evolving, genes and of a larger selection of related taxa may be better suited to a reconstruction of these relationships.

The second clade included species of the genus *Pintomyia*, and of the *Verrucarum* and *Migonei* groups of species sensu Young and Duncan (1994) and of genera *Pintomyia*, *Migonemyia* and *Ewandromyia* sensu Galati (1995) (Tables 2 and 3). The species group *Migonei* included *Lutzomyia migonei* and *Lutzomyia cortelezzii* (Table 2). None of the analyses clustered these two species in a monophyletic clade. In the 28S Bayesian analysis, *L. migonei* constituted the basal lineage of the second large clade of *Lutzomyina*. *Lutzomyia cortelezzii* was the sister taxon of *Lutzomyia fisheri* (subgenus *Pintomyia*), and the two taxa constituted the basal branch leading to the *Verrucarum* group of species. According to Galati's classification, *L. migonei* and *L. cortelezzii* did not belong to the same genus, *L. migonei* was placed in genus *Migonemyia* (subgenus *Migonemyia*) and *L. cortelezzii* was included in genus *Evandromyia* (subgenus *Barretomyia*). Galati (1995) placed the *Verrucarum* group of species in the genus *Pintomyia* (subgenus *Pifanomyia*). When the clade II structure in Fig. 3B was compared with the same taxa in Fig. 1B, two common features were noted: (1) *L. migonei* is the basal lineage of the clade and (2) the next diverging lineage (genus *Pintomyia*) carries the sister branches leading to subgenus *Pintomyia* (*L. fisheri*) and *Pifanomyia* (the *Verrucarum* group). The anomalous position of *L. cortelezzii* as sister taxon of *L. fisheri* may be due to the lack of samples from the remaining genera (*Dampfomyia*, *Expapillata*, *Pressatia*, and *Trichopygomyia*).

Within subgenera *Helcocyrtomyia* and *Pifanomyia*, the taxa consistently did not cluster by their series designation (Tables 2 and 3). This may be expected, since series definitions often have been based on a very small number of morphometric characters of unknown evolutionary significance (Kreutzer et al., 1990; Feliciangeli et al., 1992; Galati and Cáceres, 1994; Galati et al., 1995a).

The fragment of 28SrDNA appeared to provide information on subgeneric and certain interspecific relationships. With few exceptions, the inferred phylogenetic tree largely supported many aspects of the cladistic reconstruction presented by Galati (1995). The radical changes proposed for nomenclature and ranking order within *Lutzomyia* may be debatable, but clearly the widely accepted classification of Young and Duncan (1994) requires a careful reconsideration. The current study has dealt with a limited, but representative subset of taxa and a defined set of informative characters. Certainly, these data may be subjected to substantial revision with the addition of more informative gene sequences, by the progressive addition of missing

Lutzomyia taxa and of samples from other Neotropical geographical areas. However, the rDNA analyses presented herein represent the first attempt at unraveling *Lutzomyia* systematic relationships by molecular phylogenetics and, as such, they provide the basis for a new set of working hypotheses concerning the classification of phlebotomine sand flies.

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References

Anderson, B.E., Neuman, M.A., 1997. Bartonella spp. as emerging human pathogens. Clin. Microbiol. Rev. 10, 203–219.

Aransay, A.M., Scoulica, E., Tselentis, Y., Ready, P.D., 2000. Phylogenetic relationships of phlebotomine sandflies inferred from small subunit nuclear ribosomal DNA. Insect Mol. Biol. 9, 157–168.

Artemiev, M.M., 1991. A classification of the subfamily Phlebotominae. Parassitologia 33, 69–77.

Bañuls, A.L., Hide, M., Tibayrenc, M., 1999. Molecular epidemiology and evolutionary genetics of *Leishmania* parasites. Int. J. Parasitol. 29, 1137–1147.

Beati, L., Keirans, J.E., 2001. Analysis of the systematic relationships among ticks of the genera *Rhipicephalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial 12S ribosomal DNA gene sequences and morphological characters. J. Parasitol. 87, 32–48.

Birtles, R.J., Fry, N.K., Ventosilla, P., Cáceres, A.G., Sánchez, E., Vizcarra, H., Raoult, D., 2002. Identification of *Bartonella bacilliformis* genotypes and their relevance to epidemiological investigations of human bartonellosis. J. Clin. Microbiol. 40, 3606–3612.

Bull, J.J., Huelsenbeck, J.P., Cunningham, C.W., Swofford, D.L., Waddell, P.J., 1993. Partitioning and combining data in phylogenetic analysis. Syst. Biol. 42, 384–397.

Depaquit, J., Perrotey, S., Lecointre, G., Tillier, A., Tillier, S., Ferté, H., Kaltenbach, M., Léger, N., 1998. Systématique moléculaire des Phlebotominae: étude pilote. C. R. Acad. Sci. Paris 321, 849–855.

Depaquit, J., Ferté, H., Léger, N., Killick-Kendrick, R., Rioux, J.-A., Killick-Kendrick, M., Hanafi, H.A., Gobert, S., 2000. Molecular systematics of the phlebotomine sandflies of the subgenus *Paraphlebotomus* (Diptera, Psychodidae, Phlebotomus) based on ITS2 rDNA sequences. Hypothesis of dispersion and speciation. Insect Mol. Biol. 9, 293–300.

Dujardin, J.P., Le Pont, F., Cruz, M., Leon, R., Tarrieu, F., Guderian, R., Echeverria, R., Tibayrenc, M., 1996. Cryptic speciation in *Lutzomyia* (*Nyssomyia*) *trapidoi* (Fairchild and Hertig) (Diptera: Psychodidae) detected by multilocus enzyme electrophoresis. Am. J. Trop. Med. Hyg. 54, 42–45.

Fairchild, G.B., 1955. The relationships and classification of the Phlebotominae (Diptera: Psychodidae). Ann. Entomol. Soc. Am. 48, 182–196.

Feliciangeli, M.D., Arredondo, C., Ward, R., 1992. Phlebotomine sandflies in Venezuela: review of the verrucarum species group (in part) of *Lutzomyia* (Diptera: Psychodidae) with description of a new species from Lara. J. Med. Entomol. 29, 729–744.

Galati, E.A.B., 1995. Phylogenetic systematics of Phlebotominae (Diptera: Psychodidae) with emphasis on American groups. Bol. Dir. Malariol. Saneamient. Ambient. 35, 133–142.

Galati, E.A.B., Cáceres, A.G., 1994. Descrição de Lutzomyia pallidothorax, sp. n. e de Lutzomyia castanea, sp. n. do Peru e análise cladística das séries do subgênero Helcocyrtomyia Barretto (Diptera: Psychodidae). Rev. Bras. Entomol. 38, 471–488.

Galati, E.A.B., Cáceres, A.G., Le Pont, F., 1995a. Descrições de duas especies novas de Phlebotominae (Diptera, Psychodidae) e considerações sobre o subgênero Pifanomyia Ortiz and Scorza. Revta bras. Ent. 39, 431–446.

Galati, E.A.B., Cáceres, A.G., Le Pont, F., 1995b. Description of *Lutzomyia* (Pifanomyia) *robusta* n. sp. (Diptera, Psychodidae, Phlebotominae) from the Peruvian and Ecuadorean interandean areas. Rev. Saúde Pública 29, 89–99.

Grimaldi, G., Tesh, R.B., 1993. Leishmaniases of the New World: current concepts and implications for future research. Clin. Microbiol. Rev. 6, 230–250.

Hadjiolov, A.A., Georgiev, O.I., Nosikov, V.V., Yavachev, L.P., 1984. Primary and secondary structure of rat 28 S ribosomal RNA. Nucleic Acids Res. 12, 3677–3693.

Hickson, R.E., Simon, C., Cooper, A., Spicer, G.S., Sullivan, J., Penny, D., 1996. Conserved sequence motifs, alignment, and secondary structure for the third domain of the animal 12S rRNA. Mol. Biol. Evol. 13, 150–169.

Hillis, D.M., Dixon, M.T., 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q. Rev. Biol. 66, 411–453.

Huelsenbeck, J.P., 2000. MrBayes: Bayesian inference of phylogeny (software). University of Rochester, New York.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Ishikawa, E.A.Y., Ready, P.D., de Souza, A.A., Day, J.C., Rangel, E.F., Davies, C.R., Shaw, J.J., 1999. A mitochondrial DNA phylogeny indicates close relationships between populations of *Lutzomyia whitmani* (Diptera: Psychodidae, Phlebotominae) from the rain-forest regions of Amazônia and Northeast Brazil. Mem. Inst. Oswaldo Cruz 94, 339–345.

Kreutzer, R.D., Palau, M.T., Morales, A., Ferro, C., Feliciangeli, D., Young, D.G., 1990. Genetic relationships among phlebotomine sand flies (Diptera: Psychodidae) in the *verrucarum* species group. J. Med. Entomol. 27, 1–8.

Lampo, M., Torgerson, D., Márquez, L.M., Rinaldi, M., García, C.Z., Arab, A., 1999. Occurrence of sibling species of *Lutzomyia longipalpis* (Diptera: Psychodidae) in Venezuela: first evidence from reproductively isolated sympatric populations. Am. J. Trop. Med. Hyg. 61, 1004–1009.

Lane, R.P., 1986. Recent advances in the systematics of phlebotomine sandflies. Insect. Sci. Applic. 7, 225–230.

Larsen, N., 1992. Higher order interactions in 23S rRNA. Proc. Natl. Acad. Sci. USA 89, 5044–5048.

Lewis, D.J., Young, D.G., Fairchild, G.B., Minter, D.M., 1977. Proposals for a stable classification of the phlebotomine sandflies (Diptera: Psychodidae). Syst. Entomol. 2, 319–332.

Lins, R.M.M.A., Oliveira, S.G., Souza, N.A., de Queiroz, R.G., Justiniano, S.C.B., Ward, R.D., Kyriacou, C.P., Peixoto, A.A., 2002. Molecular evolution of the cacophony IVS6 region in sandflies. Insect Mol. Biol. 11, 117–122.

Maddison, D.R., Maddison, W.P., 2000. MacClade 4: analysis of phylogeny and character evolution. Sinauer Associates, Sunderland, MA (CD-ROM).

Martins, A.V., Williams, P., Falcão, A.L., 1978. American sand flies (Diptera: Psychodidae, Phlebotominae). Academia Brasileira de Ciências, Rio de Janeiro.

Mazzoni, C.J., Gomes, C.A., Souza, N.A., de Queiroz, R.G., Justiniano, S.C.B., Ward, R.D., Kyriacou, C.P., Peixoto, A.A., 2002. Molecular evolution of the period gene in sandflies. J. Mol. Evol. 55, 553–562.

Mukhopadhyay, J., Ghosh, K., Ferro, C., Munstermann, L.E., 2001. Distribution of phlebotomine sand fly genotypes (*Lutzomyia shannoni*, Diptera: Psychodidae) across a highly heterogeneous landscape. J. Med. Entomol. 38, 260–267.

Ogusuku, E., Canales, J.J., Pérez, J.E., 1997. Descripción de Lutzomyia gonzaloi n. sp. y de L. monzonensis n. sp. (Diptera: Psychodidae: Phlebotominae) y dos nuevos registros de Phlebotominae para el Perú. Rev. Peru Entomol. 40, 71–78.

Ogusuku, E., Chevarría, L., Porras, R., Pérez, J.E., 1999. Descripción de Lutzomyia munaypata sp. n. y L. quillabamba sp.n. (Diptera: Psychodidae) del Departamento de Cusco, Perù. Rev. Peru. Entomol. 41, 45–52.

Pimenta, P.F.P., Saraiva, E.M.B., Rowton, E., Modi, G.B., Garraway, L.A., Beverley, S.M., Turco, S.J., Sacks, D.L., 1994. Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. Proc. Natl. Acad. Sci. USA 91, 9155–9159.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Swofford, D.L., 2000. PAUP* Phylogenetic analysis using parsimony (* and other methods). Version 4.0. Sinauer Associates, Sunderland, MA.

Testa, J.M., Montoya-Lerma, J., Cadena, H., Oviedo, M., Ready, P.D., 2002. Molecular identification of vectors of *Leishmania* in Colombia: mitochondrial introgression in the *Lutzomyia townsendi* series. Acta Trop. 84, 205–218.

Theodor, O., 1965. On the classification of American Phlebotominae. J. Med. Entomol. 2, 171–197.

Torres-Espejo, J.M., Cáceres, G.A., Le Pont, F., 1995. Description de deux nouvelles espèces de phlébotomes du sous-genre Helcocyrtomyia, du piémont andin bolivien (Diptera, Psychodidae). Parasite 2, 157–162.

Townsend, C.H.T., 1913. A Phlebotomus: the practically certain carrier of verruga. Science 38, 194–195.

Uribe Soto, S.I., Lehmann, T., Rowton, E.D., Vélez, I.D.B., Porter, C.H., 2001. Speciation and population structure in the morphospecies *Lutzomyia longipalpis* (Lutz and Neiva) as derived from the mitochondrial ND4 gene. Mol. Phylogenet. Evol. 18, 84–93.

Williams, P., 1993. Relationships of phlebotomine sand flies (Diptera). Mem. Inst. Oswaldo Cruz 88, 177–183.

Young, D.G., Duncan, M.A., 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, Associated Publishers—American Entomological Institute, Gainsville, FL.