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GENETIC RELATIONSHIPS AMONG SOME SPECIES GROUPS WITHIN THE GENUS *LUTZOMYIA* (DIPTERA: PSYCHODIDAE)

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Abstract. Molecular data on *Lutzomyia* are very scarce, despite the fact that this genus includes all the species involved in the transmission of leishmaniasis in America. We examine the genetic relationships among eight morphologic groups within the *Lutzomyia* genus and two *Brumptomyia* species, using nine enzyme loci and the last 285 basepairs of the mitochondrial cytochrome b gene. The structure of the genetic variation among the species analyzed indicated a closer genetic relationship among members of a morphologic group than between members of different groups. The lower levels of variation recorded among these groups compared with that between *Brumptomyia* and *Lutzomyia* suggest a subgeneric status for all of these groups, including *Psychodopygus*. A maximum likelihood tree for the allozyme data and a neighbor-joining consensus tree for the mitochondrial DNA sequences showed a general agreement with morphologic groups, with only minor differences. *Nyssomyia*, *Verrucarum* and *Micropygomyia* formed separate monophyletic groups. *Lutzomyia* could not be separated from *Psathyromyia,* and both *Migonei* species, *L. dubitans* and *L. migonei,* grouped in different clades according to the host species they are found on.

INTRODUCTION

Lutzomyia is the largest phlebotomine genus and contains more than 400 species and subspecies, including all American sand flies involved in the transmission of leishmaniasis.[1] Classification within this genus is still controversial because the accepted taxonomic groupings are based on morphologic characters that exhibit a great deal of variation among species, but fail to separate distinct groups.[2–5] For example, a single morphologic character may have a specific rank in one group, and a group rank in another.[4] Also, a species may appear more closely related to one group according to one sex but to a different group according to the other sex. The absence of a common character within groups results in several opinions on the weightings of each character, and therefore the stability of taxonomic groups. Conversely, there are controversial opinions about the supraspecific rank of some of these groups, as is the case for *Psychodopygus*. Ready and others[5] have argued that *Psychodopygus* differs sufficiently from other New World species to conform its own genus separated from the *Lutzomyia*; however, other investigators disagree with this position.[1,2]

Although phylogenetic relationships based on molecular data have been constructed for many *Phlebotomus* species,[6] for *Lutzomyia*, the molecular evidence comes from few representative species. For example, using the D2 domain of ribosomal DNA, Dujardin and others[7] suggested the monophyly of three *Lutzomyia* species (*L. longipalpis*, *L. migonei*, and *L. youngi*) in relation to some species from the *Phlebotomus* and *Sergentomyia* genera. Moreover, based on isozyme evidence, Dujardin and others[8] suggested a closer relationship between *Lutzomyia* and *Phlebotomus,* both genera involved in the transmission of *Leishmania*, than between *Phlebotomus* and other Old World genera. More recently, two studies exploring the relationship between species groups within the *Lutzomyia* genera were published.[9,10] One of these studies questioned the monophyletic status of the *Lutzomyia* subgenus and the *Migonei* group using a gene involved in the production of acoustic signals used during courtship in *Lutzomyia*.[9] The other study also failed to find support for the monophyly of these two groups using a different gene involved in both biologic rhythms and courtship song.[10] However, since molecular data is still limited for New World species, the genetic relationships among accepted morphologic groups within the *Lutzomyia* genus are poorly known.

DNA sequences and allozyme loci provide two independent genetic markers by which the cohesion of morphologic taxonomic groupings may be tested. Here we examine the genetic relationships among 20 species of *Lutzomyia* and *Brumptomyia* using nine allozyme loci and the last 285 basepairs of the mitochondrial cytochrome b gene. A previous study suggested the topologies of trees based on this gene reflect the phylogenetic relationship between species rather than populations.[11] In the transmission of different forms of leishmaniasis, phlebotomine species form transmission cycles with specific associations between *Leishmania* parasites, vector species and vertebrate hosts, and unique epidemiologic patterns. Thus, the genetic relationships between *Lutzomyia* vectors and non-vectors species seem fundamental for the understanding of the evolution of zoonotic transmission cycles.[12]

MATERIALS AND METHODS

Study sites. Adult sand flies were collected from eight locations across the northern basin of Venezuela and were transported to the laboratory in liquid nitrogen (Table 1). The head and distal abdominal tergites were separated and stored in 70% ethanol, whereas the remaining thorax and proximal tergites were stored at –70°C. The head and abdominal tergites were digested at 60°C for 10 minutes in either 10% KOH or Nesbitt solution (40 grams of chloralhydrate crystals, 10 mL of HCl, and 40 mL of water), then cleared in 100% phenol. Specimens were mounted in Berlese medium and examined under the light microscope. Species diagnosis was based mainly on the morphology of female structures (cibarium, pharynx, teeth, and spermathecae), and of the male terminalia.[1]

Allozyme analysis. Ninety-nine individuals from nine species were processed using standard vertical acrylamide gel electrophoresis as previously described.[12] An *Aedes aegypti* (L.) (Rockefeller strain) was included in each electrophoresis as a standard reference. Two continuous buffer systems were used to maximize allelic separation, Tris-citrate, pH 7.1, and

TABLE 1
Taxonomic relationships among the 20 phlebotomine sandfly species used in this study

Genus	Subgenus or species group	Species	Source
Lutzomyia	*Lutzomyia*	*Lutzomyia longipalpis* (Lutz & Neiva)	Altagracia 9°52′N, 66°23′W
		Lutzomyia pseudolongipalpis (Arrivillaga & Feliciangeli)	La Rinconada 9°59′N, 69°55′W
		Lutzomyia gomezi (Nitzulescu)	El Ingenio 10°31′N, 66°34′W
	Migonei	*Lutzomyia migonei* (Franca)	El Ingenio
		Lutzomyia dubitans (Sherlock)	El Ingenio
	Verrucarum	*Lutzomyia ovallesi* (Ortiz)	El Ingenio
		Lutzomyia townsendi (Ortiz)	Altos de Pipe 10°20′N, 66°55′W
	Psathyromyia	*Lutzomyia abonnenci* (Floch & Chassignet)	El Pilón 9°45′N, 68°34′W
		Lutzomyia punctigeniculata (Floch & Abonnenc)	El Ingenio
		Lutzomyia shannoni (Dyar)	El Pilón
	Nyssomyia	*Lutzomyia hernandezi* (Ortiz)	El Vigia 8°43′N, 71°39′W
		Lutzomyia whitmani (Antunes and Coutinho)	Ready et al. 1997 Accession Number: U80966
	Psychodopygus	*Lutzomyia panamensis* (Shannon)	San Esteban 10°26′N, 68°01″W
	Micropygomyia	*Lutzomyia absonodonta* (Feliciangeli)	Mapire 7°44′N, 64°42′W
		Lutzomyia cayennensis (Floch and Abonnenc)	El Ingenio
		Lutzomyia venezuelensis (Floch and Abonnenc)	Rancho Grande 10°22′N, 67°41′W
	Oswaldoi	*Lutzomyia trinidadensis* (Newstead)	El Ingenio
	Ungrouped	*Lutzomyia rangeliana* (Ortiz)	Altagracia
Brumptomyia		*Brumptomyia beaupertuyi* (Ortiz)	Rancho Grande
		Brumptomyia devenanzii (Ortiz and Scorza)	Altos de Pipe

Tris-borate-EDTA, pH 8.9. Five enzyme loci were resolved in Tris-citrate, pH 7.1: adenylate kinase (*Ak*, E.C.2.7.4.3), arginine kinase (*Ark*, E.C.2.7.3.3), isocitrate dehydrogenase (*Idh*, E.C. 1.1.1.42), glycerol-3-phosphate dehydrogenase (*Gpd*, E.C. 1.1.1.8), malate dehydrogenase (*Mdh*, E.C. 1.1.1.37) and phosphoglucomutase (*Pgm*, E.C.5.4.2.2). Four loci were resolved in Tris-borate-EDTA pH 8.9: fumarate hydratase (*Fum*, E.C.4.2.1.2), glucose-6-phosphate isomerase (*Gpi*, E.C.5.3.1.9), hexokinase (*Hk*, E.C.2.7.1.1) and malic enzyme (*Me*, E.C.1.1.1.40). In addition, allele frequencies previously published from 157 individuals from nine other species were included in the analysis.[13]

Allozyme data was analyzed using the Fortran program BIOSYS-1.[14] Genotypic frequencies were tested for Hardy-Weinberg equilibrium using a chi-square test, and Rogers genetic distances were calculated between pairs of species within groups, between pairs of species from different groups, and between pairs of species from different genera. After testing for normality of their distribution and homogeneity of their variances, the genetic distances were compared using analysis of variance (ANOVA) and Bonferroni post-hoc comparisons.[15] The cohesion of the subgeneric species groups classically accepted was explored using a maximum likelihood tree obtained from the program Phylip.[16] For this analysis, we excluded groups for which only one species was available because the maximum likelihood analysis would force these species, in the absence of partners from their own groups, into other groups.

Sequencing of DNA. A total of 26 specimens from 13 species were sequenced for the last 285 basepairs of the mitochondrial cytochrome b gene. The cytochrome b sequence from *L. whitmani*[3] was obtained from the National Center for Biotechnology Information (Bethesda, MD) (http://www.ncbi.nlm.nih.gov), accession number U80966.

Total genomic DNA was extracted from each sand fly using a proteinase K solution. Each sand fly (thorax plus abdominal tergites) was rehydrated for approximately 10 minutes in 500 μL of distilled water, or until it sank to the bottom of the water column. Proteinase K was dissolved in distilled water to give a concentration of 20 mg/ml and stored at -20°C until use. Ten milliliters of extraction buffer was prepared using 100 μL of the proteinase K dilution, 100 μL of 1M Tris-HCl, pH 8.3, 500 μL of 1 M KCl, 50 μL of 1% Tween 20 solution, 50 μL of 1% Nonidet P40, and 9.2 mL of double-distilled water. Individual sand flies were transferred into 100 μL of the proteinase K solution and ground using a pestle. Samples were vortexed and then incubated at 50°C overnight for approximately 15 hours, then held at 95°C for 10 minutes to denature the proteinase K. Samples were allowed to cool in a refrigerator, and then stored at -20°C until amplification by a polymerase chain reaction (PCR).

A volume of 1–3 μL of extracted DNA was added to each 50-μL PCR, which contained 10× PCR buffer (without $MgCl_2$), 1.5 mM $MgCl_2$, 500 μM dNTP mixture, 300 ng of each primer (forward and reverse), and one unit of *Taq* polymerase. A drop of mineral oil was added to the top of each reaction. The PCR was performed in a Perkin-Elmer (Norwalk, CT) thermal cycler according to the method of Ready and others,[3] and included an initial denaturation step at 94°C for three minutes; five cycles at 94°C for 30 seconds, 40°C for 30 seconds, and 72°C for 1.5 minuters; 35 cycles at 94°C for 30 seconds, 44°C for 30 seconds, and 72°C for 1.5 minutes; a final

extension period at 72°C for 10 minutes; and cooling to room temperature.

The forward primer (CB3-PDR) was 5′-CA(T/C)ATTCAACC(A/T)GAATGATA-3′ and the reverse primer (N1N-PDR) was 5′-GGTA(C/T)(A/T)TTGCCTC-GA(T/A)TTCG(T/A)TATGA-3′; further descriptions of these primers are available.[3] Positive reactions were purified using a High Pure PCR Product Purification Kit (catalog no. 1732668; Boehringer Mannheim, Indianapolis, IN). DNA was sequenced with both the CB3-PDR and N1N-PDR primers for each individual sand fly on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA) using the *Taq* FS dye rhodamine sequencing kit (Applied Biosystems). Sequences were confirmed using a basic alignment search tool (BLAST) (www URL http://www.ncbi.nlm.nih.gov), and by comparing the sequences obtained from the CB3-PDR (forward) primer to the complemented and reversed sequences obtained from the ND3-PDR (reverse) primer. DNA sequences were aligned using the program ClustalX,[17] and compared with the translated amino acid alignment. To explore how the different taxonomic levels (species, subgenus, and genus) contribute to the variation of F_{ST}, we performed an analysis of molecular variance (AMOVA)[18] using the program ARLEQUIN version 2.[19] The statistical significance of these contributions was tested by a permutation test implemented in the program. Genetic relationships were investigated using a neighbor-joining bootstrap consensus procedure with Kimura two-parameter distances using the program Phylip.[16]

RESULTS

Allozyme data. Allele frequencies varied among species (Appendix 1). Some loci were shared by groups of species, whereas for other loci we detected fixed differences between several species. Allele *Me* was the most variable locus with nine alleles, and *Ark* the least variable with only three alleles. Allele $Hk^{0.89}$ separated both *Brumptomyia* species from all *Lutzomyia* species, and $Me^{0.83}$ was unique to *L. panamensis.* All other alleles were shared by species groups (Appendix 1).

Genetic variability measurements also differed among species. Mean heterozygosities ranged from 1.5% to 18.9% with 1.0–1.7 alleles per locus except for *L. shannoni*, which had fixed alleles for all loci. All populations complied with Hardy-Weinberg equilibrium expectations ($P \geq 0.05$), indicating random mating within populations.

Genetic distances varied from 0.233 to 0.979 (Table 2). The largest distances were recorded between *L. rangeliana* (ungrouped) and *L. shannoni* (*Psathyromyia* group), and the smallest between *L. venezuelensis* and *L. absonodonta,* both members of the *Micropygomyia* group (Table 2). Genetic distances were normally distributed (intragroup Kolgomorov-Smirnov [K-S] = 0.115, P = 0.967; intergroup K-S = 0.047, P = 0.991; intergenera K-S = 0.135, P = 0.471) and their variances were homogeneous (χ^2 = 2, $P > 0.1$). Mean genetic distances for species within and between taxonomic groups are shown in Figure 1. The ANOVA showed that the taxonomic status of the species was a significant source of variation in the genetic distance between species ($F_{3,147}$ = 9.55, $P < 0.001$). The post hoc Bonferroni test indicated that the genetic distance between species from different subgeneric groups within the *Lutzomyia* genus was significantly larger than that between species within each group (Figure 1). Conversely, we could not detect a significant difference in the mean genetic distances between species from different genera (*Brumptomyia* versus *Lutzomyia*) and between subgeneric groups (Figure 1). That is, allozyme variation increased significantly from species to subgenera but not from subgenera to genera.

A similar pattern of distribution of variation among hierarchical taxonomic levels was found in the *F* statistics. Although the largest variance component in F_{ST} is explained by differentiation between species (81.1%), there is an additional 19.9% that may be attributed to differences between subgeneric groups. However, once the species and subgenera level are considered, differences between genera had no contribution to the variance of F_{ST}.

The maximum likelihood tree showed two distinct groups (Figure 2). The first group includes all the species of the

TABLE 2
Pairwise Rogers distance based on nine allozymic loci between 18 phlebotomine sandfly species from Venezuela*

	bea	*dev*	*ran*	*lon*	*plon*	*mig*	*dub*	*tow*	*pun*	*abo*	*sha*	*pan*	*atr*	*abs*	*cay*	*ven*	*yen*	*trin*
bea	–																	
dev	0.432	–																
ran	0.850	0.783	–															
lon	0.873	0.774	0.788	–														
plon	0.863	0.653	0.880	0.557	–													
mig	0.661	0.681	0.697	0.832	0.680	–												
dub	0.789	0.578	0.665	0.769	0.746	0.621	–											
tow	0.609	0.398	0.624	0.743	0.524	0.570	0.487	–										
pun	0.753	0.854	0.943	0.630	0.429	0.519	0.996	0.763	–									
abo	0.806	0.793	0.928	0.350	0.468	0.690	0.901	0.766	0.558	–								
sha	0.885	0.885	0.979	0.440	0.579	0.542	0.882	0.864	0.439	0.423	–							
pan	0.687	0.576	0.837	0.737	0.518	0.747	0.794	0.576	0.644	0.580	0.563	–						
atr	0.832	0.828	0.570	0.670	0.728	0.697	0.587	0.699	0.729	0.725	0.817	0.694	–					
abs	0.671	0.460	0.675	0.650	0.530	0.729	0.568	0.275	0.747	0.680	0.774	0.451	0.609	–				
cay	0.961	0.751	0.609	0.730	0.584	0.580	0.506	0.546	0.708	0.736	0.617	0.676	0.527	0.459	–			
ven	0.764	0.554	0.554	0.556	0.537	0.633	0.551	0.422	0.645	0.679	0.660	0.519	0.492	0.233	0.420	–		
yen	0.709	0.686	0.770	0.891	0.823	0.604	0.594	0.458	0.837	0.823	0.922	0.769	0.384	0.601	0.578	0.706	–	
tri	0.583	0.580	0.721	0.642	0.677	0.746	0.714	0.645	0.528	0.673	0.758	0.497	0.432	0.484	0.775	0.467	0.637	–

* *Brumptomyia beaupertuyi (bea), B. devenanzii (dev), Lutzomyia rangeliana (ran), L. longipalpis (lon), L. pseudolongipalpis (plon), L. migonei (mig), L. dubitanss (dub), L. townsendi (tow), L. punctigeniculata (pun), L. abonnenci (abo), L. shannoni (sha), L. panamensis (pan), L. atroclavata (atr), L. absonodonta (abs), L. cayenensis (cay), L. venezuelensis (ven), L. yencannensis (yen),* and *L. trinidadensis (tri).*

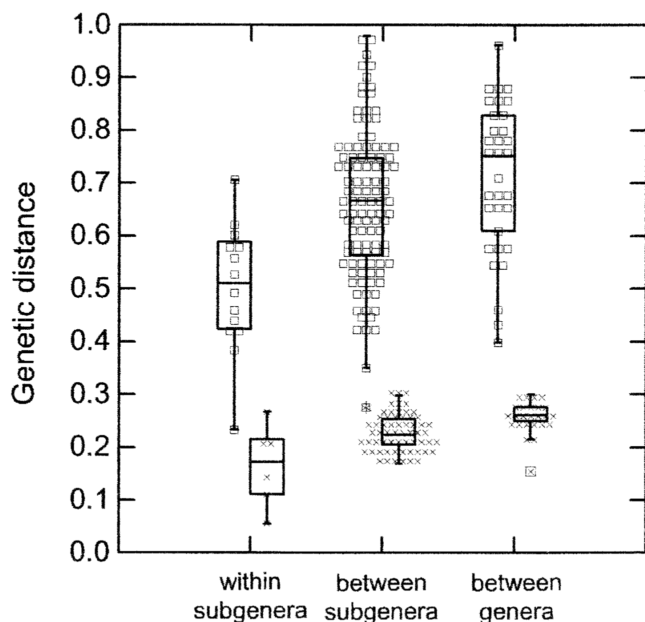

FIGURE 1. Genetic distances among species within subgeneric groups, between subgeneric groups, and between subgenera. The squares represent the Rogers distance based on nine allozyme loci and the ×s represent the Kimura two-parameter distance using the last 285 basepairs of the mitochondrial cytochrome b gene. The upper and lower limits of the boxes show the upper and lower quartiles. The horizontal line dividing each box is the median. The upper and lower limits of the vertical lines beyond the boxes show the largest and smallest observations within 1.5 interquartile ranges. Observations beyond these limits are plotted individually.

Micropygomyia group analyzed and one species of the *Migonei* group, *L. dubitans*. The second group intercalates species of the *Lutzomyia* and the *Psathyromyia* groups, including the other *Migonei* species, *L. migonei*.

DNA sequences. Cytochrome b sequences were deposited in GenBank under accession numbers AF448540– AF448546, and AY316731–AY316736. DNA sequences were successfully aligned and confirmed by eye using the amino acid alignment as a guide (Appendix 2). In every case, the two individuals that were sequenced from each species showed no sequence variation. The Kimura two-parameter genetic distances between species varied from 0.0554 to 0.3002 (Table 3). The smallest distance was recorded between both species from the *Nyssomyia* subgenus (*L. hernandezi* and *L. whitmani*) whereas the largest distances were between species from different genera (*L. gomezi* and *B. devenanzii*). The mean genetic distance between species was larger between groups than within groups, and the mean distance between genera was larger than that between groups within each genus (Figure 1). An AMOVA indicated that 45.0% of the variation in the molecular distance may be explained by differentiation between species, 39.3% to differentiation between subgenera, and the residual 15.6%, to differences between genera. Although the latter variance component is low, a permutation test on the genetic distance matrix showed that such a variance component could not be accounted for by random variation ($P < 0.0279$). This result indicates that variation in the DNA sequence analyzed increased significantly from species to subgenus, and from subgenus to genus.

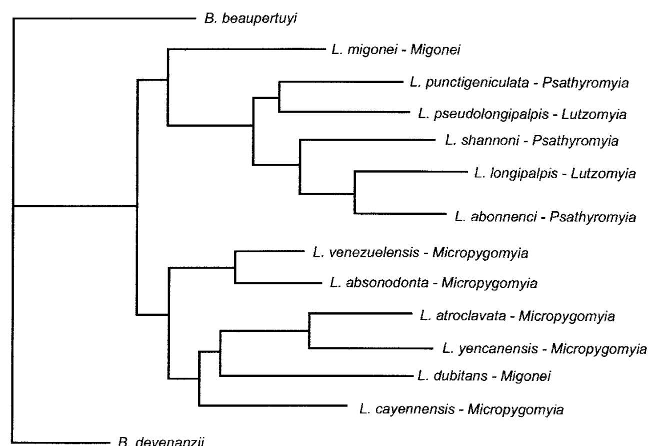

FIGURE 2. Maximum likelihood tree obtained from nine allozyme loci for 12 *Lutzomyia* and two *Brumptomyia* species from Venezuela. The input order was randomize with global rearrangements of species. *Brumptomyia beaupertuyi* was used as the outgroup.

The bootstrap consensus tree using neighbor-joining on the Kimura two-parameter distances showed two distinct evolutionary lineages with a high bootstrap value (99%) for the *Brumptomyia* and *Lutzomyia* genera (Figure 3). At the subgeneric level, four groups were formed with high bootstrap confidence (> 80%). The *Verrucarum* and the *Nyssomyia* species each formed a monophyletic group, and the two cryptic species belonging to the *Lutzomyia* subgenus, *L. longipalpis* and *L. pseudolongipalpis*, similarly formed a monophyletic group. However, the status of *L. gomezi*, also from the *Lutzomyia* subgenus, was not resolved. *Lutzomyia panamensis* from the *Psychodopygus* subgenus, which has been proposed as a distinct genus rather than subgenus, grouped together with the *Nyssomyia* species, with genetic distances much smaller than those between the well defined genera of *Brumptomyia* and *Lutzomyia*. All other species placements were unresolved by the cytochrome b fragment.

DISCUSSION

Three specific questions were addressed to explore the genetic relationships between the eight species groups analyzed: 1) is the genetic variation among the morphologic groups adopted by taxonomists different than that expected by random grouping of the species?, 2) are the levels of genetic variation observed among groups comparable to those found between different genera?, and 3) are the groups formed using phylogenetic trees consistent with the morphologic groups?

First, both allozyme data and DNA sequences from the mitochondrial cytochrome b gene showed levels of genetic variation between the morphologic groups that could not be explained solely by the random grouping between species. The groups classically adopted by *Lutzomyia* taxonomists accounted for a significant proportion of the genetic variation among species because the genetic distances recorded between members of a group were smaller than those recorded between members of different groups. The data indicates that morphologic species groups, to a certain extent, represent the genetic relationships among species.

Second, the smaller levels of DNA variation observed be-

TABLE 3

Kimura two-parameter distances based on the last 285 basepairs of the mitochondrial cytochrome b gene between 13 phlebotomine sandfly species from Venezuela*

	bea	*dev*	*ova*	*tow*	*lon*	*plon*	*tri*	*mig*	*cay*	*gom*	*dub*	*whi*	*her*	*pan*
bea	–													
dev	0.154	–												
ova	0.258	0.249	–											
tow	0.261	0.275	0.143	–										
lon	0.283	0.264	0.186	0.186	–									
plon	0.278	0.249	0.187	0.182	0.110	–								
tri	0.257	0.293	0.214	0.233	0.204	0.218	–							
mig	0.223	0.214	0.168	0.186	0.195	0.190	0.223	–						
cay	0.245	0.254	0.223	0.224	0.262	0.263	0.252	0.204	–					
gom	0.275	0.300	0.211	0.215	0.201	0.215	0.254	0.215	0.223	–				
dub	0.244	0.270	0.258	0.247	0.210	0.218	0.282	0.266	0.257	0.248	–			
whit	0.289	0.238	0.223	0.262	0.210	0.233	0.282	0.209	0.287	0.245	0.243	–		
her	0.283	0.263	0.237	0.252	0.205	0.232	0.266	0.199	0.297	0.261	0.223	0.055	–	
pan	0.262	0.258	0.190	0.227	0.199	0.228	0.287	0.209	0.256	0.275	0.252	0.186	0.173	–

* *Brumptomyia beaupertuyi (bea), B. devenanzii (dev), Lutzomyia ovallesi (ova), L. townsendi (tow), L. longipalpis, (lon), L. pseudolongipalpis (plon), L. trinidadensis (tri), L. migonei (mig), L. cayennensis (cay), L. gomezi (gom), L. dubitans (dub), L. whitmani (whi), L. hernandezi (her),* and *L. panamensis (pan).*

tween groups within *Lutzomyia* compared with those recorded between two of the recognized genera, *Brumptomyia* and *Lutzomyia,* suggests a subgeneric status as more adequate for these taxa than a generic one. Even for *Psychodopygus*, for which some investigators propose as a separate genus, the genetic evidence does not support a generic status for this subgenus. To treat the *Psychodopygus* as a separate genus may not adequately represent the close genetic relationships to the *Lutzomyia* species, particularly to the *Nyssomyia* group as seen in the cytochrome b gene fragment. Conversely, the allozyme data did not reveal any differences in the magnitude of the genetic variation between subgenera and genera. Allozymic differences not only tended to saturation below the genus level, but also the large variation intervals within subgenera masked the differences between subgenera and genera. We find that allozyme variation gives adequate resolution at the subgeneric level, but not at the generic level.

Third, the structure of groups shown in the trees agreed in general with the morphologic groups classically accepted, with only minor differences. The topography of the bootstrap consensus tree obtained with the DNA sequences showed no inconsistencies with the morphologic groups, despite the fact that the positions of some species were not resolved by the DNA fragment used in this study. The clades with high bootstrap values grouped species within morphologic groups, as was the case for the *Nyssomyia*, *Lutzomyia* and *Verrucarum* groups. For the *Nyssomyia group*, a close genetic relationship between *L. whitmani, L. intermedia,* and *L. umbratilis* was previously reported using the *period* gene.[10] The lack of resolution between *Lutzomyia* and *Psathyromyia* in the allozyme tree must be explored further with different genetic markers. Nonetheless, the distinct separation of the five *Micropygomyia* species from the *Lutzomyia* and *Psathyromyia* species suggests a close genetic relationship between the members of the *Micropygomyia*. Conversely, the position of both *Migonei* species suggests that this group could be polyphyletic, as suggested by the *cacophony* gene.[9] It is worth noting that one of the *Migonei* species, *L. dubitans,* who is known to feed on lizards, grouped with the *Micropygomyia* species who have also been associated with feeding on reptiles.[1] Similarly the other *Migonei* species, *L. migonei* who has been observed on domestic animals, clustered with a group well known for its domestic cycle, including the *Lutzomyia* and *Psathyromyia* subgenera. The lack of genetic data for most of the 400 species comprising the *Lutzomyia* genus with which to compare the results presented herein illustrates the need for more molecular data on this important taxonomic group.

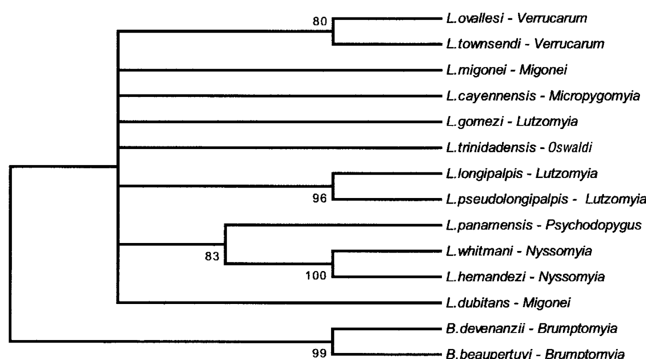

FIGURE 3. Neighbor-joining consensus tree of Kimura two-parameter distances based on the last 285 basepairs of the mitochondrial cytochrome b gene of two *Brumptomyia* and 12 *Lutzomyia* species from Venezuela. The numbers at each node represent the percent bootstrap support for 1,000 replicates; branches with bootstrap values less than 80% were collapsed.

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APPENDIX 1

Allele frequencies of nine enzyme loci from the sandflies *Brumptomyia beaupertuyi (bea), B. devenanzii (dev), Lutzomyia rangeliana (ran), L. longipalpis (lon), L. pseudolongipalpis (plon), L. migonei (mig), L. dubitanss (dub), L. townsendi (tow), L. punctigeniculata (pun), L. abonnenci (abo), L. shannoni (sha), L. panamensis (pan), L. atroclavata (atr), L. absonodonta (abs), L. cayenensis (cay), L. venezuelensis (ven), L. yencannensis (yen),* and *L. trinidadensis (tri)**

	bea	*dev*	*ran*	*lon*	*plon*	*mig*	*dub*	*tow*	*pun*	*abo*	*sha*	*pan*	*atr*	*abs*	*cay*	*ven*	*yen*	*tri*
Ak																		
N	13	11	5	53	13	30	17	5	5	4	4	16	5	10	6	5	4	11
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.36
2	1.00	0	0	0	0	0	0	0	0	0.38	0	0	0	0	0	0	0	0
3	0	1.00	0	0	1.00	0	1.00	1.00	0.10	0.50	0	1.00	1.00	1.00	1.00	1.00	1.00	0.64
4	0	0	1.00	0	0	1.00	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	1.00	0	0	0	0	0.90	0.12	1.00	0	0	0	0	0	0	0
Ark																		
N	13	14	1	59	19	30	10	5	3	2	3	13	1	6	1	7	3	12
1	0	0	0	1.00	1.00	0.03	0	0	1.00	1.00	1.00	1.00	1.00	1.00	0	1.00	0	1.00
2	1.00	1.00	0	0	0	0.97	1.00	1.00	0	0	0	0	0	0	0	0	1.00	0
3	0	0	1.00	0	0	0	0	0	0	0	0	0	0	0	1.00	0	0	0
Fum																		
N	13	14	7	44	18	28	18	7	4	5	4	16	6	14	6	9	6	13
1	0	0	0	0	0	0	0	0	0	0.10	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0.90	0	0	0	0	0	0	0.08	0
3	1.00	0.07	0.21	0	0	0.84	0	0	1.00	0	0	0	1.00	0	0	1.00	0.92	0.906
4	0	0	0.79	0	0	0	0	0	0	0	1.00	1.00	0	0	0	0	0	0
5	0	0.93	0	1.00	1.00	0.16	1.00	1.00	0	0	0	0	0	1.00	0	0	0	0.04
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.00	0	0	0
Gpi																		
N	7	7	4	53	13	29	15	6	5	5	4	9	7	14	7	6	3	6
1	0	0	0	0.01	0.89	0.03	0	0	0.10	0	0	0.33	0	0	0	0	0	0
2	0	0	0	0	0	0.86	0	0.08	0.90	0	1.00	0	0	0	0	1.00	0	0
3	0	0	0	0.88	0.11	0	0	0	0	0.60	0	0	0.86	0	0.93	0	0.67	0
4	1.00	1.00	0	0	0	0.03	0.87	0.42	0	0	0	0.67	0	0.93	0	0	0	1.00
5	0	0	0.75	0.10	0	0	0	0	0	0.40	0	0	0	0.07	0	0	0	0
6	0	0	0.25	0	0	0.07	0.13	0.42	0	0	0	0	0.14	0	0.07	0	0.33	0
7	0	0	0	0.01	0	0	0	0.08	0	0	0	0	0	0	0	0	0	0
Hk																		
N	13	14	8	59	19	30	18	7	4	5	4	16	7	13	7	9	5	13
1	0	0	0	0	0	0	0	1.00	0	0	0	0.19	0	1.00	0.64	0	1.00	0
2	0	0	0	0.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	1.00	0.93	0	0	1.00	0	0	0	0	0.03	1.00	0	0	1.00	0	1.00
4	0	0	0	0.01	1.00	1.00	0	0	1.00	1.00	1.00	0.78	0	0	0.36	0	0	0
5	1.00	1.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mdh																		
N	13	12	7	38	12	30	17	7	5	5	4	13	5	10	5	9	5	13
1	0	0	0	0	0	0	0	0	1.00	0	0	0	0	0	0	0	0	0.92
2	0	0	1.00	0	0	1.00	1.00	1.00	0	0	0	0	1.00	1.00	1.00	1.00	0.90	0
3	0	0	0	0	0	0	0	0	0	0	0	0.96	0	0	0	0	0	0.08
4	0	0	0	0.99	0.71	0	0	0	0	0.90	1.00	0.04	0	0	0	0	0	0
5	0	1.00	0	0	0	0	0	0	0	0.10	0	0	0	0	0	0	0.10	0
6	0	0	0	0.01	0.29	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0.92	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0.08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Me																		
N	13	14	6	29	16	11	16	6	2	4	3	16	1	1	1	7	6	13
1	0	0	0	0	0	0	0	0	0	0	0	0	1.00	0	0	0	1.00	1.00
2	0	0	0	0	0	0	0	0	0	0	0	1.00	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	1.00	0	0.93	0	0
4	0	0	1.00	0	0	0	0	0	0	0	0	0	0	0	0	0.07	0	0
5	0	0	0	0	0.06	0	1.00	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0.03	0.94	0.41	0	1.00	0.75	0.12	0	0	0	0	0	0	0	0
7	0	0	0	0.97	0	0	0	0	0.25	0.88	1.00	0	0	0	0	0	0	0
8	0	1.00	0	0	0	0.59	0	0	0	0	0	0	0	0	0	0	0	0
9	1.00	0	0	0	0	0	0	0	0	0	0	0	0	0	1.00	0	0	0

APPENDIX 1
Continued

	bea	*dev*	*ran*	*lon*	*plon*	*mig*	*dub*	*tow*	*pun*	*abo*	*sha*	*pan*	*atr*	*abs*	*cay*	*ven*	*yen*	*tri*
Gpd																		
N	1	1	6	1	1	1	13	1	1	2	4	1	4	1	5	1	5	1
1	1.00	1.00	0	1.00	1.00	1.00	0	1.00	1.00	1.00	1.00	1.00	0	1.00	0	1.00	0	1.00
2	0	0	1.00	0	0	0	0	0	0	0	0	0	1.00	0	0.40	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.50	0	1.00	0
4	0	0	0	0	0	0	1.00	0	0	0	0	0	0	0	0.10	0	0	0
Idh																		
N	1	1	1	14	1	30	11	1	5	5	4	1	6	1	5	1	4	1
1	1.00	1.00	1.00	0	0	0	0	1.00	0	0	0	1.00	0.42	1.00	0	1.00	0.50	1.00
2	0	0	0	0	0	1.00	1.00	0	0	0	1.00	0	0.58	0	1.00	0	0.50	0
3	0	0	0	1.00	0	0	0	0	0	1.00	0	0	0	0	0	0	0	0
4	0	0	0	0	1.00	0	0	0	1.00	0	0	0	0	0	0	0	0	0

* *Ak* = adenylate kinase; *Ark* = arginine kinase; *Fum* = fumarate hydratase, *Gpi* = glucose-phosphate isomerase; *Hk* = hexokinase; *Mdh* = malate dehydrogenase; *Me* = malicenzyme; *Gpd* = glycerol-3-phosphate dehydrogenase; *Idh* = isocitrate dehydrogenase.

APPENDIX 2

Multiple sequence alignment of the last 285 basepairs of the mitochondrial cytochrome b gene in New World phlebotomine sand flies*

	1												
B. devenanzii	CCC	AAT	AAA	CTG	GGG	GGA	GTA	ATT	GCT	CTT	GTT	ATA	TCT
B. beaupertuyi	..T	...	...	..T	..A	...	..T	..C	..C	...	...	...	..A
L. ovallesi	...	...	...	T.A	..A	...	...	...	...	T.A	...	...	..A
L. townsendi	..T	...	...	T.A	..A	...	...	...	...	T.A	...	...	...
L. longipalpis	..T	...	...	T.A	...	..T	..T	...	...	...	...	...	..A
L. pseudolongipalpis	..T	...	...	T.A	..A	...	..T	...	..A	...	..A	...	..A
L. trinidadensis	..T	...	...	...	...	...	...	...	...	...	...	...	..A
L. migonei	..T	...	...	..T	..T	...	...	...	..C	T.A	..A	...	..A
L. cayennensis	..A	...	..G	T.A	..A	...	...	...	..A	T.A	...	...	...
L. gomezi	..T	...	...	T.A	..A	...	...	...	...	..A	...	...	...
L. dubitans	..A	...	...	T.A	...	...	..T	..C	...	...	...	...	...
L. whitmani	..T	...	...	..T	..A	..T	..G	..C	..C	T.A	...	...	..A
L. hernandezi	..T	...	...	..T	...	..T	...	..C	..C	T.A	...	...	..A
L. panamensis	..A	...	...	..T	..A	...	...	...	...	...	...	...	...

	79												
B. devenanzii	AAA	TCT	CAA	GGA	TTA	CAA	TTT	TAT	CCT	TTA	AAT	CAA	ATT
B. beaupertuyi	...	...	...	...	...	...	...	..C	..C	...	..C	...	..C
L. ovallesi	...	ATA	...	..C	...	...	...	...	..A	...	...	...	...
L. townsendi	...	A.C	...	...	C.T	...	...	...	...	...	...	...	...
L. longipalpis	...	AGC	...	...	C.T	...	...	...	..A	A.T	...	...	..C
L. pseudolongipalpis	...	AG.	...	...	C.T	...	...	...	..A	A..	...	...	...
L. trinidadensis	...	.TC	...	..T	C.T	...	...	...	...	...	..C	...	...
L. migonei	...	A..	...	...	...	...	...	...	..A	...	...	...	...
L. cayennensis	...	ATA	...	...	...	...	...	..C	...	...	...	...	...
L. gomezi	..G	AGT	...	..C	C.T	...	...	..C	..A	G..	...	...	...
L. dubitans	..T	...	...	..G	C.T	...	..C	...	...	A.T	GC.	...	...
L. whitmani	...	AA.	...	...	C.C	...	...	..C	..A	A.T	...	...	...
L. hernandezi	...	AA.	...	...	C.C	...	...	...	..A	A.T	...	...	...
L. panamensis	...	AA.	...	...	...	...	...	...	..A	...	...	...	..C

	157												
B. devenanzii	TGA	ATT	GGG	GCT	CGT	CCT	GTA	GAA	GAC	CCC	TAC	GTT	CTT
B. beaupertuyi	...	...	..A	...	..G	...	...	...	A..	...	..T	A.C	..A
L. ovallesi	...	...	..A	..C	..C	...	..C	...	...	...	..T	A.C	T.A
L. townsendi	...	...	..A	..C	...	..A	...	...	...	..T	..T	A..	T.A
L. longipalpis	...	...	..A	..A	..G	...	..T	..G	...	..T	..T	A..	T.A
L. pseudolongipalpis	...	...	..A	...	..A	..A	..T	...	...	...	..T	A..	..A
L. trinidadensis	...	...	..T	..A	..G	...	...	...	...	..T	..T	A..	T.A
L. migonei	...	...	..A	..A	..C	...	..T	...	...	..A	..T	A..	T.A
L. cayennensis	...	...	..T	...	..C	...	...	...	...	..T	..T	A..	T.A
L. gomezi	...	...	...	..A	..C	...	..T	...	...	...	..T	A..	A..
L. dubitans	...	...	..A	..C	..A	...	..T	...	...	..T	..T	A..	...
L. whitmani	...	...	..A	..A	..G	...	..T	...	...	...	..T	A.C	...
L. hernandezi	...	..C	..A	..A	..G	...	..T	...	..T	..T	..T	A..	...
L. panamensis	...	...	..A	..C	..A	..A	..T	..G	.CA	..T	..T	A.C	A..

	235												
B. devenanzii	ATT	CTA	AAT	CCT	TTA	ATT	TCT	AAA	ATT	TGA	GAT	AAT	AAT
B. beaupertuyi	..C	A..	...	...	...	...	...	...	...	...	...	...	...
L. ovallesi	..C	A..	...	..G	C.T	G..	...	...	..C	...	...	..A	...
L. townsendi	..C	AC.	.CC	...	...	G..	...	...	...	...	...	..A	...
L. longipalpis	...	A.T	...	...	A.T	G.C	..A	...	..C	...	...	..A	.C.
L. pseudolongipalpis	..C	A.T	..C	...	A.T	..C	..A	...	...	...	...	..A	.CC
L. trinidadensis	...	..C	...	..A	A..	...	A.C	T.T	T.A	...	...	..A	...
L. migonei	T..	A..	...	..A	A..	...	..A	.TT	...	...	...	..A	...
L. cayennensis	...	A.C	...	...	...	...	...	TT.	TA.	...	...	..A	...
L. gomezi	...	A.T	...	..A	G..	G.C	...	...	...	...	..C	..A	..C
L. dubitans	...	A..	.GC	...	A.C	.GA	...	...	...	...	...	.CA	..C
L. whitmani	...	A.T	...	...	A..	G.A	..A	...	...	...	...	.G.	TA.
L. hernandezi	...	A.T	..C	...	A..	G.A	..A	...	...	...	...	...	TA.
L. panamensis	...	T..	..C	...	A..	G..	..A	...	...	...	...	...	TA.

Appendix 2
Continued

	40												
B. devenanzii	ATC	GCA	ATT	TTA	TTT	TTC	CTG	CCT	TTC	CTG	CAC	TTA	AAT
B. beaupertuyi	..T	...	..C	...	..C	..T	..T	..C	..T	T.A	..T	...	...
L. ovallesi	..T	...	..C	C..	...	..A	..C	..A	A.T	..T	..T	A..	.GA
L. townsendi	..T	..T	..C	C..	...	..A	T..	..A	A.T	..T	..T	A..	.G.
L. longipalpis	..T	..T	...	...	...	..T	..C	..A	A.T	..T	..T	ACT	.G.
L. pseudolongipalpis	..T	..T	...	...	...	..T	..C	..A	..T	..C	..T	AC.	.G.
L. trinidadensis	...	..T	...	...	..C	..T	..C	..G	A.T	..C	..T	AAT	.GA
L. migonei	..T	...	...	...	...	..T	..T	..C	..T	..T	..T	A..	.GA
L. cayennensis	..T	..C	...	...	...	..T	T.A	..C	..T	T.A	...	CA.	.GA
L. gomezi	..T	..T	...	...	..C	...	T.A	..C	A..	ACC	..T	A..	.GA
L. dubitans	..T	...	...	...	...	..T	..T	...	A..	T.A	..T	...	.G.
L. whitmani	..T	...	...	C.G	...	..T	..C	...	A.T	T.A	..T	A..	.GA
L. hernandezi	..T	...	...	C.C	...	..T	..T	...	A.T	T.A	..T	A..	.GA
L. panamensis	..T	...	..C	C..	...	..T	..T	..A	A.T	..T	..T	AC.	.GA

	118												
B. devenanzii	TTA	TTT	TGA	TAT	ATA	GTA	ATT	ATT	ATT	ATC	CTT	TTA	ACC
B. beaupertuyi	...	..C	...	...	...	...	...	...	..C	...	..C	...	..A
L. ovallesi	...	...	...	...	...	T..	...	...	G.A	..T	T.A	..G	..A
L. townsendi	...	...	...	...	C..	T..	...	...	G.A	C.T	T.A	...	...
L. longipalpis	...	...	...	...	...	T.T	...	...	G.A	..T	T.A	...	..A
L. pseudolongipalpis	...	...	...	...	...	T.T	...	...	G.A	..T	T.A	...	..A
L. trinidadensis	...	...	...	...	...	T..	..A	...	G.A	..T	T.A	...	..T
L. migonei	...	...	...	...	...	..T	...	.C.	...	..T	T.A	...	...
L. cayennensis	C..	...	...	...	...	T.T	G..	...	G.A	..T	...	...	..A
L. gomezi	...	...	...	...	...	T.T	G..	...	G..	...	..A	...	..T
L. dubitans	G..	...	...	...	...	T..	...	...	G.A	..T	T.A	...	..A
L. whitmani	...	...	...	..C	...	T..	...	...	G..	..T	T.A	...	..T
L. hernandezi	...	...	...	...	...	T..	...	...	G..	..T	T.A	..G	..T
L. panamensis	...	...	...	...	...	T.T	...	...	G.A	..T	T.A	...	..A

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B. devenanzii	ACT	GGC	CAA	ATT	CTT	ACT	GTT	CTC	TAT	TTT	GCT	TAT	TAT
B. beaupertuyi	...	..T	..G	...	..A	...	...	..T	...	...	...	...	...
L. ovallesi	..A	...	...	...	T.A	..A	...	A.T	..C	...	TTA	..C	...
L. townsendi	..A	..T	...	...	T.A	...	...	G.T	..C	..C	CTA	...	..C
L. longipalpis	...	..T	...	...	T.A	...	..A	..T	...	...	TTA	...	...
L. pseudolongipalpis	..A	..T	...	...	T.A	...	..C	..T	..C	...	TTA	...	...
L. trinidadensis	..A	..A	...	...	T.A	..A	...	G..	..C	...	TTA	...	...
L. migonei	...	..T	...	...	T.A	...	...	A.T	..C	...	ATA	...	..C
L. cayennensis	..A	..A	...	..C	..C	...	A..	A.T	...	...	ATA	...	..C
L. gomezi	...	..T	...	..C	T.A	..A	...	A.T	..C	...	TTA	..C	..C
L. dubitans	...	..A	...	..C	...	..C	..A	A.T	..C	...	TTA	...	.T.
L. whitmani	..C	..A	...	T.A	T.A	...	...	A.T	..C	...	CT.	...	...
L. hernandezi	...	..A	...	T.A	T.A	...	...	A.T	..C	...	CT.	...	...
L. panamensis	...	...	...	T.A	T.G	..A	A..	A.T	...	...	ATA	...	...

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B. devenanzii	TTA	AAC	AAT	TAA
B. beaupertuyi	...	C..	...	...
L. ovallesi	...	...	C.A	...
L. townsendi	...	..T	C.A	..G
L. longipalpis	...	..T	T..	..G
L. pseudolongipalpis	C.T	...	C.C	..G
L. trinidadensis	C.T	...	.CA	...
L. migonei	...	..T	C.A	...
L. cayennensis	...	..T	...	...
L. gomezi	...	..T	...	..G
L. dubitans	...	..T	...	...
L. whitmani	...	GCA	...	...
L. hernandezi	C..	GCA	...	...
L. panamensis	...	TCA	...	...

* *B. = Brumptomyia; L. = Lutzomyia.*