Genetic Variability Among Populations of the Sand Fly *Lutzomyia* (*Lutzomyia*) *longipalpis* (Diptera: Psychodidae) from Central America

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ABSTRACT Eleven Central American populations of *Lutzomyia longipalpis* (Lutz & Neiva) were analyzed for genetic variation at 16 enzyme loci. The aim was to study the genetic structure among populations within this region and to identify demes that may represent different sibling species. Genotypic frequencies within populations agreed with Hardy-Weinberg expectations, indicating that there were no sympatric sibling species among these 11 populations. Levels of genetic distance between pairs of populations were very low (<0.02). Some substructing was evident, because after genotypes of all populations mere pooled, 7 of the 16 enzyme loci deviated from Hardy-Weinberg expectations. Estimates of effective migration rates among populations (*Nm*) were low (3.7), indicating that gene flow was restricted. These data explained observed genetic substructuring when all genotypes were pooled.

KEY WORDS *Lutzomyia longipalpis*, genetic variation, enzyme loci, demes

VISCERAL LEISHMANIASIS (KALA-AZAR) is of major public health importance in South and Central America (Grimaldi et al. 1989). Approximately 1.6 million people reside in highly endemic areas with 16,000 clinical cases reported annually (Ashford et al. 1992). The causative agent is *Leishmania donovani chagasi* Cunha & Chagas (Ward 1977, 1985; Lainson et al. 1987; Grimaldi et al. 1989). The principal arthropod vector in South and Central America is the sand fly *Lutzomyia (Lutzomyia) longipalpis* (Lutz & Neiva) (Lainson et al. 1985, LePont and Dejeux 1985, Zeledon 1985, Young and Lawyer 1987, Corredor et al. 1989).

Lutzomyia longipalpis is a species complex (Ward et al. 1983, 1988; Lanzaro et al. 1993). However, the number of species within the complex, their distribution, and their role in the transmission of visceral leishmaniasis are not well understood.

In some areas of Central America, an atypical cutaneous leishmaniasis caused by *L. d. chagasi* has been reported (Zeledon et al. 1989, Ponce et al. 1991). Saliva of *L. longipalpis* from different geographical areas differ in composition and in their capacity to enhance *Leishmania* infections (Warburg et al. 1994). One of the pharmacological agents in the saliva of *L. longipalpis*, maxadilan, is a potent protein vasodilator (Lerner et al. 1991, 1992). The amounts of maxadilan differ significantly among the 3 species described by Lanzaro et al. (1993). Flies from Brazil have the largest amounts of maxadilan in their saliva, flies from Costa Rica have the least, and flies from Colombia are intermediate (Warburg et al. 1994). It is in Central America, where flies have the least maxadilan, that atypical cutaneous leishmaniasis has been reported. It has been hypothesized that maxadilan may play an important role in the visceralization of *L. d. chagasi* infection (Warburg et al. 1994, Lanzaro and Warburg 1995). If this is true, the occurrence of visceral and atypical cutaneous leishmaniasis may be associated with specific, genetically distinct vectors.

Recently, Hamilton et al. (1996) analyzed male sex pheromones of *L. longipalpis* populations from Honduras and Costa Rica and found all but 1 of them to be the same. The populations in Hamilton's study were from areas endemic for atypical cutaneous leishmaniasis or mixed atypical cutaneous and visceral disease. In 1 population from Liberia, Costa Rica, where only atypical cutaneous leishmaniasis is endemic, they found 3 different pheromones present. Different sex pheromones previously have been found for reproductively isolated *L. longipalpis* in Brazil (Ward et al. 1989). Based on these earlier observations, Hamilton et al. (1996) concluded that 2 or possibly 3 distinct populations exist sympatrically at this site (Liberia, Costa Rica).

The current project was initiated to study the genetic structure and estimate levels of genetic diversity among populations of *L. longipalpis* in Central America. With genetic analysis we hoped to

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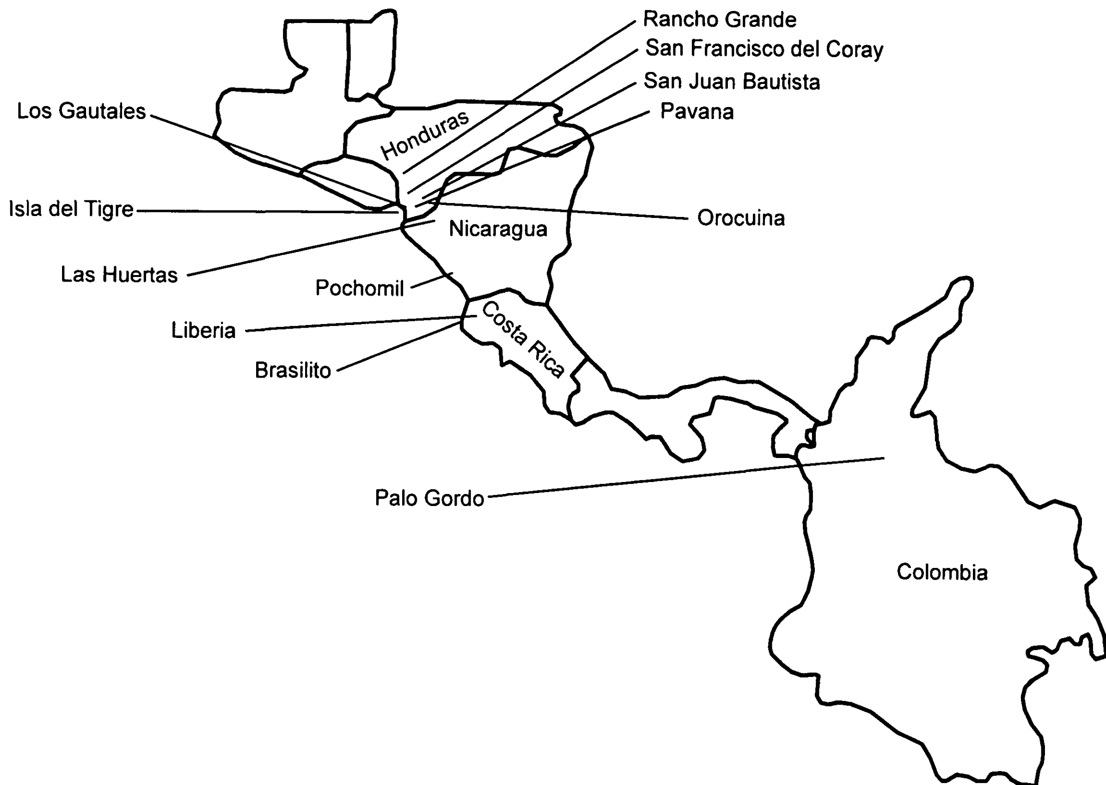

Fig. 1. Collection sites.

learn the extent of variation, estimate degrees of relatedness among populations, determine if there is >1 sibling species within the study area, and, if multiple species are present, to establish their distributions.

Materials and Methods

Sand Fly Populations. Samples from 11 populations of *L. longipalpis* from Nicaragua, Costa Rica, and Honduras were collected at hosts (human and domestic animal) in areas endemic for leishmaniasis. Collection sites (Fig. 1) are described in detail in Table 1. Laboratory strains from Colombia and Brazil were included for comparison. The Colombia colony was derived from flies collected in 1989 by R. B. Tesh near Melgar, Tolima department (4° 11' N, 74° 18' W). The Brazil colony was from flies collected in caves at Lapinha near Belo Horizonte, Minas Gerias (20° 0' S, 44° 0' E). A field-collected strain of *Lutzomyia evansi* (Nuñez-Tovar) was collected from Pochomil in Nicaragua (Fig. 1) and included for interspecific comparison. Field-collected sand flies were identified to species by morphological characteristics and stored in liquid ni-

Table 1. Central American field populations of *L. longipalpis* sampled, approximate geographical locations, dates of collection, and form of leishmaniasis found at the collection sites

Population	Location	Date	Disease
Costa Rica			
1. Nazareth, Liberia	10° 38.181' N, 85° 26.727' W	May 1996	Atypical cutaneous
2. Brazilito	10° 24.819' N, 86° 47.455' W	May 1996	Atypical cutaneous
Honduras			
3. Pavana	13° 25.352' N, 87° 20.019' W	July 1995	Visceral
4. San Juan Bautista	13° 34.576' N, 87° 16.221' W	July 1995	Atypical cutaneous
5. Orocuina	13° 29.041' N, 87° 06.476' W	July 1995	Visceral
6. San Francisco del Coray	13° 40.024' N, 87° 30.857' W	July 1995	Visceral
7. Tiger Island	13° 17.300' N, 87° 37.591' W	July 1995	Mixed
8. Los Guatales	13° 21.503' N, 87° 37.290' W	July 1995	Cutaneous
9. Rancho Grande	14° 02.634' N, 87° 41.218' W	July 1995	Unknown
Nicaragua			
10. Pochomil	11° 46.016' N, 86° 30.000' W	May 1996	Visceral
11. Las Huertas	13° 03.136' N, 86° 55.862' W	May 1996	Visceral

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Table 2. Enzyme loci

Enzyme	E.C. no.	Abbreviation	Buffer[a]
Aconitase-2	4.4.1.3	*Acon-2*	CA-8
Aldehyde dehydrogenase	1.2.1.3	*Aldh*	C
Fumarase	4.41.2	*Fum*	CA-8
α-Glycerophosphate	1.1.1.8	*α-Gpd*	CA-7
Glutamate oxaloacetate transaminase-1	2.6.1.1	*Got-1*	CA-7
Glutamate oxaloacetate transaminase-2	2.6.1.1	*Got-2*	CA-7
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	*G3pdh*	CA-7
Hexokinase	2.7.1.1	*Hk*	TBE
Isocitrate dehydrogenase-1	1.1.1.42	*Idh-2*	C
Malic acid dehydrogenase-1	1.1.1.37	*Mdh-1*	C
Malic acid dehydrogenase-2	1.1.1.37	*Mdh-2*	C
Malic enzyme-1	1.1.1.40	*Me-1*	C
Phosphoglucoisomerase	5.3.1.9	*Pgi*	TBE
Phosphoglucomutase	2.7.5.1	*Pgm*	TBE
α, α-Trehalase	3.2.1.28	*Tre*	CA-7

E.C. = enzyme commission.

[a] CA-8: gel buffer = 0.074 M Tris, 0.009 M citric acid, pH 8.45 (undiluted), electrode buffer = 1.37 M Tris, 0.314 M citric acid, pH 8.1 (diluted 1:3 for the cathode and 1:4 for the anode) (Steiner and Josslyn 1979); C: gel buffer = 0.002 M citric acid, pH 6.0 (undiluted), electrode buffer = 0.04 M citric acid, pH 6.1 (undiluted) (pH for buffer C is adjusted with N-(3-aminopropyl)-morpholine) (Clayton and Tretiak 1972); CA-7: gel buffer = 0.009 M Tris, 0.003 M citric acid, pH 7.0 (undiluted), electrode buffer = 0.135 M Tris, 0.04 M citric acid, pH 7.0 (undiluted); TBE: 0.1 M Tris, 0.05 M boric acid, 0.002 M EDTA, pH 8.6 (undiluted) (gel and electrode buffer identical) (Selander et al. 1971).

trogen. In the laboratory, they were stored at -80°C until used in isozyme assays.

Isozyme Assays. Sand flies were assayed for genetic variation at 16 isozyme loci by starch gel electrophoresis (Steiner and Joslyn 1979). Sand flies were homogenized individually in 8 μl of distilled water using a micropipette tip. The preparation was frozen briefly at -80°C to rupture cellular membranes and release enzymes. The homogenate was fractionated on 12.5% (wt:vol) horizontal starch gels. Four different buffer systems were used to maximize electrophoretic separation and to improve visualization of histochemically stained products. The 16 enzymes assayed are presented in Table 2. Bands within staining regions were assumed to be controlled by single genes. Migration distances of bands at the same loci were assumed to be different alleles and multiple bands at the same locus in an individual were assumed to be heterozygous. Loci were designated with positive or negative codes depending on whether they migrated to the cathode or anode. Alleles were scored on the basis of migration distance relative to the most common allele (rf. = 1.00).

Data Analysis. Genotypes of individuals within populations were analyzed for genetic variability, for compliance to Hardy-Weinberg expectations, and for genetic relatedness using a FORTRAN-77 computer program BIOSYS-1 (Swofford and Selander 1989). The effective migration rates (*Nm*) were calculated from the equation, $Nm = (1-F_{st})/4F_{st}$, which was derived from the original formula $F_{st} = 1/(4\,Nm + 1)$ (Wright 1931).

Results

Genetic Variability. Three of 16 enzymes (18.8%)—*Mdh, Idh,* and *Got*—were coded by 2 loci; 1 migrated to the anode and the other to the cathode. The number of polymorphic loci per population ranged from 1 (6.3%) to 3 (18.8%). A locus was considered polymorphic if the frequency of the most common allele was ≤0.95. The most frequent alleles at all loci were the same for all populations. The mean number of alleles per locus (±SEM) per population varied from 1.3 ± 0.1 to 1.9 ± 0.2 (range, 1-5 alleles per locus).

The expected average heterozygosities for all populations are presented in Table 3, assuming populations were in Hardy-Weinberg equilibrium. Heterozygosities were low, ranging from 0.016 to 0.057. The average for all populations pooled was 0.044.

Population Structure. Assuming that mating was random within populations, the heterozygotes at each polymorphic locus were tested for goodness-of-fit to the Hardy-Weinberg equilibrium by chi-square tests. At loci with >2 alleles, genotypes were pooled by considering all alleles except the most common one as a single allele. This was done because the chi-square test is problematic when the expected frequencies of some classes are low. Significant deviations from Hardy-Weinberg were detected at *Gpi* from Brasilito, Costa Rica; *Gpi* and *Hk* from San Juan Bautista, Honduras; *Mdh-1* from Liberia, Costa Rica; *Hk* from Los Guatales, Honduras; and *Aldh, Gpi,* and *Tre* from Las Huertas, Nicaragua. Although chi-square values were significant, discrepancies between expected and observed values were very small and most likely were caused by sampling errors. No significant deviations from Hardy-Weinberg were observed for those loci at any of the other populations.

To determine if all 11 populations were panmictic, data were pooled and all loci were tested for goodness-of-fit to Hardy-Weinberg equilibrium. Deviations from Hardy-Weinberg equilibrium were

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Table 3. Genetic variability of Central American populations of *L. longipalpis* at 16 enzyme loci

Location	Mean sample size/locus (±SE)	Mean no. alleles/locus (±SE)	% loci polymorphic[a]	Mean heterozygosity: Direct count (±SE)	Mean heterozygosity: Hardy-Weinberg expected (±SE)
Brasilito	59.7 (6.6)	1.6 (0.2)	6.3	0.021 (0.011)	0.028 (0.016)
Rancho Grande	34.8 (6.0)	1.6 (0.2)	6.3	0.017 (0.009)	0.016 (0.009)
Orocuina	41.1 (4.8)	1.6 (0.2)	6.3	0.032 (0.019)	0.030 (0.018)
Pavana	13.9 (1.6)	1.3 (0.1)	6.3	0.023 (0.015)	0.022 (0.014)
San Juan Bautista	14.1 (1.7)	1.4 (0.1)	18.8	0.037 (0.017)	0.047 (0.017)
San Francisco Del Coray	31.7 (4.0)	1.6 (0.2)	18.8	0.040 (0.014)	0.039 (0.013)
Liberia	60.1 (6.4)	1.8 (0.3)	12.5	0.039 (0.028)	0.044 (0.030)
Tiger Island	34.1 (4.1)	1.6 (0.2)	18.8	0.063 (0.031)	0.065 (0.033)
Los Guatales	26.2 (2.3)	1.7 (0.3)	18.8	0.043 (0.023)	0.047 (0.024)
Las Huertas	44.9 (4.7)	1.9 (0.2)	12.5	0.037 (0.015)	0.057 (0.018)
Pochomil	14.6 (1.5)	1.4 (0.2)	12.5	0.023 (0.011)	0.022 (0.011)

[a] A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95.

observed at 7 of the 16 loci, indicating that these populations were not panmictic. Values for F_{st} statistics (Wright 1978) for 7 Honduran populations and for all Central American populations combined were 0.0761 and 0.0655, respectively. These values were low but positive, which also indicated that populations were not panmictic. Estimates of effective migration (*Nm*) were low—3.0 for the Honduras populations and 3.6 for all 11 populations. These values indicated restricted gene flow among populations, but were high enough to prevent the evolution of significant levels of genetic divergence.

Genetic Differentiation. Genetic similarity and difference were estimated between populations using the *I* and *D* statistics of Nei (1978). Populations were grouped by country of origin. Genetic distance and similarity coefficients showed low levels of genetic differentiation among populations and countries.

Cluster analysis was conducted on the *D* matrix values of the pairwise comparisons among all populations using the unweighted pair group algorithms using arithmetic averages of Sneath and Sokal (1973). Laboratory populations from near Melgar, Tolima department (4° 11' N, 74° 18' W), Colombia, and from Lapinha caves near Belo Horizonte, Minas Gerais (20° 0' S, 44° 0' E), Brazil, were included for comparison. The resulting dendrogram (Fig. 2) shows that all Central American populations clustered together below the 0.02 level of genetic distance (*D*). The disease manifestation (visceral or atypical cutaneous) associated with each population is indicated in Fig. 2 and there was no indication of clustering of populations by disease.

Discussion

Our isozyme data did not support the existence of >1 species of *L. longipalpis* in Central America. There also were no apparent associations between disease manifestation and multilocus isozyme profile. This does not rule out an association between the composition of saliva and visceralization of leishmanial parasites, because this may involve only 1 or very few genes. Isozyme genotypes at loci within individual populations were in close agreement with Hardy-Weinberg expectations, indicating that there was no evidence of substructuring. Our observations were, for the most part, similar to those of Hamilton et al. (1996), who reported male sex pheromone homogeneity among all populations studied, except 1. In Liberia, Costa Rica, they identified 2 or possibly 3 different male sex pheromones and concluded that there were at least 2 or possibly 3 distinct populations in this area. The discrepancy between our findings concerning the Liberia population of *L. longipalpis* and those of Hamilton et al. (1996) are hard to explain at this time. A possible explanation for this discrepancy may lie with genetics of the male pheromones. Phillips et al. (1986) performed gas-chromatographic analysis on spot extracts from 4 colonies. Glands of only 1 of these contained a single compound, the remaining 3 colonies contained 2 chemically distinct compounds. Studies by Hamilton and Ward (1991) indicated that there are least 6 different pheromone compounds produced by different strains of *L. longipalpis*. Because extractions are done on pools of adult males, it is not known if extracts from a single male contain

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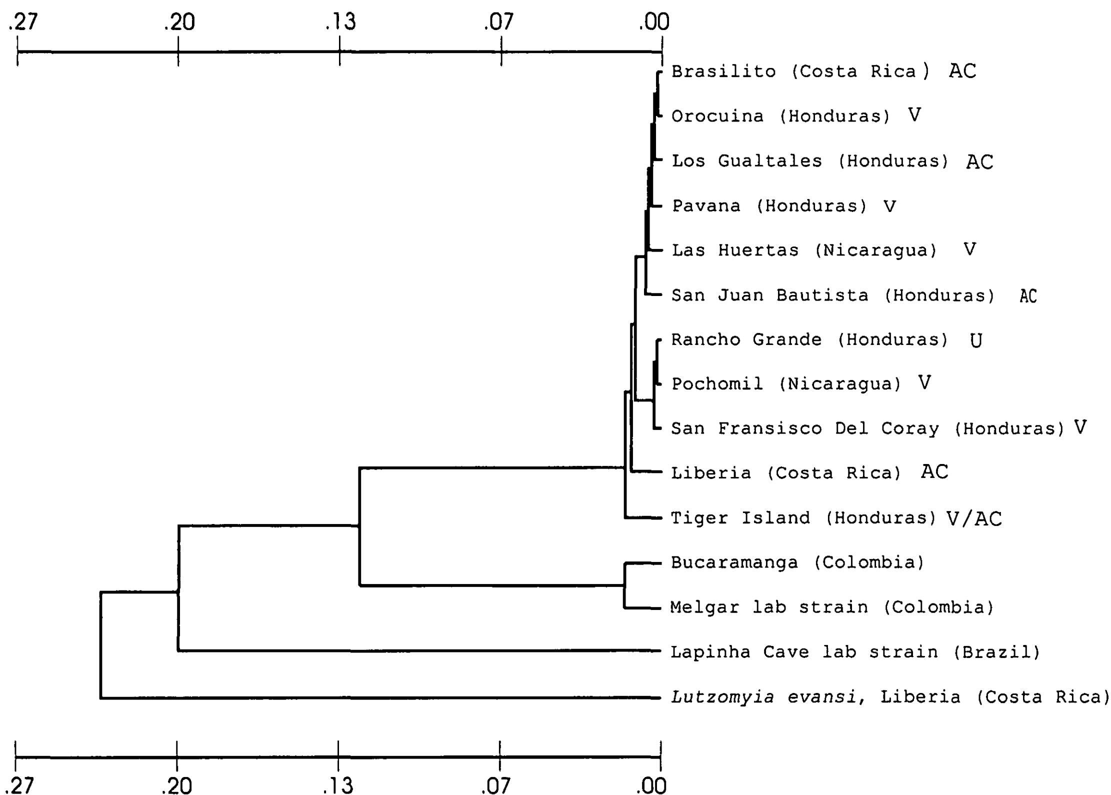

Fig. 2. Dendrogram constructed from values for *D* for field-collected Central American populations of *L. longipalpis*. Laboratory colonies from Colombia and Brazil; a field population from Palo Gordo (near Bucaramanga), Colombia; and a field population of *L. evansi* from Liberia, Costa Rica, are included for comparison. Disease manifestation indicated for Central American populations as follows: V, visceral leishmaniasis, AC, atypical cutaneous leishmaniasis, U, disease unknown. South American populations are all visceral leishmaniasis.

>1 compound. It is possible that some of these compounds are inherited in a Mendelian manner and are codominant in the heterozygous state. If so, it may explain the existence of multiple forms of male sex pheromones in a single randomly mating and genetically homogeneous population.

Alternatively, there may be some divergences in sampling procedures. There is a possibility of spatial or temporal separation among distinct populations within the same area. We probably did not sample at the exact same sites or times as Hamilton et al. (1996). Sand flies are relatively localized and rarely disperse >300 m (Alexander 1989, Morrison 1993).

We also observed that genetic variability among Central American populations were lower than those reported for other field populations of *Lutzomyia* species (Lanzaro and Warburg 1995). Observed and expected mean heterozygosities for all populations were between 0.016 and 0.057, lower than the range (0.057–0.071) reported by Lanzaro et al. (1993) for laboratory strains of *L. longipalpis*. Some of the highly variable enzymes, *Mpi, Sodh, Pep,* and *Ald,* did not stain consistently in field-collected specimens. Because of the very low numbers of individuals that did stain, these enzymes were excluded from the final analysis and this undoubtedly contributed to low estimates of genetic variability. It also is possible that heterozygosity is atypically high in laboratory populations resulting from the occurrence of balanced lethal systems within these populations, as described for *Aedes aegypti* by L. Munstermann (1994).

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