

MALE SEX PHEROMONES AND THE PHYLOGEOGRAPHIC STRUCTURE OF THE *LUTZOMYIA LONGIPALPIS* SPECIES COMPLEX (DIPTERA: PSYCHODIDAE) FROM BRAZIL AND VENEZUELA

PHILLIP C. WATTS, J. GORDON C. HAMILTON, RICHARD D. WARD, HARRY A. NOYES, NATALY A. SOUZA, STEPHEN J. KEMP, M. DORA FELICIANGELI, REGINALDO BRAZIL, AND RHAYZA D. C. MAINGON*

School of Biological Sciences, Liverpool University, Liverpool, United Kingdom; School of Life Sciences, Keele University, Staffordshire, United Kingdom; Departamento de Entomologia, Oswaldo Cruz Institute, Rio de Janeiro, Brazil; Centro Nacional de Referencia de Flebotomos, BIOMED, Universidad de Carabobo, Maracay, Venezuela; Departamento de Bioquímica e Biología Molecular, Laboratorio de Bioquímica e Fisiología de Insetos, Rio de Janeiro, Brazil

Abstract. *Lutzomyia longipalpis*, a sibling complex, is the main vector of *Leishmania chagasi/infantum*. Discriminating between siblings is important as they may differ in vectorial capacity. *Lutzomyia longipalpis* populations display distinct male sex pheromone chemotypes. We investigated the phylogeographic pattern of variation at microsatellite loci from 11 populations from Brazil and Venezuela related to their male pheromone. Temporal genetic differentiation was mostly not significant at the same site. Spatial genetic differentiation was, however, strong, although there was only a weak relationship between genetic differentiation and the geographic distance separating the samples ($r^2 < 0.10$); geographic separation explained a much greater (54–97%) percentage of the genetic differences among populations when samples with the same pheromone type were analyzed separately. A cluster analysis showed five groups: *Lu. cruzi* (Brazil) and *Lu. pseudolongipalpis* (Venezuela) as separate species, two (mostly 9-methyl-germacrene-B) Venezuelan and Brazilian groups, and a very distinct cluster of Brazilian cembrene populations.

INTRODUCTION

The sand fly *Lutzomyia longipalpis* (Lutz & Neiva 1912) (Diptera: Psychodidae: Phlebotominae) is widespread throughout Central and South America, between central Mexico and northern Argentina,¹ where it is often associated with the peri-domestic environment of rural communities. *Lutzomyia longipalpis* is the principal vector of the parasite *Leishmania chagasi* (Cunha and Chagas 1937) that is currently believed to be imported *Leishmania infantum* from the Mediterranean.² *Lutzomyia longipalpis* has received more scientific attention than any other New World sand fly since *L. chagasi/infantum*, which is responsible for recurrent large-scale epidemics of potentially fatal visceral leishmaniasis.^{3–9} One subject of debate is the taxon status of *Lu. longipalpis*.¹⁰ The value of this taxonomic research was highlighted by the observation of dissimilar clinical manifestations of leishmaniasis caused by genetically similar *L. chagasi/infantum* in different areas of Central and South American. The clinical pleomorphism observed was thought to reflect variations in levels of the *Lu. longipalpis* salivary vasodilatory protein maxadilan.^{11–14}

With the important exception of *Lu. pseudolongipalpis* (Arrivillaga & Feliciangeli 2001),¹⁵ morphological characters have not consistently provided useful characteristics for distinguishing *Lu. longipalpis* sibling species. In this context, Mangabeira first reported that *Lu. longipalpis* (*Phlebotomus longipalpis*) was either highly polymorphic or more than one species.¹⁶ He also proposed that the north and northeastern Brazilian populations of *Lu. longipalpis* that differ in the number of pairs of pale patches (spots) on the abdomen of males could be a separate species.¹⁶ However, it has been subsequently demonstrated that some populations whose males were characterized by different spot morphology could interbreed while *Lu. longipalpis* from different areas but with

similar spot phenotype did not always interbreed.¹⁷ In *Lu. longipalpis*, the spots are the visual manifestation of glandular areas underlying the paler cuticle surface that release sex pheromones that have been shown to influence the sexual behavior of *Lu. longipalpis*.^{18,19} Four male sex pheromone chemotypes have been identified in Brazilian populations: (S)-9-methyl-germacrene-B (9MGB), 3-methyl- α -himachalene (3MAH) together with α -himachalene (AH), cembrene diterpene (CEMB), and a fourth cembrene isomer.^{19–24} Male copulation/courtship songs are strikingly distinct between populations in which males have different pheromone chemotypes.^{18,25,26} Furthermore, populations with distinct pheromone types and copulation love songs have shown sequence differences for the speciation²⁷ *period* gene.^{28,29} Since love songs and male attractant pheromones are used in maintaining speciation by providing pre-zygotic mating barriers,²⁷ their genes are likely to be significant components in the genetic structuring of the *Lu. longipalpis* group.

Genetic evidence for a *Lu. longipalpis* species complex has been reported over large distances, usually between countries, but the taxonomic status of *Lu. longipalpis* within a particular geographic region remains more controversial.^{15,27–42} Among Venezuelan populations, differences in genetic and morphological characteristics have resulted in the first formal description of a new species from the *Lu. longipalpis* species complex, *Lu. pseudolongipalpis* Arrivillaga & Feliciangeli 2001.¹⁵ In contrast, variation at allozyme loci between populations of *Lu. longipalpis* from different areas within Brazil has been interpreted as consistent with a single but genetically heterogeneous species.^{30,32,35–40} More recently, the distribution of mitochondrial DNA haplotypes in Central and South American *Lu. longipalpis* led the investigators to conclude that although there are several *Lutzomyia* clades, there is probably only a single, recently established Brazilian clade.^{38,39} The results of these studies conflict with those of breeding experiments,^{17,19} pheromone analyses,^{19–23} and those on the pattern of genetic variation in the *periodicity* and *cacophony* genes between Brazilian populations of *Lu. lon-*

* Address correspondence to Rhayza D. C. Maingon, School of Life Sciences, Keele University, Staffordshire, ST5 5BG, United Kingdom. E-mail: r.d.c.maingon@biol.keele.ac.uk

gipalpis, which together have provided convincing evidence for the presence of separate clades in Brazil.^{28,29,41–43} Furthermore, for at least one Brazilian location in the northeastern state of Ceará (Sobral), there is cumulative unambiguous evidence (lack of cross-mating in the laboratory, distinct pheromones and copulation songs, genetic variation) for reproductive isolation between two sympatric populations that can best be described as *bona fide* species,^{17,19,29,42} although the contemporary level of gene flow needs to be reassessed to ascertain whether speciation is relatively recent.⁴³ Part of the apparent misunderstanding about the phylogeographic structure of this taxon appears to have arisen from differential mutation rates among the genetic markers used and from studies largely conducted without considering pre- and post-cross-mating barriers.

Accurate epidemiologic data is required for efficient control of any disease. Therefore, establishing correct taxonomic distinctions between putative sibling species of *Lu. longipalpis* is likely to contribute to the basic foundation upon which a successful management strategy to control New World visceral leishmaniasis may be built. Furthermore, the degree of gene flow between *Lu. longipalpis* siblings is likely to affect the spread of insecticide resistance genes, as has been suggested for malaria associated mosquitoes in sub-Saharan Africa.⁴⁴

The objective of this study was to use variation at microsatellite loci to investigate genetic differences between populations of *Lu. longipalpis* from South America (Venezuela and Brazil), with particular emphasis on the male sex pheromone type. We have also included samples from two taxonomically recognized species to provide a context to the pattern and strength of genetic differences between *Lu. longipalpis* siblings from Brazil and Venezuela with contrasting sex pheromones.

MATERIALS AND METHODS

Sand fly populations. Sand flies were collected from 11 localities in Brazil and Venezuela between 1997 and 2001 (Table 1 and Figure 1); three of these localities (Guayabita, *Lu. pseudolongipalpis* at La Rinconada, Curarigua, and Sobral) were sampled twice. The Brazilian populations of Jaco-

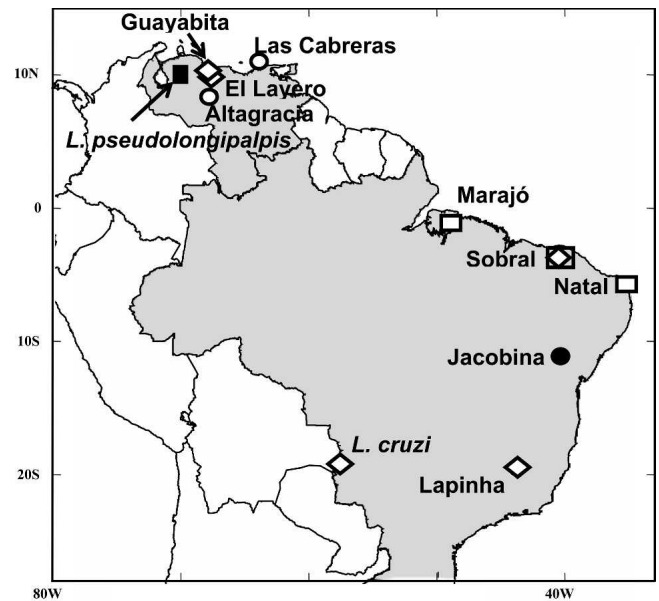


FIGURE 1. Geographic distribution of samples of the sand flies *Lutzomyia longipalpis*, *Lu. cruzi*, and *Lu. pseudolongipalpis* collected from Brazil and Venezuela. ◇, □, ■, and ● indicate that males release (S)-9-methyl-germacrene-B, cembrene, 3-methyl-α-himachalene (3MAH) or (3MAH plus α-himachalene) sex pheromones, respectively, and ○ indicate that the type of sex pheromone is unknown.

bina, Lapinha, Marajó, Natal, and *Lu. cruzi* were laboratory-established colonies derived from isofemale lines from females collected at the sites indicated in Table 1 and reared in the laboratory as described.⁴⁵ The Jacobina colony was used at approximately 120 generations whereas the Lapinha, Marajó, Natal, and *Lu. cruzi* colonies were 100, 20, 16, and 3 generations, respectively.

The field-collected sand fly populations included two populations in sympatry at Sobral, Ceará State, Brazil: Sobral 1S (one pair of pale spots; 9MGB), and Sobral 2S (two pairs of spots; CEMB); in which the spot phenotype coincides, and therefore can be related, with the pheromone chemotype. Previous reports on the Sobral *Lu. longipalpis*,^{26,29,42} have indicated that the 9MGB and CEMB chemotypes at Sobral

TABLE 1

Species, sampling location and sampling date, source of individuals and type of male sex pheromone for populations of the sand fly genus *Lutzomyia* included in this study

Species	Locality	Sample date	Source	Pheromone
Venezuela				
<i>L. longipalpis</i>	Altagracia, Guárico State	08/01/01	Wild	Not known
<i>L. longipalpis</i>	El Layero, Guárico State	07/12/01	Wild	9-methyl-germacrene-B
<i>L. longipalpis</i>	Guayabita, Aragua State	12/09/97	Wild	9-methyl-germacrene-B
		07/21/01		
<i>L. longipalpis</i>	Las Cabrerías, Nueva Esparta State	01/25/01	Wild	Not known
<i>L. pseudolongipalpis</i>	La Rinconada, Curarigua Lara State	12/11/97	Wild	3-methyl-α-himachalene
		11/03/00		
Brazil				
<i>L. longipalpis</i>	Jacobina Bahia State	07/30/01	Colony	3-methyl-α-himachalene/α-himachalene
<i>L. longipalpis</i>	Lapinha, Minas Gerais State	08/30/01	Colony	9-methyl-germacrene-B
<i>L. cruzi</i>	Corumba and Ladario, Mato Grosso do Sul State	09/12/01	Colony	9-methyl-germacrene-B
<i>L. longipalpis</i>	Marajó, Pará State	08/30/01	Colony	Cembrene
<i>L. longipalpis</i>	Natal, Rio Grande do Norte State	10/01	Colony	Cembrene
<i>L. longipalpis</i>	Sobral, Ceará State	11/04/99	Wild	Cembrene
		04/06/01		9-methyl-germacrene-B

are different species, and as such were treated as separate population samples in this study. Females from the Sobral sample were allocated a pheromone type using the assignment test described.⁴² The Sobral samples were collected between November 1 and 4, 1999 and again between April 3 and 6, 2001 (Table 1).

The Venezuelan *Lu. longipalpis* were collected using Centers of Disease Control (CDC) light traps on December 9, 1997 and again on July 21, 2001 at Guayabita in Aragua State; on July 12, 2001 in El Layero and August 1, 2001 in Altigracia in Guárico State; and on January 18–25, 2001 in Las Cabrerías on Margarita Island in Nueva Esparta State. *Lutzomyia pseudolongipalpis* Arrivillaga and Feliciangeli 2001 samples included males caught in CDC light traps on December 11, 1997 in La Rinconada, Curarigua in Lara State, and a smaller field collection of females collected at the same original site using CDC light traps in October 2000. The pheromone type at each location has been identified for all but two of the samples (Altigracia and Las Cabrerías).

Sand fly specimens were preserved in 98% (v/v) ethanol until DNA extraction.

Genotyping of sand flies. DNA was extracted from decapitated sand flies, homogenized in 100 μ L of lysis buffer (80 mM NaCl, 172 mM sucrose, 130 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.5% [w/v] sodium dodecyl sulfate), followed by incubation at 65°C for 30 minutes and protein precipitation with potassium acetate.^{46,47} The DNA was precipitated with ethanol and resuspended in 30 μ L of sterile water. One microliter of DNA was used for a polymerase chain reaction (PCR) in a 10- μ L reaction volume that contained 75 mM Tris-HCl, pH 8.9, 20 mM (NH₄)₂SO₄, 0.01% [v/v] Tween 20, 0.2 mM of each dNTP, 3.0 mM MgCl₂, 100 ng of each primer, and 0.25 units of *Taq* polymerase (ABgene, Epsom, United Kingdom). We genotyped all sand flies at five dinucleotide microsatellite loci, LIST6-002, LIST6-004, LIST6-006, LIST6-012, and LIST6-029, which were isolated from the genome of a *Lu. longipalpis* colony derived from Jacobina, Brazil.⁴⁷ Thermal cycling conditions of loci are described elsewhere.⁴⁷ For allele visualization, the forward primers flanking the microsatellite loci LIST6-002, LIST6-004, LIST6-006, and LIST6-012 were 5' labeled (Invitrogen Ltd., Paisley, United Kingdom) with D2, D4, D4, and D3 fluorescent dyes, respectively. The forward primer flanking LIST6-029 was 5' labeled with IRD700 (MWG Biotech UK Ltd., Inc., Milton Keynes, United Kingdom). The PCR products from LIST6-002, LIST6-004, LIST6-006, and LIST6-012 were pooled along with a 400-basepair (D1) size standard (Beckman Coulter, Fullerton, CA) and separated by capillary electrophoresis through a denaturing polyacrylamide gel on a Ceq2000XL automated sequencer (Beckman Coulter). Alleles were sized using the cubic model of analysis in the Ceq2000XL fragment analysis software (Beckman Coulter). Genotypes at the LIST6-029 locus were obtained by separating alleles on a 6% (w/v) denaturing polyacrylamide gel on a LiCor4200 apparatus (MWG Biotech UK Ltd.) and sized alongside a *microSTEP*-24a ladder (Microzone Ltd., Haywards Heath, United Kingdom).

Data analysis. Deviation from genotypic linkage equilibrium among all locus-pair combinations was estimated.⁴⁸ Observed and expected genotype frequencies at each locus were tested for deviation from Hardy-Weinberg equilibrium (HWE) conditions for every sample (different sample dates

from the same location were tested separately) using the HWE test available in ARLEQUIN VERSION 2.001.⁴⁹ Genetic diversity was characterized from the number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), and Wright's inbreeding coefficient (f).⁵⁰ All statistics were calculated for each locus and each sample separately, and also across all loci for N_a and f . N_a , H_o , and H_e were calculated using the program POPULATIONS version 1.2.28.⁵¹

The extent of genetic differentiation between samples was investigated by calculating fixation indices based on an infinite allele model because allele sizes were not consistent with a stepwise mutational model and F_{ST} -based approaches are considered to be more robust when less than 20 loci are used.⁵² Genetic differentiation between samples was assessed from the unbiased estimator (θ) of Weir and Cockerham⁵³ of the F_{ST} of Wright⁵⁰ calculated using ARLEQUIN version 2.001.⁴⁹ The significance of the estimates of genetic differentiation among samples was assessed with 10,000 permutations of genotypes. We first estimated the extent of genetic differences between sand flies collected from the same location but on different sampling dates, and then estimated pairwise genetic differentiation between all locations but with samples from the same location and different collection dates pooled (to increase the sample size at each site).

Isolation by distance genetic structure was examined from the correlation of pairwise estimates of genetic differentiation between samples (samples from the same location but different sample dates were treated separately) against the corresponding logarithms of geographic distance (log km) separating the populations.⁵⁴ ARLEQUIN version 2.001⁴⁹ was used for this test, with genetic distance defined as $\theta/(1 - \theta)$.⁵⁵ A Mantel test (10,000 permutations) was used to assess the significance of any correlation between the genetic and geographic distances using the permutation procedure implemented by ARLEQUIN version 2.001.⁴⁹ Partial correlations were also undertaken for the 9MGB and the CEMB samples separately (there were too few 3MAH samples for a meaningful analysis). A neighbor-joining tree⁵⁵ of pairwise estimates of θ between pooled samples was constructed using POPULATIONS version 1.2.28.⁵¹ The robustness of the tree was evaluated from 500 bootstrap resamplings across individuals.

RESULTS

Genetic diversity. Individual genotype data obtained from 493 sand flies from 11 different locations in South America (Figure 1) yielded 89 alleles across all 5 microsatellite loci. Summary characteristics for all samples and microsatellite loci are provided in Tables 1 and 2, respectively. The number of alleles per locus varied from 11 at LIST6-029 to 23 at LIST6-004 over all individuals, with between 1 and 13 alleles per sampled population. The mean number of alleles per sampled population and over all loci ranged between 2.2 and 9.2, although it was never greater than 6 in any colony-raised sample (Table 2). The number of alleles was correlated with sample size (Pearson product-moment correlation [ρ], $r = 0.631$, degrees of freedom [df] = 15, $P = 0.009$) and this relationship was significant only for the wild-caught sand fly samples (ρ wild-caught, $r = 0.771$, df = 10, $P = 0.005$; colony $r = 0.677$, df = 4, $P = 0.209$) when the samples were ana-

TABLE 2
Description of genetic variation, at 5 microsatellite loci in 493 sand flies from 11 *Lutzomyia longipalpis* populations collected in Brazil and Venezuela and one *Lutzomyia cruzi* population*

Year <i>n</i>	GUY					LPS			SOG					SOC		
	ALT 19	LAS 32	ELY 26	1997 20	2001 25	1997 11	2000 9	JAC 70	LAP 21	LCZ 29	1999 62	2001 37	MAR 21	NAT 23	1999 57	2001 31
LIST6-002																
<i>N_a</i>	5	4	3	4	5	4	5	5	5	5	9	8	6	6	13	12
<i>H_a</i>	0.563	0.500	0.462	0.600	0.720	0.636	0.778	0.900†	0.800	0.448	0.710	0.702	0.286†	0.304†	0.429†	0.581†
<i>H_o</i>	0.675	0.430	0.541	0.558	0.693	0.688	0.778	0.614	0.733	0.543	0.859	0.813	0.481	0.616	0.823	0.827
<i>f</i>	0.100	–	0.149	–	–	0.014	–	–	–	0.171	0.162	0.140	0.365	0.512	0.479	0.292
		0.235		0.078	0.040		0.047	0.471	0.145							
LIST6-004																
<i>N_a</i>	5	2	4	2	4	1	2	7	5	5	11	12	1	2	7	11
<i>H_a</i>	0.158†	0.419	0.360	0.400	0.320	–	0.125	0.743	0.714	0.758	0.710	0.811	–	0.174	0.088	0.177†
<i>H_o</i>	0.606	0.405	0.313	0.328	0.318	–	0.241	0.778	0.739	0.704	0.894	0.876	–	0.202	0.136	0.321
<i>f</i>	0.731	–	–	–	–	–	0.000	0.042	–	–	0.199	0.075	–	–	0.267	0.448
		0.037	0.152	0.226	0.126			0.014	0.091					0.073		
LIST6-006																
<i>N_a</i>	8	8	6	9	9	1	1	7	6	4	8	6	4	6	9	9
<i>H_a</i>	0.316†	0.451†	0.720	0.789	0.792	–	–	0.643	0.750	0.310	0.645	0.417	0.810†	0.957	0.351†	0.305†
<i>H_o</i>	0.684	0.641	0.757	0.869	0.805	–	–	0.700	0.652	0.309	0.678	0.529	0.574	0.716	0.486	0.409
<i>f</i>	0.541	0.299	0.027	0.089	0.012	–	–	0.082	–	–	0.023	0.186	–	–	0.259	0.228
									0.213	0.108			0.478	0.346		
LIST6-012																
<i>N_a</i>	6	3	3	4	5	2	2	3	2	8	4	5	6	6	11	9
<i>H_a</i>	0.444	0.406	0.538	0.650	0.520	0.545	0.444	0.508	0.333	0.793	0.451	0.594	0.500†	0.670	0.807	0.726
<i>H_o</i>	0.554	0.498	0.520	0.627	0.544	0.519	0.627	0.518	0.396	0.751	0.507	0.567	0.763	0.796	0.836	0.819
<i>f</i>	0.145	0.187	–	–	–	–	0.158	0.006	0.162	–	0.111	–	0.327	0.108	0.033	0.113
			0.085	0.038	0.016	0.053				0.057		0.050				
LIST6-029																
<i>N_a</i>	4	5	4	4	7	3	4	5	3	5	6	4	4	4	6	5
<i>H_a</i>	0.263†	0.313†	0.231†	0.150†	0.280†	0.364	0.333†	0.522	0.563	0.517	0.414†	0.382†	0.556	0.435	0.351†	0.356†
<i>H_o</i>	0.475	0.609	0.564	0.606	0.797	0.325	0.778	0.579	0.587	0.628	0.644	0.689	0.492	0.729	0.566	0.522
<i>f</i>	0.443	0.468	0.573	0.737	0.639	–	0.556	0.078	0.029	0.172	0.333	0.431	–	0.365	0.379	0.320
						0.127							0.133			
All loci																
<i>N_a</i>	5.6	4.4	4.0	4.6	6.0	2.2	2.8	5.4	4.2	5.4	7.6	7.0	4.2	4.8	9.2	9.2
<i>f</i>	0.397	0.177	0.122	0.121	0.142	–	0.217	–	–	0.022	0.168	0.156	0.036	0.134	0.281	0.255
						0.040		0.048	0.054							

* *n* = sample size; *N_a* = number of alleles; *H_a* = observed heterozygosity; *H_o* = expected heterozygosity; *f* = inbreeding statistic; † indicates a significant (*P* < 0.05 after sequential Bonferroni correction, *k* = 5) deficit of heterozygotes from expected HWE proportions; – = locus monomorphic and no test carried out; ALT = Atlagracia; LAS = Las Cabrerías; ELY = El Layero; GUY = Guayabita; JAC = Jacobina; LAP = Lapinha; LCZ = L. cruzi; SOG = Sobral (9MGB); MAR = Marajó; NAT = Natal; SOC = Sobral (CEMB).

lyzed separately according to their origin. Despite the apparent effects of sample size and origin upon the number of alleles, there were no differences overall in either the number of alleles, expected heterozygosity, or observed heterozygosity at any locus between wild-caught and colony sand fly samples ($P > 0.05$ for all tests, by Mann-Whitney U test for all tests) except for H_o at LIST6-029 ($P = 0.002$, by Mann-Whitney U test).

Just less than one third (23 of 76) of the locus sample combinations tested showed a significant deviation from expected HWE conditions after a sequential Bonferroni correction⁵⁶ for multiple testing within samples ($\alpha = 0.05$, $k = 10$), which for all but one case (Jacobina LIST6-002) was due to a deficit of heterozygotes (Table 2). At least one significant heterozygote deficit was observed at each locus, although almost half (10 of 23) of the deviations from HWE were observed at LIST6-029. Three (*Lu. pseudolongipalis* 1997, Lapinha, and *Lu. cruzi*) of the 16 samples demonstrated no deviations from expected HWE conditions (Table 2). The inbreeding coefficient varied considerably from -0.471 to 0.737 at individual microsatellite loci and between -0.048 and 0.397 over all loci (Table 2).

Linkage disequilibrium between loci was significant ($P < 0.05$) for 18 out of the 145 possible pairwise tests (15 tests were not made because loci were monomorphic), although only 8 of these comparisons were significant ($P < 0.05$) after a within-sample ($k = 10$) sequential Bonferroni correction.⁵⁶ The Natal sample, demonstrated a relatively disproportionate amount of linkage disequilibrium (3 significant locus comparisons, data not shown), but because the majority of samples were in linkage equilibrium, all loci were retained for the data analyses.

Temporal genetic differentiation. Only *Lu. pseudolongipalis* showed significant genetic differences between samples collected on different sampling dates (Sobral CEMB, $\theta = 0.0018$, $P = 0.472$; Sobral 9MGB, $\theta = 0.0049$, $P = 0.281$; Guayabita, $\theta = 0.0219$, $P = 0.052$; *Lu. pseudolongipalis*, $\theta = 0.160$, $P < 0.001$). Since this difference was likely to be a statistical effect of the small number of individuals collected from La Rinconada, Curarigua (see Discussion), genotypes from the same locality were pooled for all samples for the analyses of spatial genetic differentiation.

Spatial genetic differentiation. Genetic differentiation between samples was generally high, with θ values ranging from

0.024 (Las Cabrerías-El Layero) to 0.558 (Marajó *Lu. pseudolongipalis*); the pairwise estimates of θ were significant ($P < 0.05$) for all comparisons. All estimates of θ ($P < 0.05$) involving *Lu. cruzi* and *Lu. pseudolongipalis* were greater than 0.176 and 0.388 , respectively. Genetic differentiation between the two samples with different sex pheromones at Sobral was as great (or higher) as many of the comparisons between samples that are separated by considerable distances (Table 3).

Although there was a significant ($P = 0.0014$) correlation between genetic differentiation ($\theta/1 - \theta$) and the geographic distance (log km) between sample localities ($r = 0.31$) for all samples, its effect was rather weak with distance, accounting for less than 10% of the variation in genetic differences among samples (Figure 2a and Table 4). This relatively explanatory capability of geographic distance is a result of large genetic differences between samples with different pheromones at small and intermediate geographic distances (Figure 2). There was a marked increase in the strength of isolation by distance genetic structure when the samples with CEMB and 9MGB pheromones were analyzed separately, with geographic separation accounting for 97% and 54%, respectively, of the genetic variation between samples (Table 4). The correlation between geographic distance and genetic differentiation was significant only for the 9MGB samples ($P = 0.0028$) after a sequential Bonferroni correction ($\alpha = 0.05$, $k = 3$), most likely because the small numbers of CEMB samples available to examine this relationship decreased the statistical power of this test.

A neighbor-joining tree⁵⁵ of θ showed five groups: *Lu. pseudolongipalis* by itself (3MAH); a well-supported (mostly 9MGB) clade containing Altagracia, Guayabita, El Layero, and Las Cabrerías from Venezuela; *Lu. cruzi* by itself (a 9MGB chemotype); a well-supported and distinct CEMB clade that consists of the Marajó, Sobral (CEMB), and Natal samples; and a group containing the remaining samples (Sobral 9MGB, Jacobina 3MAH plus AH, and Lapinha MGB) from Brazil. Phylogeographic trees based upon other genetic distance metrics produced identical tree topologies.

DISCUSSION

Our analysis of the phylogeographic structure of South American *Lutzomyia* produced four main outcomes: 1) much

TABLE 3

Pair-wise estimates of genetic differentiation (θ), between samples of 11 populations of the *Lutzomyia longipalpis* species complex from Brazil and Venezuela*

	Venezuela					Brazil					
	ALT	LAS	ELY	GUY	LPS	JAC	LAP	LCZ	SOC	SOG	MAR
LAS	0.1809										
ELY	0.1575	0.0235									
GUY	0.1537	0.0420	0.0361								
LPS	0.4951	0.5217	0.5122	0.4610							
JAC	0.2929	0.3092	0.2965	0.2753	0.4372						
LAP	0.3361	0.3677	0.3574	0.3192	0.4165	0.1983					
LCZ	0.3407	0.3863	0.3675	0.3424	0.4507	0.3150	0.3464				
SOC	0.3466	0.3861	0.3718	0.3673	0.4571	0.3118	0.3406	0.3197			
SOG	0.2271	0.2539	0.2405	0.2307	0.3883	0.1420	0.1698	0.1760	0.2745		
MAR	0.4246	0.4466	0.4403	0.4013	0.5584	0.2832	0.4164	0.3832	0.1505	0.2997	
NAT	0.3696	0.3880	0.3924	0.3347	0.4632	0.3170	0.3581	0.2809	0.1496	0.2790	0.1864

* All pair-wise comparisons are significant ($P < 0.05$) after a sequential Bonferroni correction ($k = 11$). ALT = Altagracia; LAS = Las Cabrerías; ELY = El Layero; GUY = Guayabita; LPS = *Lutzomyia pseudolongipalis*; JAC = Jacobina; LAP = Lapinha; LCZ = *Lutzomyia cruzi*; SOC = Sobral CEMB; SOG = Sobral 9MGB; MAR = Marajó; NAT = Natal.

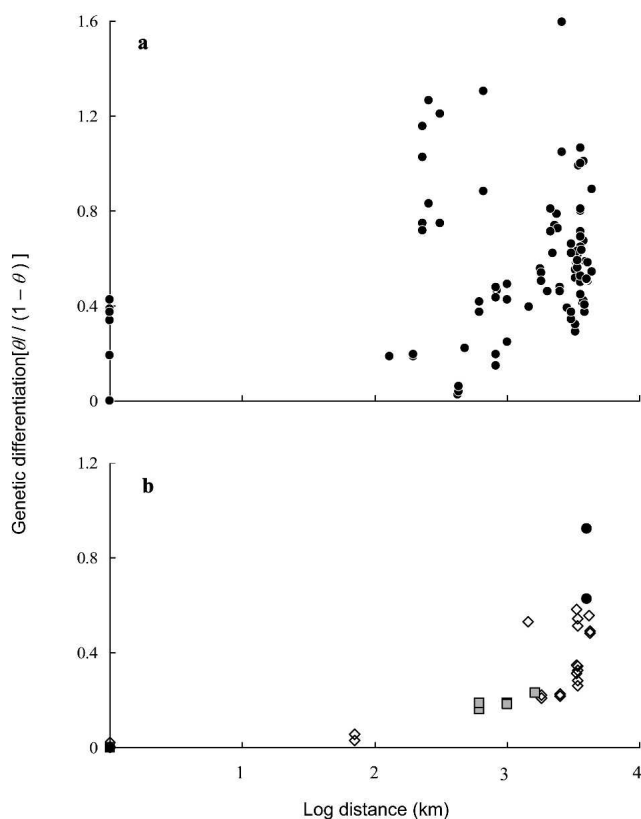


FIGURE 2. Relationship between geographic distance between samples (log km) and the corresponding estimate of pairwise genetic distance measured by $[\theta/(1 - \theta)]$ for samples (samples from the same site but collected during different years were treated separately) of the *Lutzomyia longipalpis* species complex from Brazil and Venezuela. **a**, Pairwise comparisons. **b**, Comparisons between samples that possess the same type of sex pheromone only. 9MGB \diamond = (S)-9-methyl-germacrene-B; \square = cembrene; \bullet = 3-methyl- α -himachalene.

of the genetic differentiation between populations reflects geographic separation, 2) the type of sex pheromone released by the male overrides geographic distance effects upon the phylogeographic structure of *Lu. longipalpis*, 3) these data provide further support for the taxonomic descriptions of *Lu. cruzi* and *Lu. pseudolongipalpis*, and 4) the data agree with reported differences in male copulation songs, a second phenotype likely to be involved in sexual selection in *Lu. longipalpis*.^{25,26}

Microsatellite loci have become the mainstay of modern population genetics,⁵⁷ but it is relevant that two processes may reduce genetic variability and therefore affect the rela-

TABLE 4

Mantel test results of the relationship between genetic distance $[\theta/(1 - \theta)]$ and the geographic separation (log km) for pairs of samples of the *Lutzomyia longipalpis* species complex from Brazil and Venezuela*

	<i>b</i>	<i>R</i>	<i>r</i> ²	<i>P</i>
All samples	0.0994	0.3076	0.0947	0.0014
9-methyl-germacrene	0.1234	0.7354	0.5409	0.0028
Cembrene	0.0643	0.9825	0.9652	0.0390

* *b* = regression slope; *R* = correlation coefficient; *r*² = coefficient of determination; *P* = significance of correlation coefficient.

tionships between our samples. First, longer (and typically more polymorphic) alleles may be restricted to the focal species (used to isolate the microsatellites).⁵⁸ We found no evidence for this ascertainment bias because although there was significant variation (different sampling dates were pooled) in allele length among samples (*df* = 11, *P* < 0.001 for all loci, by Kruskal-Wallis test), the size ranges of alleles were significantly longer in the Jacobina sample at only two loci and generally overlapped between most samples. Second, this study like many others used sand flies from experimental colonies that may have lost genetic variation through a founder effect. The influence of colonization upon genetic variability and its potential effect upon understanding the genetic relationships of *Lu. longipalpis* has been questioned, with genetic differences noted for some⁵⁹ but not all^{60,61} comparisons between field and colony samples. We could not explicitly test for a loss of variation (there are too few loci for a meaningful bottleneck analysis), but noted that although we observed fewer alleles in the laboratory populations, there were no statistically robust differences in genetic variability between field and colony samples. Furthermore, although Mukhopadhyay and others⁵⁹ reported a reduction in genetic variability in laboratory-reared *Lu. longipalpis* from Lapinha compared with its field counterpart, the two sampled populations clustered together but separately from other Brazilian populations in a genetic distance-based dendrogram.⁵⁹ We believe that although there was undoubtedly a reduction of genetic variation in the colonized material, relative to the field sampled sand fly populations, the magnitude of this reduction did not appreciably affect the phylogeographic substructuring shown in Figure 2. A similar phylogenetic tree to that shown in Figure 3 was obtained when the two long-term colony populations (Lapinha and Jacobina) were excluded from the analysis. This indicates that the effect of colonization was negligible in this instance. However, the results presented



FIGURE 3. Phylogeographic structure of the *Lutzomyia longipalpis* species complex based on an unrooted neighbor-joining tree inferred from θ .⁴⁸ Dashed line separates Brazilian and Venezuelan samples. Only bootstrap values greater than 50% are shown. \diamond , \square , \bullet , and \bullet indicate that males release (S)-9-methyl-germacrene-B, cembrene, 3-methyl- α -himachalene (3MAH) or (3MAH plus α -himachalene) sex pheromones, respectively, and \circ indicates that the type of sex pheromone is unknown.

here would be strengthened by the use of more loci and additional field-caught sample sets.

There was apparent temporal genetic stability in all but in one field population, *Lu. pseudolongipalpis*, which was caught at its original description site (La Rinconada, Curarigua), which is approximately 20 km from El Paso where a cryptic subspecies of *Lu. longipalpis* has been detected.³³ Although we may have inadvertently collected separate species during different sampling trips, and this cannot be ruled out without further sampling, it is considered unlikely because the significant variation in θ is due to differences in allele frequency (probably because of small sample sizes and sampling error) at a single locus (LIST6-002), rather than different alleles *per se*. Furthermore, the phylogenetic structure based upon samples from individual years was identical to that with pooled groups and displayed 100% bootstrap support for the two *Lu. pseudolongipalpis* samples on their own distinct branch. Although fine-scale genetic differences between temporal collections of the same population might exist, the genetic stability found in this study allowed us to increase sample size through pooling collections made on different dates in the same location.

Geographic distance between *Lu. longipalpis* populations was a relatively poor predictor of the level of genetic differentiation among populations when all samples were considered regardless of male sex pheromone chemotype (Figure 2a and Table 4). We note that not only are there genetic differences between Brazilian *Lu. longipalpis per se*, but that the type of sex pheromone shapes the genetic structure of this sand fly taxon. This conclusion is apparent from the contrasting patterns of isolation by distance with and without regard to the sex pheromone (Figure 2) and the phylogeographic clustering by pheromone, particularly the striking separation of the sympatric Sobral samples between two Brazilian species or clades that are based on different chemotypes (Figure 3). Three independent studies^{29,42,43} showed strong genetic differentiation between sympatric populations with 9MGB and CEMB pheromones at Sobral, although the magnitude of these differences in one of the studies was lower at Sobral than among other members of the *Lutzomyia* species complex.⁴³ Although two previous reports described genetic differentiation between a sample from Sobral and other Brazilian populations, because neither study distinguished between pheromone types, it is difficult to interpret their data with respect to speciation processes relevant to *Lu. longipalpis*.^{31,61}

The smaller genetic differences among Venezuelan samples (except *Lu. pseudolongipalpis*) compared with those from Brazil reflects the variation in geographic scale between samples (Table 3 and Figure 3). To what extent the genetic divergence between Venezuelan samples represents restricted gene flow or the presence of sibling species is unclear without breeding experiments. All sex pheromone-typed Venezuelan populations (with the exception of *Lu. pseudolongipalpis*) were of the wide spread 9MGB type (also in Honduras, Guatemala, Colombia, and Bolivia, as well as the Brazilian Lapinha population).^{19,62,63} The collection sites for the Venezuelan study collections (El Layero, Las Cabrerías, Guayabita, and La Rinconada in Venezuela) were located in tropical dry forest type of vegetation. The role of the Chivacoa Gap in isolating the Laran Clade (*Lu. pseudolongipalpis*) from other *Lu. longipalpis* populations

has been previously discussed,³⁸ although isolation is not complete since *Lu. pseudolongipalpis* (3MAH) was found in sympatry with a 9MGB population at El Paso, only 8 km from La Rinconada, Curarigua.³³ The Brazilian CEMB populations were located in drier sites along the northeastern region (Marajó and São Luis Islands, Natal, João Pessoa, Las Jaibas-Maceió, Alagoas, and Santarem).^{19,24,63–65} The Jacobina population (the only known 3MAH/AH population) lies in a narrow valley between two mountain ranges east of the Chapada Diamantina Ridge and well separated from the nearest sex pheromone-typed populations. However, at Sobral (a dry caatinga region in northeastern Brazil), two genetically differentiated 9MGB and CEMB populations share the same ecologic habitat.^{19,42} It would be interesting to estimate the relative contribution of pre-mating and post-mating barriers to gene flow between these sympatric populations.

This albeit incomplete contemporary geographic distribution of the pheromone chemotypes and the weak correlation between genetic and geographic distances described begs an explanation. The simplest one, based on ecologic distribution, would assume 9MGB as the ancestral chemotype in *Lu. longipalpis* across the savannahs of South America, followed by subsequent speciation to either 3MAH/AH or CEMB. Forest refuges arose in the dry climatic periods of the cyclical oscillations of cool versus warm ~100,000-year intervals characteristic of the Pleistocene and Recent epochs of the Quaternary period and have been shown to foster increased rates of speciation and biodiversity.⁶⁶

Given the size of Brazil, the presence of sibling species in this region should not be surprising. However, the controversy regarding the taxonomy of *Lu. longipalpis* generally results from the pattern of genetic differentiation among Brazilian samples, which has been interpreted as representing members of the same biologic species or clade.^{12,30,32,35–40} However, genetic divergence in the *per* intron and in the *cac* IVS6 intron among the populations from Jacobina, Lapinha, and Natal supports the occurrence of sibling species or near species in Brazil.^{28,29,41,42} Overall, the pattern and magnitude of genetic differences between Brazilian sand fly samples reported here is consistent with other studies, importantly including male songs and sex pheromones that indicate that *Lu. longipalpis* exists as four or five cryptic species or clades within Brazil. These species (siblings, clades, or races) appear to be sibling 1, cembrene pheromone, and burst-type copulation song in northeastern Natal, Marajó, and Sobral CEMB populations; sibling 2, 9MGB pheromone and song pattern I pulse copulation song, at Lapinha Cave; sibling 3, 9MGB pheromone and song pattern II pulse copulation song at Sobral; sibling 4, (3MAH plus AH) pheromone and polycyclic pulse copulation song at Jacobina; and a possible sibling 5, cembrene isomer pheromone at Jaibas in Minas Gerais State.^{16–24} The present number of known siblings in the South American subcontinent would be six, including the 3MAH-producing *Lu. pseudolongipalpis* Arrivillaga & Feliciangeli 2001 from La Rinconada and El Paso in the Lara State of Venezuela.¹⁵ Current genetic data have been interpreted as consistent with a single species of *Lu. longipalpis* in Central America, but given the presence of several cryptic species in Venezuela and Brazil, we believe that these data should be treated with caution.^{10–14,30–32,67} Our data supports only one (*Lu. pseudolongipalpis*) of the four (cis and trans Andean, *Lu. pseudolongipalpis* or Laran, and Brazilian) clades sug-

gested by isoenzyme and mitochondrial analyses.^{38,39} These analyses were interpreted to support a single clade in Brazil, despite a population collected on the Brazilian side of the Roraima formation that clustered with the trans-Andean clade and not within the Brazilian clade.^{38,39} In these reports, it was concluded that the large genetic distance between the four clades supports vicariance (separation or division of a group of organisms by a geographic barrier) as the most significant force in their evolution.^{38,39} As shown in Figure 2, our data for six Brazilian *Lu. longipalpis* populations contests this conclusion.

Furthermore, most studies have overlooked the distinct CEMB pheromone clade with regard to its effect on the patterns and processes of genetic evolution in Brazilian sand fly populations. For example, an absence of isolation by distance genetic structure has been reported,^{36,37,40} although three of the populations examined (Lapinha, Jacobina, and Santarém) produced different sex pheromones.¹⁹ Where there are sufficient samples, as observed for the 9MGB samples in Figure 3, we demonstrated a stronger pattern of isolation by distance (the Venezuelan and the Brazilian clades) between populations with similar sex pheromones than that observed between all populations. Although genetic differences between Brazilian samples are expected to arise through geographic separation within a particular sex pheromone clade, speciation processes (i.e., pre-zygotic barriers) other than vicariance are clearly shaping evolution within the *Lu. longipalpis* complex. From the pattern of sample clustering (Figure 3), we suspect that males from Las Cabrerias and possibly Altagracia possess 9MGB or a related sex pheromone.

Lutzomyia pseudolongipalpis is as distinct from the main Venezuelan clade of *Lu. longipalpis* as is *Lu. cruzi* from the Brazilian *Lu. longipalpis* samples (Figure 3). Female *Lu. cruzi* (a 9MGB population) and *Lu. longipalpis* are indistinguishable from each other, both transmit *L. chagasi/infantum* to humans, and have been found in sympatry in Mato Grosso do Sul State in Brazil.^{68,69}

Genetic differences between *Lu. pseudolongipalpis* and other Venezuelan sand fly populations have been reported,^{33,34} and our data support the recent taxonomic redescription of *Lu. longipalpis* from La Rinconada, Curarigua.¹⁵ It is significant that *Lu. pseudolongipalpis* does not cluster with the other 3MAH sample (Jacobina, Brazil). Although this could largely be an effect of geographic separation, genetic differences are also expected from the male sex pheromone chemistry of these samples because in contrast to *Lu. pseudolongipalpis*, males from the Jacobina *Lu. longipalpis* population produce the unmethylated α -himachalene (AH) in addition to 3MAH (Table 1).⁶² The Jacobina (3MAH plus AH) population mapped within the Brazilian 9MGB cluster in the phylogenetic tree in Figure 3. This agrees with previous *per* gene intron analyses, which indicated either introgression or the presence of common ancestral polymorphisms between these two homosesquiterpene-producing populations.²⁸

To conclude, it is important to recognize that genetic differences between allopatric populations are in most instances insufficient evidence for the presence of cryptic speciation. Our immediate aim was not to define the taxonomic status of *Lu. longipalpis*, but to reinforce the importance of sex pheromone type (and other behavioral traits) with respect to the pattern of genetic differentiation in *Lutzomyia* sp., and emphasize that if these traits are ignored then phylogeographic

patterns attributed to geography will be confounded by speciation due to putative mate-recognition systems. The identification of candidate mating barriers and their genetic determinants is a prerequisite to progress our understanding of the mechanisms that drive speciation within *Lu. longipalpis*. Considerable progress in this area is being achieved in the *Drosophila* genus and in the *Anopheles gambiae* complex, whose genomes have been sequenced.^{27,44,70}

Investigating the epidemiology of leishmaniasis in the light of these new taxonomic distinctions would seem an appropriate step towards controlling this disease. It is interesting that Brazil has most of the cases of visceral leishmaniasis in the New World and that of the 3,000–4,000 cases of visceral leishmaniasis reported in Brazil annually during 1999–2000, more than 90% were contributed by the northeastern states of Piauí, Maranhão, Bahia, and Ceará,^{4–6,9} and the northern states of Pará, Rio Grande do Norte, and Roraima,^{7,8} which is precisely the area occupied by one of the cembrene isomer producing siblings (CEMB clade in Figure 3) of *Lu. longipalpis*.

Received May 27, 2004. Accepted for publication May 23, 2005.

Acknowledgments: We thank Jose Wellington and Lindbergh Souza (Fundação Nacional de Saúde, Ceará, Brazil) and Arturo Bravo (BIOMED, Maracay, Venezuela) for technical assistance in the field. We are also grateful to Emma Jones for sand fly decapitations.

Financial support: This study was supported by the Wellcome Trust (London, United Kingdom), Universidad de Carabobo (Maracay, Venezuela) (project CDCH-FCS-2001-005), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Brazil), and Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (Brazil).

Authors' addresses: Phillip C. Watts and Stephen J. Kemp, School of Biological Sciences, Liverpool University, Crown Street, Liverpool, L69 7ZB, United Kingdom, Telephone: 44-151-795-4512, Fax: 44-151-795-4408, E-mails: p.c.watts@liv.ac.uk and s.j.kemp@liv.ac.uk. J. Gordon C. Hamilton, School of Life Sciences, Keele University, Staffordshire, ST5 5BG, United Kingdom, Telephone: 44-178-258-3026, Fax: 44-178-258-3516, E-mail: bia28@keele.ac.uk. Richard D. Ward, School of Life Sciences, Keele University, Staffordshire, ST5 5BG, United Kingdom, Telephone: 44-178-258-3417, Fax: 44-178-258-3516, E-mail: bia40@keele.ac.uk. Harry A. Noyes, School of Biological Sciences, Liverpool University, Crown Street, Liverpool, L69 7ZB, United Kingdom, Telephone: 44-151-794-7334, Fax: 44-151-795-4408, E-mail: harry@liv.ac.uk. Nataly A. Souza, Departamento de Entomologia, Oswaldo Cruz Institute, Av. Brasil 4365, Manguinhos, CEP 21045-900, Rio de Janeiro, Brazil, Telephone: 55-21-598-4320, Fax: 55-21-257-34468, E-mail: souzana@ioc.fiocruz.br. M. Dora Feliciangeli, Centro Nacional de Referencia de Flebotomos, BIOMED, Universidad de Carabobo, Nucleo Aragua, Maracay, Apartado 4873, Venezuela, Telephone and Fax: 58-243-242-0559, E-mail: mdora@telcel.net.ve. Reginaldo Brazil, Departamento de Bioquímica e Biología Molecular, Laboratorio de Bioquímica e Fisiología de Insetos, Av. Brasil 4365, Rio de Janeiro 21 045-900, Brazil, E-mail: rpbrasil@ioc.fiocruz.br. Rhayza D. C. Maingon, School of Life Sciences, Keele University, Staffordshire, ST5 5BG, United Kingdom, Telephone: 44-178-258-4219, Fax: 44-178-258-3516, E-mail: r.d.c.maingon@biol.keele.ac.uk.

Reprint requests: Rhayza D. C. Maingon, School of Life Sciences, Keele University, Staffordshire, ST5 5BG, United Kingdom.

REFERENCES

1. Young DG, Duncan MA, 1994. *Guide to the Identification and Geographic Distribution of Lutzomyia Sand Flies in Mexico, West Indies, Central and South America (Diptera: Psychodidae)*. Gainesville, FL: American Entomologic Institute.
2. Mauricio IL, Howard MK, Stothard JR, Miles MA, 1999. Geno-

- mic diversity in the *Leishmania donovani* complex. *Parasitology* 119: 237–246.
3. Ashford RW, Desjeux P, de Raadt P, 1992. Estimation of population at risk of infection and number of cases of leishmaniasis. *Parasitol Today* 8: 104–105.
 4. Arias JR, Monteiro PS, Zicker F, 1996. The re-emergence of visceral leishmaniasis in Brazil. *Emerg Infect Dis* 2: 145–146.
 5. Author unknown, 2003. *Brazil: Health Situation Analysis and Trends Summary*. Accessed April 5, 2004. Available from <http://www.paho.org>
 6. Franke CR, Ziller M, Staubach C, Latif M, 2002. Impact of the El Niño/southern oscillation on visceral leishmaniasis, Brazil. *Emerg Infect Dis* 8: 914–917.
 7. Guerra JAO, Barros MLB, Ferreira Fé N, Guerra MVF, Castellon E, Gomes Paes M, Sherlock IA, 2004. Leishmaniose visceral entre índios no Estado de Roraima, Brasil. Aspectos clinicoepidemiológicos de casos observados no período de 1989 a 1993. *Rev Soc Bras Med Trop* 37: 305–311.
 8. Marzochi MC de A, Marzochi KBF, 1994. Tegumentary and visceral leishmaniasis in Brazil – Emerging anthroponosis and possibilities for their control. *Cad Saude Publica* 10: 359–375.
 9. Alves Gama ME, Lopes Costa JM, Castro Gomes CM, Pereira Corbett CE, 2004. Subclinical form of American visceral leishmaniasis. *Mem Inst Oswaldo Cruz* 99: 889–893.
 10. Uribe S, 1999. The status of the *Lutzomyia longipalpis* species complex and possible implications for *Leishmania* transmission. *Mem Inst Oswaldo Cruz* 94: 729–734.
 11. Warburg A, Saraiva E, Lanzaro GC, Titus RG, Neva F, 1994. Saliva of *Lutzomyia longipalpis* sibling species differs in its composition and capacity to enhance leishmaniasis. *Philos Trans R Soc Lond B Biol Sci* 345: 223–230.
 12. Lanzaro GC, Warburg A, 1995. Genetic variability in phlebotomine sandflies: possible implications for leishmaniasis epidemiology. *Parasitol Today* 11: 151–154.
 13. Noyes HA, Chance M, Ponce C, Ponce E, Maingon RDC, 1997. *Leishmania chagasi*: genotypically similar parasites from Honduras cause both visceral and cutaneous leishmaniasis in humans. *Exp Parasitol* 85: 264–273.
 14. Yin H, Norris DE, Lanzaro GC, 2000. Sibling species in the *Lutzomyia longipalpis* complex differ in levels of mRNA expression for the salivary peptide, maxadilan. *Insect Mol Biol* 9: 309–314.
 15. Arrivillaga JC, Feliciangeli MD, 2001. *Lutzomyia pseudolongipalpis*: The first new species within the *longipalpis* (Diptera: Psychodidae: Phlebotominae) complex from La Rinconada, Curarigua, Lara State, Venezuela. *J Med Entomol* 38: 783–790.
 16. Mangabeira O, 1969. Sobre a sistemática e biologia dos *Phlebotomus* do Ceará. *Rev Bras Malariol Doenças Trop* 12: 3–26.
 17. Lane RP, Ward RD, 1984. The morphology and possible function of abdominal patches in males of two forms of the leishmaniasis vector *Lutzomyia longipalpis* (Diptera: Phlebotominae). *Cah ORSTOM Ser Entomol Med Parasitol* 22: 245–249.
 18. Ward RD, Ribeiro A, Ready PD, Murtagh A, 1983. Reproductive isolation between different forms of *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae) the vector of *Leishmania donovani chagasi* Cunha & Chagas and its significance to Kala-azar distribution in South America. *Mem Inst Oswaldo Cruz* 78: 269–280.
 19. Ward RD, Phillips A, Burnet B, Marcondes CB, 1988. The *Lutzomyia longipalpis* complex: reproduction and distribution. Service MW, ed. *Biosystematics of Haematophagous Insects*. Oxford, United Kingdom: Oxford University Press, 258–269.
 20. Hamilton JGC, Dawson GW, Pickett JA, 1996a. 9-Methylgermacrene B, a novel homosequiterpene from sex pheromone glands of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Lapinha, Brazil. *J Chem Ecol* 22: 1477–1491.
 21. Hamilton JGC, Dawson GW, Pickett JA, 1996b. 3-Methyl- α -himachalene; sex pheromone of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Jacobina, Brazil. *J Chem Ecol* 22: 2331–2340.
 22. Hamilton JGC, Hooper AM, Ibbotson HC, Kurosawa S, Mori K, Muto S, Pickett JA, 1999(a). 9-Methylgermacrene-B is confirmed as the sex pheromone of the sandfly *Lutzomyia longipalpis* from Lapinha, Brazil, and the absolute stereochemistry is defined as S. *Chem Comm* 2335–2336.
 23. Hamilton JGC, Hooper AM, Mori K, Pickett JA, Sano S, 1999(b). 3-Methyl- α -himachalene is confirmed, and the relative stereochemistry defined, by synthesis as the sex pheromone of the sandfly *Lutzomyia longipalpis* from Jacobina, Brazil. *Chem Comm* 355–356.
 24. Hamilton JGC, Brazil RP, Maingon RDC, 2004. A fourth chemotype of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Jaíba, Minas Gerais State, Brazil. *J Med Entomol* 41: 1021–1026.
 25. Souza NA, Ward RD, Hamilton JGC, Kyriacou CP, Peixoto AA, 2002. Copulation songs in three siblings of *Lutzomyia longipalpis* (Diptera: Psychodidae). *Trans R Soc Trop Med Hyg* 96: 102–103.
 26. Souza NA, Vigoder FM, Araki AS, Ward RD, Kyriacou CP, Peixoto AA, 2004. Analysis of the copulation songs of *Lutzomyia longipalpis* in six populations from Brazil. *J Med Entomol* 41: 906–913.
 27. Coyne JA, Orr HA, 2004. Studying speciation. Coyne JA, Orr HA. Eds. *Speciation*. Sunderland, MA: Sinauer Associates Inc., 65–82.
 28. Bauzer LGSR, Souza NA, Ward RD, Kyriacou C, Peixoto AA, 2002a. The *period* gene and genetic differentiation between three Brazilian populations of *L. longipalpis*. *Insect Mol Biol* 11: 315–323.
 29. Bauzer LGSR, Gestó JSM, Souza NA, Ward RD, Hamilton JGC, Kyriacou C, Peixoto AA, 2002b. Molecular divergence in the *period* gene between two putative sympatric species of the *Lutzomyia longipalpis* complex. *Mol Biol Evol* 19: 1624–1627.
 30. Lanzaro GC, Ostrovska K, Herrero MV, Lawyer PG, Warburg A, 1993. *Lutzomyia longipalpis* is a species complex: genetic divergence and interspecific hybrid sterility among three populations. *Am J Trop Med Hyg* 48: 839–847.
 31. Mutebi JP, Rowton E, Herrero MV, Ponce C, Belli A, Valle S, Lanzaro GC, 1998. Genetic variability among populations of the sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae) from Central America. *J Med Entomol* 35: 169–174.
 32. Yin H, Mutebi JP, Marriott S, Lanzaro GC, 1999. Metaphase karyotypes and G-banding in sandflies of the *Lutzomyia longipalpis* complex. *Med Vet Entomol* 13: 72–77.
 33. Lampo M, Torgerson D, Márquez LM, Rinaldi M, García CZ, 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.
 34. Arrivillaga JC, Rangel YN, Oviedo M, Feliciangeli MD, 2000. Correlated morphologic and genetic diversity among *Lutzomyia longipalpis* (Diptera: Psychodidae) collections in Venezuela. *J Am Mosq Control Assoc* 16: 171–174.
 35. Mukhopadhyay J, Ghosh K, Azevedo CR, Rangel EF, Munstermann LE, 1998. Genetic polymorphism of morphological and biochemical characters in a Natal, Brazil, population of *Lutzomyia longipalpis* (Diptera: Psychodidae). *J Am Mosq Control Assoc* 14: 277–282.
 36. Mutebi JP, Alexander B, Sherlock I, Wellington J, Souza AA, Shaw J, Rangel EF, Lanzaro GC, 1999. Breeding structure of the sandfly *Lutzomyia longipalpis* (Lutz & Neiva) in Brazil. *Am J Trop Med Hyg* 61: 149–157.
 37. Azevedo ACR, Monteiro FA, Cabello PH, Souza NA, Rosa-Freitas MG, Rangel EF, 2000. Studies on populations of *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) in Brazil. *Mem Inst Oswaldo Cruz* 95: 305–322.
 38. Arrivillaga JC, Norris DE, Feliciangeli MD, Lanzaro GC, 2002. Phylogeography of the neotropical sand fly *Lutzomyia longipalpis* inferred from mitochondrial DNA sequences. *Infect Genet Evol* 2: 83–95.
 39. Arrivillaga J, Mutebi JP, Pinango H, Norris D, Alexander B, Feliciangeli MD, Lanzaro GC, 2003. The taxonomic status of genetically divergent populations of *Lutzomyia longipalpis* (Diptera: Psychodidae) based on the distribution of mitochondrial and isozyme variation. *J Med Entomol* 40: 615–627.
 40. Hodgkinson VH, Birungi J, Quintana M, Deitze R, Munstermann LE, 2003. Mitochondrial cytochrome b variation in populations of the visceral leishmaniasis vector *Lutzomyia lon-*

- gipalpis* across eastern Brazil. *Am J Trop Med Hyg* 69: 386–392.
41. Lins RMMA, Oliveira SG, Souza NA, de Queiroz RG, Justiniano SCB, Ward RD, Kyriacou CP, Peixoto AA, 2002. Molecular evolution of the cacophony IVS6 region in sandflies. *Insect Mol Biol* 11: 117–122.
 42. Maingon RDC, Ward RD, Hamilton JGC, Noyes HA, Souza N, Kemp SJ, Watts PC, 2003. Genetic differentiation of two sibling species of *Lutzomyia longipalpis* (Diptera: Psychodidae) that produce distinct male sex pheromones in Sobral, Ceará State, Brazil. *Mol Ecol* 12: 1879–1894.
 43. Bottechia M, Oliveira SG, Bauzer LGSR, Souza NA, Ward RD, Garner KJ, Kyriacou CP, Peixoto AA, 2004. Genetic divergence in the cacophony IVS6 intron among five Brazilian populations of *Lutzomyia longipalpis*. *J Mol Evol* 58: 754–761.
 44. Krzywinski J, Besansky NJ, 2003. Molecular systematics of *Anopheles*: from subgenera to subpopulations. *Annu Rev Entomol* 48: 111–139.
 45. Modi GB, Tesh RB, 1983. A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae). *J Med Entomol* 20: 568–569.
 46. Collins FH, Mendez MA, Rasmussen MO, Mehaffey PC, Besansky NJ, Finnerty V, 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *Am J Trop Med Hyg* 37: 37–41.
 47. Watts PC, Boyland E, Noyes HA, Maingon RDC, Kemp SJ, 2001. Polymorphic dinucleotide microsatellite loci in the sandfly *Lutzomyia longipalpis* (Diptera: Phlebotominae). *Mol Ecol Notes* 2: 60–61.
 48. Raymond M, Rousset F, 1995. GENEPOP, Version 1.2. Population genetics software for exact tests and ecumenicism. *J Hered* 86: 248–249.
 49. Schneider S, Roessli D, Exoffier C, 2000. *ARLEQUIN Version 2000. Genetics and Biometry*. Geneva, Switzerland: University of Geneva.
 50. Wright S, 1951. The genetical structure of populations. *Ann Eugen* 15: 323–354.
 51. Langella O, 2002. *POPULATIONS 1.2.28. Population Genetic Software (Individuals or Populations Distances, Phylogenetic Trees)*. Available from <http://www.cnrs-gif.fr/pge/bioinfo/populations/index.php>
 52. Gaggiotti OE, Lange O, Rassmann K, Gliddon C, 1999. A comparison of two indirect methods for estimating average levels of gene flow using microsatellite data. *Mol Ecol* 8: 1513–1520.
 53. Weir BS, Cockerham CC, 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* 38: 1358–1370.
 54. Rousset F, 1997. Genetic differentiation and estimation of gene flow from *F*-statistics under isolation by distance. *Genetics* 145: 219–228.
 55. Saitou N, Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425.
 56. Rice WR, 1989. Analyzing tables of statistical tests. *Evolution* 43: 223–225.
 57. Sunnucks P, 2000. Efficient genetic markers for population biology. *Trends Ecol Evol* 15: 199–203.
 58. Ellegren H, Moore S, Robinson N, Byrne K, Ward W, Sheldon BC, 1997. Microsatellite evolution – a reciprocal study of repeat lengths at homologous loci in cattle and sheep. *Mol Biol Evol* 14: 854–860.
 59. Mukhopadhyay J, Rangel EF, Ghosh K, Munstermann LE, 1997. Patterns of genetic variability in colonized strains of *Lutzomyia longipalpis* (Diptera: Psychodidae) and its consequences. *Am J Trop Med Hyg* 57: 216–221.
 60. Lanzaro GC, Alexander B, Mutebi JP, Montoya-Lerma J, Warburg A, 1998. Genetic variation among natural and laboratory colony populations of *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae) from Colombia. *Mem Inst Oswaldo Cruz* 93: 65–69.
 61. Uribe-Soto SI, Lehmann T, Rowton ED, Velez ID, Porter CH, 2001. Speciation and population structure in the morphospecies *Lutzomyia longipalpis* (Lutz & Neiva) as derived from the mitochondrial ND4 gene. *Mol Phylogenet Evol* 10: 84–93.
 62. Hamilton JGC, Maingon RDC, Alexander B, Ward RD, Brazil RP, 2005. Analysis of the sex pheromone extract of individual male *Lutzomyia longipalpis* from six regions in Brazil. *Med Vet Entomol* 19: (in press).
 63. Hamilton JGC, Brazil RP, Campbell-Lendrum D, Davies CR, Kelly DW, Pessoa FAC, de Queiroz RG, 2002. Distribution of putative male sex pheromones among *Lutzomyia* sandflies (Diptera: Psychodidae). *Ann Trop Med Parasitol* 96: 83–92.
 64. Mares MA, Genoways HH, 1981. South American Landscapes and their mammals. *The Pymatuning Symposia in Ecology, Special Publication Series*. Volume 6. Pittsburgh: University of Pittsburgh Press, 53–75.
 65. Huber O, 1987. Neotropical savannas: their flora and vegetation. *Trends Ecol Evol* 2: 67–71.
 66. Haffer J, 1969. Speciation in Amazonian forest birds. *Science* 165: 131–137.
 67. Hamilton JGC, Ward RD, Dougherty MJ, Maingon R, Ponce C, Ponce E, Noyes H, Zeledon R, 1996. Comparison of the sex-pheromone components of *Lutzomyia longipalpis* (Diptera: Psychodidae) from areas of visceral and atypical cutaneous leishmaniasis in Honduras and Costa Rica. *Ann Trop Med Parasitol* 90: 533–541.
 68. dos Santos S, Arias JR, Hoffmann MP, Grotta Furlan MB, Wilson FF, Pereira C, Ferreira L, 2003. The presence of *Lutzomyia longipalpis* in a focus on American visceral leishmaniasis where the only proven vector is *Lutzomyia cruzi*, Corumbá, Mato Grosso do Sul State. *Rev Soc Bras Med Trop* 36: 633–634.
 69. Brazil RP, Hamilton JGC, 2002. Isolation and identification of 9-methylgermacrene-B as the putative sex pheromone of *Lutzomyia cruzi* (Mangabeira, 1938) (Diptera: Psychodidae). *Mem Inst Oswaldo Cruz* 97: 435–436.
 70. Greenberg AJ, Moran JR, Coyne JA, Wu CI, 2003. Ecological adaptation during incipient speciation revealed by precise gene replacement. *Science* 302: 1754–1757.