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

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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 in 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 chemotypes, 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, 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. 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. One subject of debate is the taxonomic status of Lutzomyia longipalpis. 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 America. The clinical pleomorphism observed was thought to reflect variations in levels of the L. longipalpis salivary vasodilatory protein maximilian.

With the important exception of Lu. pseudolongipalpis (Arrivillaga & Feliciangeli 2001), 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. 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. 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. 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. Four male sex pheromone chemotypes have been identified in Brazilian populations: (S)-9-methyl-germacrene-B (9MGB), 3-methyl-α-himachalene (3MAH), and a fourth cembrene iso-mer. Male copulation/courtship songs are strikingly distinct between populations in which males have different pheromone chemotypes. Furthermore, populations with distinct pheromone types and copulation love songs have shown sequence differences for the period gene. Since love songs and male attractant pheromones are used in maintaining speciation by providing pre-zygotic mating barriers, 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. 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. 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. 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. The results of these studies conflict with those of breeding experiments, pheromone analyses and those on the pattern of genetic variation in the periodicity and cacophony genes between Brazilian populations of Lu. lon-
Lutzomyia longipalpis, which together have provided convincing evidence for the presence of separate clades in Brazil. 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, although the contemporary level of gene flow needs to be reassessed to ascertain whether speciation is relatively recent. 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-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.

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 Jacobina, 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. 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 sympathy 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,*, [26,29,43] have indicated that the 9MGB and CEMB chemotypes at Sobral

![Figure 1. Geographic distribution of samples of the sand flies Lutzomyia longipalpis, *Lu. cruzi*, and *Lu. pseudolongipalpis* collected from Brazil and Venezuela.](image-url)

**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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality</th>
<th>Sample date</th>
<th>Source</th>
<th>Pheromone</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. longipalpis</em></td>
<td>Altagracia, Guárico State</td>
<td>08/01/01</td>
<td>Wild</td>
<td>9-methyl-germacrene-B</td>
</tr>
<tr>
<td><em>L. longipalpis</em></td>
<td>El Larrayo, Guárico State</td>
<td>07/12/01</td>
<td>Wild</td>
<td>9-methyl-germacrene-B</td>
</tr>
<tr>
<td><em>L. longipalpis</em></td>
<td>Guayabita, Aragua State</td>
<td>12/09/97</td>
<td>Wild</td>
<td>9-methyl-germacrene-B</td>
</tr>
<tr>
<td><em>L. longipalpis</em></td>
<td>Las Cabreras, Nueva Esparta State</td>
<td>07/21/01</td>
<td>Wild</td>
<td>9-methyl-germacrene-B</td>
</tr>
<tr>
<td><em>L. pseudolongipalpis</em></td>
<td>La Rinconada, Curarigua Lara State</td>
<td>12/11/97</td>
<td>Wild</td>
<td>3-methyl-α-himachalene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11/03/00</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. longipalpis</em></td>
<td>Jacobina Bahia State</td>
<td>07/30/01</td>
<td>Colony</td>
<td>3-methyl-α-himachalene/α-himachalene</td>
</tr>
<tr>
<td><em>L. longipalpis</em></td>
<td>Lapinha, Minas Gerais State</td>
<td>08/30/01</td>
<td>Colony</td>
<td>9-methyl-germacrene-B</td>
</tr>
<tr>
<td><em>L. cruzi</em></td>
<td>Corumba and Ladario, Mato Grosso do Sul State</td>
<td>09/12/01</td>
<td>Colony</td>
<td>9-methyl-germacrene-B</td>
</tr>
<tr>
<td><em>L. longipalpis</em></td>
<td>Marajó, Pará State</td>
<td>08/30/01</td>
<td>Colony</td>
<td>Cembrene</td>
</tr>
<tr>
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<td>Natal, Rio Grande do Norte State</td>
<td>10/01</td>
<td>Colony</td>
<td>Cembrene</td>
</tr>
<tr>
<td><em>L. longipalpis</em></td>
<td>Sobral, Ceará State</td>
<td>11/04/99</td>
<td>Wild</td>
<td>Cembrene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>04/06/01</td>
<td></td>
<td>9-methyl-germacrene-B</td>
</tr>
</tbody>
</table>
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. 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 Alttagracia in Guárico State; and on January 18–25, 2001 in Las Cabreras 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 (Alttagracia and Las Cabreras).

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 μL 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 μL of sterile water. One microliter of DNA was used for a polymerase chain reaction (PCR) in a 10-μL reaction volume that contained 75 mM Tris-HCl, pH 8.9, 20 mM (NH₄)₂SO₄, 0.01% [w/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., Milton Keynes, United Kingdom). The PCR products from LIST6-002, LIST6-004, LIST6-006, and LIST6-012 were pooled with a 400-base pair (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 (*Na*), observed heterozygosity (*Hobs*), expected heterozygosity (*Hexp*), and Wright’s inbreeding coefficient (*f*).50 All statistics were calculated for each locus and each sample separately, and also across all loci for *Na* and *f*. *Na*, *Hexp*, and *Hobs* 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 *FST*-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 *FST* of Wright54 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 θ/(1 – θ).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 [r], *r* = 0.631, degrees of freedom [df] = 15, *P* = 0.009) and this relationship was significant only for the wild-caught sand fly samples (w 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*

<table>
<thead>
<tr>
<th>Year</th>
<th>ALT</th>
<th>LAS</th>
<th>ELY</th>
<th>GUY</th>
<th>LPS</th>
<th>SOG</th>
<th>SOC</th>
<th>1997</th>
<th>2001</th>
<th>2001</th>
</tr>
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<td>2001</td>
<td></td>
<td></td>
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<td></td>
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</table>

**N** = sample size; **N** = number of alleles; **H** = observed heterozygosity; **H** = expected heterozygosity; **f** = inbreeding statistic; † indicates a significant (P < 0.05) after sequential Bonferroni correction; ‡ indicates a significant (P < 0.05) after sequential Bonferroni correction; * indicates a significant (P < 0.05) after sequential Bonferroni correction; 5) deficit of heterozygotes from expected HWE proportions; — locus monomorphic and no test carried out; ALT = Altagracia; LAS = Las Cabreras; ELY = El Layero; GUY = Guayabita; LPS = Lutzomyia pseudolongipalpis; 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 $H_s$ 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 correction\(^6\) 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. pseudolongipalpis 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.\(^6\) 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. pseudolongipalpis 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. pseudolongipalpis, $\theta = 0.160, P < 0.001$). 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 $\theta$ values ranging from 0.024 (Las Cabreras-El Layero) to 0.558 (Marajó Lu. pseudolongipalpis); the pairwise estimates of $\theta$ were significant ($P < 0.05$) for all comparisons. All estimates of $\theta$ ($P < 0.05$) involving Lu. cruzi and Lu. pseudolongipalpis 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 planar capacity 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 tree\(^5\) of $\theta$ showed five groups: Lu. pseudolongipalpis by itself (3MAH); a well-supported (mostly 9MGB) clade containing Altgracia, Guayabita, El Layero, and Las Cabreras from Venezuela; Lu. cruzi by itself (a 9MGB chemotype); a well-supported and distinct CEMB clade that consists of the Marajo, 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

<table>
<thead>
<tr>
<th></th>
<th>Venezuela</th>
<th>Brazil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALT</td>
<td>LAS</td>
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<tr>
<td>LAS</td>
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<tr>
<td>NAT</td>
<td>0.3696</td>
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</table>

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

Microsatellite loci have become the mainstay of modern population genetics, but it is relevant that two processes may reduce genetic variability and therefore affect the relationships between our samples. First, longer (and typically more polymorphic) alleles may be restricted to the focal species (used to isolate the microsatellites). 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 some but not all 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 others 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. 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 2](image-url)

**Figure 2.** Relationship between geographic distance between samples (log km) and the corresponding estimate of pairwise genetic distance measured by [0/(1 - θ)] 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 = (S)-9-methyl-germacrene-B; □ = cembrene; ● = 3-methyl-α-himachalene.

![Figure 3](image-url)

**Figure 3.** Phylogeographic structure of the *Lutzomyia longipalpis* species complex based on an unrooted neighbor-joining tree inferred from *G. m. morsitans* Dashed line separates Brazilian and Venezuelan samples. Only bootstrap values greater than 50% are shown. ◊, □, 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 ○ indicates that the type of sex pheromone is unknown.

**Table 4**

Mantel test results of the relationship between genetic distance [0/(1 - θ)] and the geographic separation (log km) for pairs of samples of the *Lutzomyia longipalpis* species complex from Brazil and Venezuela.

<table>
<thead>
<tr>
<th></th>
<th>b</th>
<th>R</th>
<th>r²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples</td>
<td>0.0994</td>
<td>0.3076</td>
<td>0.0947</td>
<td>0.0014</td>
</tr>
<tr>
<td>9-methyl-germacrene</td>
<td>0.1234</td>
<td>0.7354</td>
<td>0.5409</td>
<td>0.0028</td>
</tr>
<tr>
<td>Cembrene</td>
<td>0.0643</td>
<td>0.9825</td>
<td>0.9652</td>
<td>0.0390</td>
</tr>
</tbody>
</table>

*b* = regression slope; *R* = correlation coefficient; *r²* = coefficient of determination.

*P* = significance of correlation coefficient.
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 captured at its original description site (La Rinconada, Curari-
gua), which is approximately 20 km from El Paso where a cryptic subspecies of *Lu. longipalpis* has been detected. 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 0 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 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. 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*. 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 (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). The collection sites for the Venezuelan study collections (El Layero, Las Cabreras, 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, although isolation is not complete since *Lu. pseudolongipalpis* (3MAH) was found in sympathy with a 9MGB population at El Paso, only 8 km from La Rinconada, Curariagua. The Brazilian CEMB populations were located in drier sites along the northeastern region (Marajó and São Luis Islands, Natal, João Pessoa, Las Jaibas-Macéio, Alagoas, and Santarém). 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. 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 savannas 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.

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 clades. However, genetic divergence in the *per* intron and in the *cac IV*S6 intron among the populations from Jacobina, Lapinha, and Natal supports the occurrence of sibling species or near species in Brazil. 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, cebrenbe 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, cebrenbe isomer pheromone at Jaibas in Minas Gerais State. 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. 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. Our data supports only one (*Lu. pseudolongipalpis*) of the four (cis and trans Andean, *Lu. pseudolongipalpis* or Laran, and Brazilian) clades sug-
gusted by isoenzyme and mitochondrial analyses. 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. 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. 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, although three of the populations examined (Lapinha, Jacobina, and Santarém) produced different sex pheromones. 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., prezygotic 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 Cabreras 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.

Genetic differences between *Lu. pseudolongipalpis* and other Venezuelan sand fly populations have been reported, and our data support the recent taxonomic redefinition of *Lu. longipalpis* from La Rinconada, Curarigua. 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). 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.

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 gambiense* complex, whose genomes have been sequenced.

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, and the northern states of Pará, Rio Grande do Norte, and Roraima, which is precisely the area occupied by one of the cembrene isomer producing siblings (CEMB clade in Figure 3) of *Lu. longipalpis*.

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19. Hamilton JGC, Dawson GW, Pickett JA, 1996a. 9-Methyl

20. Hamilton JGC, Dawson GW, Pickett JA, 1996b. 3-Methyl-α

21. Hamilton JGC, Hooper AM, Ibbotson HC, Kuroswa 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.

22. 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.

type of Lutzomyia longipalpis (Diptera: Psychodidae) from Ja


29. Lanzaro GC, Ostrovská H, Herrero MV, Lawyer PG, Warburg A, 1993. Lutzomyia longipalpis is a species complex; genetic divergence and interspecific hybrid sterility among three popula


34. Mukhopadhyay J, Ghosh K, Azevedo CR, Rangel EF, Munster


36. Azevedo ACR, Monteiro FA, Cabello PH, Souza NA, Rosa


39. Hodgkinson VH, Birungi J, Quintana M, Deitze R, Munster
mann LE, 2003. Mitochondrial cytochrome b variation in populations of the visceral leishmaniasis vector Lutzomyia lon-