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# CRYPTIC SPECIATION IN *LUTZOMYIA (NYSSOMYIA) TRAPIDOI* (FAIRCHILD & HERTIG) *(DIPTERA: PSYCHODIDAE)* DETECTED BY MULTILOCUS ENZYME ELECTROPHORESIS

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*Abstract.* *Lutzomyia trapidoi* is the major vector of cutaneous leishmaniasis in Ecuador. In the framework of an epidemiologic study, female *Lu. trapidoi* sand flies were captured on human bait in La Tablada and Paraiso Escondido. Some coloration heterogeneity among the specimens caught led us to look for the existence of cryptic species using multilocus enzyme electrophoresis. In 196 specimens studied, five of seven enzyme loci proved to be variable, making it possible to check for departures from panmixia both by Hardy-Weinberg statistics and linkage disequilibrium analysis. Two discrete groups were clearly distinguished, which could be differentiated by the diagnostic locus glycerophosphate dehydrogenase. The two groups occurred in sympatry within each locality. Genetic distances measured between these two groups were consistent with values usually found between distinct species. These results suggest the existence of at least two sibling species in Paraiso Escondido as well as La Tablada. The epidemiologic relevance of these results is discussed.

Differences in color within *Lutzomyia trapidoi* have been reported in Colombia (Pacific Coast) and in Ecuador (Pichincha Province), where specimens appeared darker than elsewhere,[1] and an isozymic survey in Colombia resulted in postulation of some speciation within the *Lu. trapidoi* taxon.[2] In the framework of epidemiologic surveys, similar morphologic variation was recorded among specimens caught in sympatry in two northern areas of Ecuador. The presence of numerous intermediate forms between dark and clear made it difficult to unambiguously separate them. Since morphologic variation could be an indication of cryptic speciation, we performed a multilocus enzyme electrophoresis (MLEE) analysis in selected samples offering both guarantees of sympatry and anthropophily. Sympatry was required for simplifying the interpretation of any possible genotypic disequilibrium. Anthropophily ensured that we collected epidemiologically important subpopulations of *Lu. trapidoi.*

## MATERIALS AND METHODS

**Insects.** On the March 29 and 31 and April 2, 1992, 347 female sand flies were collected manually from human bait catches placed in the tree canopy (25 meters high) and stored in individual glass vials. Captures were performed in two ecologically distinct regions: Paraiso Escondido (PE) and La Tablada (LT). La Tablada (altitude = 150 m, Esmeraldas province) is located in the hills of the coastal cordillera and PE (altitude = 300 m, Pichincha province) is located in the coastal plain between the Andean foothills and the cordillera. Immediately after capture, genitalia were dissected for morphologic diagnosis, whereas the remaining parts of the insects were stored in liquid nitrogen. They were not separated into dark and clear forms because of the presence of intermediate forms, but special care was devoted to the recognition of the related species *Lu. ylephiletor* (Fairchild & Hertig)[2] and *Lu. edentula* (de Leon).[1] Neither species were found in the above collection sample.

**Isoenzyme electrophoresis.** After transportation from Ecuador to France, insect samples in liquid nitrogen were subjected to MLEE on cellulose acetate plates (Helena Laboratories, Beaumont, TX). Each sand fly was squashed in 16 μl of hypotonic enzyme stabilizers[3] and then immediately subjected to electrophoresis. Samples of 8 μl allowed the survey of as many as 10 different enzyme systems. The remaining 8 μl were used as controls for further verifications. The following enzyme systems were assayed: aconitase (ACON, E.C. 4.2.1.3.), glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49), glucose phosphate isomerase (GPI, E.C. 5.3.1.9), αglycerophosphate dehydrogenase (GPD, E.C. 1.1.1.49), hexokinase (HK, E.C. 2.7.5.1), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), leucine aminopeptidase (LAP, E.C. 3.4.11 or 13), malate dehydrogenase (MDH, E.C. 1.1.1.37), peptidase 1, substrate L-leucyl-leucine-leucine (PEP1, E.C. 3.4.13), peptidase 2, substrate L-leucyl-L-alanine (PEP2, E.C. 3.4.13), 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44), and phosphoglucomutase (PGM, E.C. 2.7.5.1). Seven of these systems gave reproducible patterns, namely GPI, GPD, HK, IDH, MDH, PEP2, and PGM, for each of 196 female sand flies. Running conditions and histochemical methods were adapted from standard techniques.[4–7]

**Statistical analysis.** Estimation of single-locus (h) and total (H) expected heterozygosities, as well as their standard deviation, was based on the formulas of Nei.[8] Departures from panmixia were looked for using both single locus analysis (fixation index, Table 1) and multilocus analysis (linkage disequilibrium tests, Table 2). The standard genetic distance (Ds) of Nei[8] was used to compare gene frequency differences between geographic areas or groups, and converted into an unweighted pair group method arithmetical average phenogram (Figure 1).

## RESULTS

**Isoenzyme polymorphism.** Five of the seven enzyme systems, namely GPI, GPD, IDH, PEP2, and PGM, revealed

TABLE 1

Fixation indices at four loci in *Lutzomyia trapodoi* from La Tablada (LT) and Paraiso Escondido (PE), Ecuador*

| | PE AB (n = 99) | LT AB (n = 97) | PE A (n = 6) | PE B (n = 93) | LT A (n = 19) | LT B (n = 78) |
|---|---|---|---|---|---|---|
| GPI | 0.130 | 0.130 | −0.111 | 0.140 | −0.086 | −0.042 |
| PGM | 0.214† | 0.176 | / | 0.200 | 0.113 | 0.190 |
| IDH | 0.311‡ | 0.781§ | / | 0.187 | −0.077 | −0.033 |
| GPD | 1§ | 1§ | / | / | / | / |

* The value of the fixation index (F) at each locus was computed according to Nei:[10] $F = (4 n_{11}n_{22} - n^2_{12})/(2n_{11} + n_{12})(2n_{22} + n_{12})$, with $n_{11}$ and $n_{22}$ being the number of homozygotes and $n_{12}$ the number of heterozygotes. The deviation of F from 0 was tested by $\chi^2 = nF^2$ with one degree of freedom, and $n = n_{11} + n_{12} + n_{22}$.[10] A = individuals showing homozygosity for a GPD-1 allele attributed to one cryptic species of *Lu. trapidoi*; B = individuals showing homozygosity for a GPD-2 allele attributed to a second species of *Lu. trapidoi*; AB = individuals A and B grouped together; n = number of specimens; GPI = glucose phosphate isomerase; PGM = phosphoglucomutase; / = monomorphic locus; IDH = isocitrate dehydrogenase; GPD = glycerophosphate dehydrogenase.
† $P = 0.05$.
‡ $P < 0.01$.
§ $P < 0.001$.

TABLE 2

Linkage disequilibrium of enzymatic loci in *Lutzomyia trapidoi* from La Tablada (LT) and Paraiso Escondido (PE), Ecuador*

| | LT AB (n = 97) | PE AB (n = 99) | PE A (n = 6) | LT A (n = 19) | PE B (n = 93) | LT B (n = 78) |
|---|---|---|---|---|---|---|
| GPI-PGM | NS | NS | NS | NS | NS | NS |
| GPI-IDH | NS | NS | NS | NS | NS | NS |
| PGM-IDH | NS | NS | NS | NS | NS | NS |
| GPD-GPI | NS | NS | / | / | / | / |
| GPD-PGM | NS | NS | / | / | / | / |
| GPD-IDH | <0.001 | <0.02 | / | / | / | / |

* Linkage disequilibrium was tested using an m × n chi-square homogeneity test, with each cell containing the number of individuals with a particular phenotype (locus 1) × phenotype (locus 2) combination.[9] A = individuals showing homozygosity for a GPD-1 allele attributed to one cryptic species of *Lu. trapidoi*; B = individuals showing homozygosity for a GPD-2 allele attributed to a second species of *Lu. trapidoi*; AB = individuals A and B grouped together; n = number of specimens; GPI = glucose phosphate dehydrogenase; PGM = phosphoglucomutase; NS = nonsignificant $P$ value at the 0.05 level; IDH = isocitrate dehydrogenase; GPD = glycerophosphate dehydrogenase; / = monomorphic locus.

polymorphic patterns (polymorphism rate = 0.71 ). Different positions on the gel were attributed to distinct alleles, which were numbered 1, 2, etc., starting from the faster migrating allele. The enzyme system GPD exhibited two alleles. As observed by Petersen (unpublished data) in two Panamanian populations, GPI exhibited four alleles. Nevertheless, the proximity of the slowest alleles, GPI-3 and GPI-4, made it difficult to confidently separate them on the gel, so that we amalgamated them into a GPI-3 allele. Enzyme systems PGM and IDH exhibited four well-separated alleles. Heterozygous forms suggested a monomeric structure for PGM and a dimeric structure for IDH and GPI, whereas no heterozygous forms were observed for GPD. The frequencies of all alleles were computed (Table 3) for genetic distance calculations (Figure 1), while only two possible alleles were taken into account for the other calculations: the more frequent one on the one hand, and all other ones plotted together into a unique allele on the other hand. Both HK and MDH also exhibited fairly defined bands (one band interpreted as one locus for HK, and two bands interpreted as two loci for MDH). Nevertheless, these three putative loci exhibited no variability, and were not taken into account for population genetic analysis, except for computing genetic distances and variability. The polymorphism of PEP2 was not amenable to an allelic interpretation; however, because some distinct and reproducible profiles could be distinguished, qualitative information brought by this system was considered in the discussion.

**Sample processing.** The total lack of heterozygotes at the GPD locus led us to subdivide the sample according to the GPD genotypes: those specimens that exhibited GPD-1 were scored as A, while the remaining ones that exhibited GPD-2 were scored as B. These two subgroups were combined with collecting sites, either LT or PE, to form various subdivisions: the total sample (196 specimens) was divided into LT-AB (97 specimens) and PE-AB (99 specimens), and these geographic areas were further subdivided into A and B according to their GPD genotypes: LT-A (19 specimens), LT-B (78 specimens), PE-A (six specimens), and PE-B (93 specimens).

**Genotypic equilibrium.** Except for the GPI locus, for which results were constantly consistent with panmixia, departures from Hardy-Weinberg expectations as well as linkage disequilibrium were detected in each of the two geographic areas when plotting together the GPD-1 and GPD-2 genotypes, i.e., the subgroups A and B (columns LT-AB and PE-AB, Tables 1 and 2).

**Genetic distances.** Nei's standard genetic distance sepa-

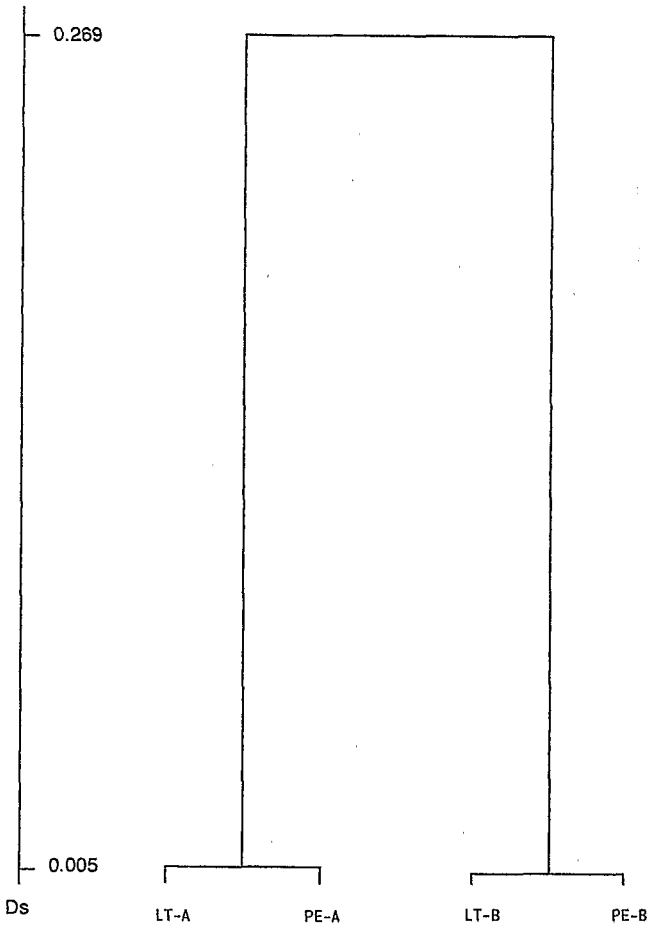


FIGURE 1. Unweighted pair group method arithmetical average tree derived from Nei's standard genetic distances (Ds) between and within cryptic species of *Lutzomyia trapidoi*. LT = La Tablada; PE = Paraiso Escondido; A and B indicate homozygosity for alternate alleles (glycerophosphate dehydrogenase-1 and -2).

TABLE 3

Allelic frequencies of enzymatic loci in *Lutzomyia trapidoi* from La Tablada (LT) and Paraiso Escondido (PE), Ecuador*

| Locus† | LT AB (n = 97) | PE AB (n = 99) | LT A (n = 19) | LT B (n = 78) | PE A (n = 6) | PE B (n = 93) |
|---|---|---|---|---|---|---|
| GPI-1 | 0.015 | 0.070 | 0.000 | 0.019 | 0.000 | 0.075 |
| GPI-2 | 0.953 | 0.878 | 0.921 | 0.961 | 0.916 | 0.876 |
| GPI-3 | 0.030 | 0.050 | 0.078 | 0.019 | 0.083 | 0.048 |
| PGM-1 | 0.038 | 0.062 | 0.000 | 0.047 | 0.000 | 0.066 |
| PGM-2 | 0.774 | 0.760 | 0.805 | 0.767 | 0.916 | 0.750 |
| PGM-3 | 0.159 | 0.171 | 0.083 | 0.178 | 0.083 | 0.177 |
| PGM-4 | 0.027 | 0.005 | 0.111 | 0.006 | 0.000 | 0.005 |
| IDH-1 | 0.162 | 0.024 | 0.545 | 0.037 | 0.400 | 0.000 |
| IDH-2 | 0.117 | 0.135 | 0.386 | 0.029 | 0.400 | 0.118 |
| IDH-3 | 0.707 | 0.746 | 0.068 | 0.917 | 0.200 | 0.782 |
| IDH-4 | 0.011 | 0.092 | 0.000 | 0.014 | 0.000 | 0.098 |
| GPD-1 | 0.217 | 0.060 | 1 | 0.000 | 1 | 0.000 |
| GPD-2 | 0.782 | 0.939 | 0.000 | 1 | 0.000 | 1 |
| MDH-1 | 1 | 1 | 1 | 1 | 1 | 1 |
| MDH-2 | 1 | 1 | 1 | 1 | 1 | 1 |
| HK | 1 | 1 | 1 | 1 | 1 | 1 |

* A = individuals showing homozygosity for a GPD-1 allele attributed to one cryptic species of *Lu. trapidoi*; B = individuals showing homozygosity for a GPD-2 allele attributed to a second species of *Lu. trapidoi*; AB = individuals A and B grouped together; n = number of specimens.

† GPI = glucose phosphate isomerase; PGM = phosphoglucomutase; IDH = isocitrate dehydrogenase; GPD = glycerophosphate dehydrogenase; MDH = malate dehydrogenase; HK = hexokinase. Alleles are numbered in the order of decreasing mobility.

rating the two geographic populations was very low (from 0.003 to 0.005), whereas subgroups A and B were separated by more than 50 times greater genetic distances (0.269) (Figure 1).

## DISCUSSION

Each sand fly population was caught by human bait within a 3-hr time period. Knowing the relatively reduced dispersing capacity of New World sand flies[9–12] and their relatively long generation time, this way of sampling ensures that the collected specimens were living in a reasonable level of sympatry, allowing us to discard a hypothesis such as spatial or temporal subdivision (Wahlund effect) in case of a lack of interbreeding. This provided optimal conditions for discussing any genotypic disequilibrium in terms of speciation.[13]

Polymorphism levels appeared to be high in the material examined for *Lu. trapidoi* since five of seven enzyme systems were found to be polymorphic. More loci per specimen would be needed to give more accurate estimates of the genetic variability in *Lu. trapidoi.* Nevertheless, previous studies suggested that such sampling may give reliable values. For instance, the estimate of genetic variability measured at 25 loci in *Phlebotomus papatasi*[14] was found to be similar when only nine loci were used.[13] In the whole sample or in its subdivisions, the mean number of alleles per locus (from 1.57 to 2.29), the proportion of variable loci (from 0.43 to 0.57), as well as Nei's unbiased estimates of gene diversity (ranging from 0.098 ± −0.063 [± SD] to 0.211 ± −0.082) should be considered as underestimates of the true genetic variability. Indeed, a polymorphic system, namely PEP2, was discarded because its genetic background was unclear. The genetic variability of *Lu. trapidoi* was comparable to that of *Lu. (Psychodopygus) carrerai* and *Lu. (P.) yucumensis,*[15, 16] which are also sylvatic vectors of human cutaneous leishmaniasis when humans invade these areas. On the other hand, *Lu. trapidoi* variability was higher than that of peridomestic sand flies such as *Lu. longipalpis* (Lutz & Neiva), the vector of visceral leishmaniasis in the Yungas (Bolivia),[17, 18] or *Lu. nuneztevari* (Ortiz) in the same area, described there as *Lu. nuneztevari anglesi*[19] (Torrez M and others, unpublished data), a presumed vector of cutaneous leishmaniasis.[17]

Departures from Hardy-Weinberg expectations as well as linkage disequilibrium are expected in populations in which mating is not random, especially when there is reproductive isolation between individuals. However, other causes may generate genetic disequilibrium, such as geographic subdivision or selection.[8] In this study, we used both within-locus and between-locus analysis to look for possible species heterogeneity. Since fixation indices were positive, all deviations from Hardy-Weinberg expectations were due to heterozygote deficiency. The Wahlund effect, i.e., geographic or spatial subdivision, would be a logical hypothesis to account for heterozygote deficiency. However, since females originating from LT (LT-AB) or PE (PE-AB) were captured at the top of the same tree on the same day, spatial subdivision would be unlikely. Also unlikely would be a selective pressure against heterozygotes at the same two loci, GPD and IDH, in two different geographic areas. Even if this hypothesis was considered, further selective pressure would be necessary to account for the linkage disequilibrium observed in each area between GPD and IDH (LT-AB and PE-A8 in Table 2). In the same way as stated for departures from Hardy-Weinberg expectations, there is no reason to expect a linkage disequilibrium to show exactly the same patterns in two distinct geographic areas, except when genes are closely linked inside chromosomal inversions.[20] However, when each of the two sets of individuals referred to as A and B was considered separately in each locality, no departure from panmictic expectations was observed. In the present state of this research, the most parsimonious hypothesis to account for our overall results is reproductive isolation between A and B in the two localities under survey.

Values of Nei's standard genetic distance between A and B (Figure 1) were comparable with the values found between sibling species, such as *Lu. (Psychodopygus) yucumensis* and *L. (P.) carrerai,*[15, 16] or even noncryptic, closely related species, such as *P. chinensis* and *P. sichuanensis.*[21] A sampling of seven loci is not sufficient for an accurate estimate of genetic distance. Nevertheless, even if seven additional loci exhibited the same alleles with the same frequencies between A and B, the genetic distance between these two groups of individuals would remain greater than 0.10. Presently, no case of local conspecific populations separated by Nei's genetic distances up to 0.10 is known.[22] Furthermore, the values presented can be considered as an underestimation of the actual genetic distance since the PEP system, which gave distinct patterns in each of the two groups A and B, was not taken into account in genetic distance calculations. On the other hand, the values observed here between the A and B groups even in sympatric conditions are more than 50 times higher than the values observed between geographic populations (Figure 1). Thus, Nei's standard genetic distances between the A and B groups of individuals are in full agreement with the working hypothesis that these groups

represent distinct species. The GPD locus, which presents alternate alleles for each of the two A and B groups, constitutes a convenient diagnostic locus[23] in this area.

The two cryptic species could exhibit distinct vectorial capacities and different behaviors. This will have to be determined by additional studies in which the subdivision of *Lu. trapidoi* into two distinct species is taken into account. The material examined in the present study is of sylvatic origin. However, *Lu. trapidoi* has been shown to enter dwellings, being responsible for a peridomestic transmission of leishmaniasis in this area of Ecuador.[24] Thus, we propose first to verify whether the proportions of both cryptic species are the same in the peridomestic and in the sylvatic environment. In this study, the lack of interbreeding within *Lu. trapidoi* populations was demonstrated by the genotypic disequilibrium revealed in individuals living in temporal and spatial proximity.

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