Pole 2

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# GENETIC ANALYSIS OF *LEISHMANIA* PARASITES IN ECUADOR: ARE *LEISHMANIA* (*VIANNIA*) *PANAMENSIS* AND *LEISHMANIA* (*V.*) *GUYANENSIS* DISTINCT TAXA?

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*Abstract.* In the course of an epidemiologic survey in Ecuador, the following collection of *Leishmania* stocks was isolated: 28 from patients with clinical signs of leishmaniasis, 2 from sloths, 1 from a dog, and 4 from sand flies. For genetic characterization of these stocks, multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD) were used. Twenty six of the 35 stocks were identified as either *Leishmania* (*V.*) *panamensis* or *L.* (*V.*) *guyanensis*, 2 stocks were identified as *L.* (*V.*) *braziliensis*, the 2 stocks from sloths showed specific genotypes, and 5 stocks were characterized as hybrids between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis*. These data show that genetic diversity of *Leishmania* in Ecuador is high and that *L.* (*V.*) *panamensis/guyanensis* is the dominant group in this country. The genetic analysis questioned the distinctness between the two species *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis*, since MLEE and RAPD data did not indicate that *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis* correspond to distinct monophyletic lines. Population genetic analysis performed on the *L.* (*V.*) *panamensis/guyanensis* group favors the hypothesis of a basically clonal population structure.

Cutaneous and mucocutaneous leishmaniases constitute a serious public health problem in Ecuador, since the disease is endemic in 17 of 20 provinces.[1] The most frequent clinical forms are the cutaneous and mucocutaneous ones, with a large spectrum of clinical variation.[2] This clinical variability of the disease is believed to be due to the *Leishmania* species diversity encountered in Ecuador.[1] Seven *Leishmania* species are known to be responsible for infections reported in the country: *Leishmania* (*Viannia*) *braziliensis*, *L.* (*V.*) *panamensis*, *L.* (*V.*) *guyanensis*, *L.* (*Leishmania*) *mexicana*, *L.* (*L.*) *pifanoi*, *L.* (*L.*) *amazonensis*, and *L.* (*V.*) *equatorensis*.[3–6] The most frequently sampled *Leishmania* species in Ecuador are *L.* (*V.*) *guyanensis* and *L.* (*V.*) *panamensis*.[7] These two species belong to the *guyanensis* complex according to the World Health organization classification.[8] Genetic and biochemical analysis demonstrated that they are genetically very close and that only one enzymatic system could be used as a diagnostic marker able to discriminate them.[9]

This study reports the genetic analysis of 35 *Leishmania* isolates collected in the course of extensive field studies in Ecuador.[10] This genetic epidemiology study improves our knowledge on the epidemiology of the disease in this country. Moreover, the results clearly raise the question of the distinctness between *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis*.

## MATERIALS AND METHODS

**Isolate collection.** The study adhered to the ethical rules of the European Community in accordance with the Ecuadorian government. Twenty-eight stocks were isolated from patients presenting clinical signs, 2 isolates were from sloths, 1 from a dog, and 4 from sandfly vectors (Table 1). Twenty-six of these stocks were obtained at 3 study stations selected in departments with a high transmission level of leishmaniasis: La Tablada, Paraiso Escundido, and Zumba (Figure 1). La Tablada and Paraiso Escundido on the Pacific coast were selected based on their geographic location (rural areas not easily accessible) and the endemic character of the disease. Paraiso Escundido is located in the interior part of the country in humid tropical forest, and La Tablada is a littoral village in a dry tropical forest. The third site, Zumba, is located in the southern part of the country in the Amazonian plain. Some parasites were isolated from 2 other sites: 1 stock came from Vozandes Hospital in Quito and 5 other stocks came from Augusto Egas Hospital in Santo Domingo (Table 1). The origin of 3 stocks is unknown.

To better ascertain the species attribution of the stocks surveyed, 11 reference stocks pertaining to different species were added to the study: 3 *L.* (*V.*) *guyanensis* (MHOM/FG/84/H166, MHOM/BR/78/M5378, and MHOM/GF/85/LEM669), 2 *L.* (*V.*) *panamensis* (MCHO/PA–755 and MHOM/PA/71/LS94), 3 *L.* (*V.*) *braziliensis* (MHOM/PE/89/LH754, MHOM/BO/84/Lpz595, and MHOM/BR/75/M2903), 1 *L.* (*V.*) *colombiensis* (IHAR/CO/86/CL500), 1 *L.* (*V.*) *equatorensis* (MCHO/EC/82/LSP01), and 1 *L.* (*L.*) *infantum* (MHOM/MA(BE)/67/ITMAP63).

**Isolation and culture of parasites.** Human and canine stocks were isolated by classical techniques of biopsy and aspirated materials. Stocks PL2 and PL21 were taken from sloths by hepatic puncture. To identify the parasites from an isolate, the technique of Armijos and others was used.[4] The parasites were bulk-cultured in RPMI 1640 medium with 10% fetal calf serum. They were harvested by centrifugation (8,000 × *g* for 15 min at 4°C) and washed twice in phosphate-buffered saline, pH 7.3.

**Preparation of samples and isoenzyme electrophoresis.** Technical conditions for preparation of samples, electrophoresis, and staining procedures have been described by Ben Abderrazak and others.[11] Cellulose acetate electrophoresis was used. Fifteen enzyme systems were used: aconitase (ACON: EC 4.2.1.3), glucose-6-phosphate dehydrogenase (G6PD: EC 1.1.1.49), glucose phosphate isomerase (GPI: EC 5.3.1.9), glutamate oxaloacetate transaminase (GOT: EC 2.6.1.1), glutamate pyruvate transaminase (ALAT: EC 2.6.1.2), isocitrate dehydrogenase (IDH: EC 1.1.1.42), ma-

TABLE 1

World Health Organization codes of *Leishmania* stocks, hosts, clinical features, localities, isolation dates, and multilocus enzyme electrophoresis (MLEE) identification of Ecuadorian stocks

| Stocks (WHO code) | Host | Clinical feature | Locality | Isolation date | MLEE identification |
|---|---|---|---|---|---|
| MHOM/EC/92/E72 | Human | ? | Santo Domingo | 2/1/92 | *Leishmania (Viannia) braziliensis* |
| MHOM/EC/92/E94* | Human | ? | Santo Domingo | 2/28/92 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/92/E91* | Human | ? | Santo Domingo | 9/28/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E29 | Human | Simple cutaneous lesion (arm) | Santo Domingo | 11/30/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/92/ST6 | Human | ? | Santo Domingo | 8/4/92 | *L. (V.) Panamensis/guyanensis* |
| MCHO/EC/91/PL21 | Sloth | | La Tablada | 11/27/91 | ? |
| MCHO/EC/92/PL2 | Sloth | | La Tablada | 2/21/92 | ? |
| ITRA/EC/92/EK747 | *Lutzomyia trapidoi* | | La Tablada | 8/17/92 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/92/A8044* | Human | ? | La Tablada | 8/04/92 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E18 | Human | Multiple cutaneous lesions (arms) | La Tablada | 11/26/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E3* | Human | Simple cutaneous lesion (leg) | La Tablada | 11/24/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E4* | Human | Simple cutaneous lesion (arm) | La Tablada | 11/24/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E6 | Human | Multiple cutaneous lesions (leg, arms) | La Tablada | 11/25/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E19* | Human | Multiple cutaneous lesions (arm) | La Tablada | 11/26/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/92/E10 | Human | Simple cutaneous lesion (leg) | La Tablada (Cacao) | 11/25/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E11 | Human | Multiple cutaneous lesions (face, arm, leg) | La Tablada (Cacao) | 11/25/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E14 | Human | Multiple cutaneous lesions (leg) | La Tablada (Crisanto) | 11/26/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E12* | Human | Simple cutaneous lesion (shoulder) | La Tablada (Crisanto) | 11/25/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E1* | Human | Multiple cutaneous lesions (leg, face) | La Tablada (Crisanto) | 11/24/91 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/91/E30* | Human | Simple cutaneous lesion (face) | Paraiso Escundido | 11/30/91 | *L. (V.) panamensis/guyanensis* |
| MCAN/EC/92/DOG1* | Dog | | Paraiso Escundido | 1/9/92 | *L. (V.) panamensis/guyanensis* |
| ITRA/EC/92/EK112 | *Lutzomyia trapidoi* | | Paraiso Escundido | 10/10/91 | *L. (V.) panamensis/guyanensis* |
| ITRA/EC/92/EK665* | *Lutzomyia trapidoi* | | Paraiso Escundido | 8/11/92 | *L. (V.) panamensis/guyanensis* |
| ITRA/EC/92/EK649* | *Lutzomyia trapidoi* | | Paraiso Escundido | 8/11/92 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/92/E79 | Human | ? | Vozandes (Quito) | 2/10/92 | *L. (V.) braziliensis* |
| MHOM/EC/92/Z9 | Human | Cutaneous lesion | Zumba | 7/17/92 | Hybrid[27] |
| MHOM/EC/92/Z5 | Human | Cutaneous lesion | Zumba | 7/17/92 | Hybrid[27] |
| MHOM/EC/92/PR1* | Human | Cutaneous lesion | Zumba | 8/28/92 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/92/P5 | Human | Cutaneous lesion | Zumba (Palanda) | 6/23/94 | Hybrid[27] |
| HMOM/EC/91/E67* | Human | Cutaneous lesion | Zumba (Palanda) | 1/20/92 | *L. (V.) panamensis/guyanensis* |
| MHOM/EC/92/E102 | Human | Cutaneous lesion | Zumba (Pucapamba) | 4/16/92 | Hybrid[27] |
| MHOM/EC/92/E107 | Human | Cutaneous lesion | Zumba (Pucapamba) | 4/16/92 | Hybrid[27] |
| MHOM/EC/-/E73* | Human | Cutaneous lesion | ? | ? | *L. (V.) panamensis/guyanensis* |
| MHOM/EC–E50* | Human | Multiple cutaneous lesions (leg, arm) | ? | ? | *L. (V.) panamensis/guyanensis* |
| MHOM/EC–E49 | Human | Simple cutaneous lesion (arm) | ? | ? | *L. (V.) panamensis/guyanensis* |

* Stocks used for the random amplified polymorphic DNA analysis.

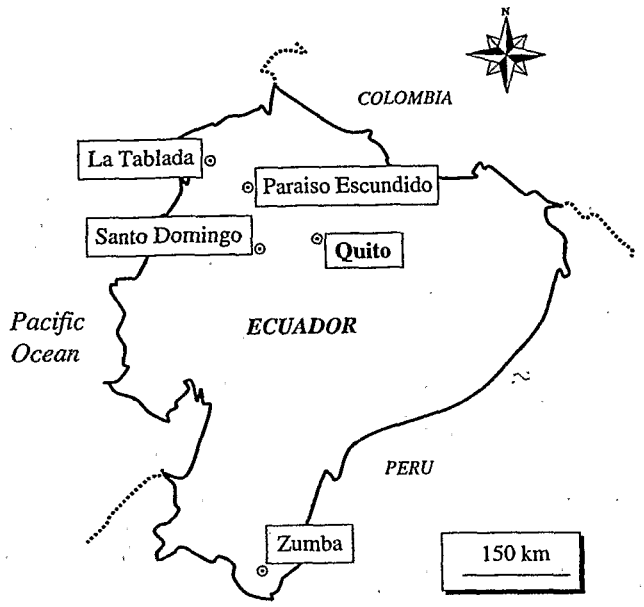


FIGURE 1. Map of Ecuador showing the locations of *Leishmania* stock sampling areas.

late dehydrogenase NAD+ (MDH: EC 1.1.1.37), malate dehydrogenase NADP+ or malic enzyme (ME: EC 1.1.1.40), mannose phosphate isomerase (MPI: EC 5.3.1.8), nucleoside hydrolases, substrate inosine and substrate deoxyinosine, I and D, respectively (NHI and NHD: EC 2.4.2.*), peptidases 1 and 2 (PEP1 and PEP2: EC 3.4.4.11 or 13), 6-phosphogluconate dehydrogenase (6PGD: EC 1.1.1.44), and phosphoglucomutase (PGM: EC 2.7.5.1). The 15 enzyme systems used made it possible to study 16 different putative loci. Two different loci could be distinguished with the NHI system, *Nhi* 1 and *Nhi* 2.

**Random amplified polymorphic DNA (RAPD) protocol.** The RAPD fingerprinting method, first described by Welsh and McClelland[12] and Williams and others,[13] has been used in the characterization of various parasitic protozoa by Tibayrenc and others.[14]

A subset of 16 stocks of this Ecuadorian sample (marked by an asterisk in Table 1) plus 5 reference stocks, pertaining either to the *L.* (*V.*) *panamensis* or to *the L.* (*V.*) *guyanensis* species (3 *L.* (*V.*) *guyanensis*: MCHO/FG/83/CAY A116, MHOM/BR/78/M5378, and MHOM/FG/85/LEM669; 2 *L.* (*V.*) *panamensis*: MCHO/PA–M4039 and MHOM/CO/83/REST417), was further analyzed by RAPD fingerprinting.

The DNA was isolated according to the protocol of Sambrook and others.[15]

Thirteen primers in a kit (Operon Technologies, Inc., Alameda, CA) were used: A10: GTGATCGCAG, B8: GTCCACACGG, F1: ACGGATCCTG, F13: GGCTGCAGAA, N13: AGCGTCACTC, N20: GGTGCTCCGT, R13: GGACGACAAG, R14: CAGGATTCCC, R15: GGACAACGAG, R16: CTCTGCGCGT, R20: CTCTGCGCGT, U15: ACGGGCCAGT, and U16: CTGCGCTGGA.

**Identification of stocks and estimation of genetic relationships.** The electrophoretic profiles of Ecuadorian stocks were compared with those of stocks used as reference for the different species. On the basis of band similarity and known diagnostic loci, a species attribution was made for each stock.

The genetic relationships among the stocks were established by computing Jaccard distances from multilocus enzyme electrophoresis (MLEE) and RAPD data for all possible pairwise comparisons:[16] $Dij = 1 - (a/a + b + c)$ where $a$ = number of bands that are common to the i and j stocks, $b$ = number of bands recorded for stock i and absent for stock j, and $c$ = number of bands recorded for stock j and absent for stock i. From the distance matrix obtained, unweighted pair-group method with arithmetic averages (UPGMA) dendrograms were designed.[17]

**Population genetic analysis.** *Leishmania* population structure in this area was explored by the analysis of linkage disequilibrium or nonrandom association of genotypes occurring at different loci. The tests proposed by Tibayrenc and others[18] were used. They are based on the null hypothesis that the population is panmictic (recombination occurs at random). Any statistical departures from the panmictic expectations show that gene flow is inhibited in the population. The d1 test calculates the combinatorial probability of sampling the most common genotype as often as, or more often than, the observed frequency. The d2 test calculates the probability of observing any genotype as often as, or more often than the most common genotype in the sample. The e test calculates the probability of observing as few or fewer genotypes in the population than observed in the sample. The f test calculates the probability of observing a linkage disequilibrium in the population as high or higher than the one observed in the sample. The d2, e, and f tests are based on computer simulations (Montecarlo tests) with $10^4$ runs. The biases generated by either spatial or temporal isolation, and the means to avoid them, have been reported by Tibayrenc and others.[19]

## RESULTS

The species attribution of each stock was based on the diagnostic loci identified by Guerrini (Guerrini F, 1993. *Génétique des Populations et Phylogénie des Leishmania du Nouveau-Monde.* PhD Thesis. Université des Sciences et Techniques du Languedoc, Montpellier France) and on the comparison with the reference stocks. Table 1 shows the species identification of the isolates by MLEE. Of the 35 stocks examined, 2 were identified as *L.* (*V.*) *braziliensis,* the 2 isolated from sloths, PL2 and PL21, had a specific genotype different from any known *Leishmania* species, 26 were identified as either *L.* (*V.*) *panamensis* or *L.* (*V.*) *guyanensis* and 5 had complex isoenzyme patterns (Table 1). The stocks identified as *L.* (*V.*) *panamensis/guyanensis* were isolated either from humans, a dog, or sand flies (*Lutzomyia trapidoi*). The 6-*Pgd* locus described in the literature[9] as a diagnostic marker to distinguish *L* (*V.*) *panamensis* from *L.* (*V.*) *guyanensis* showed many different profiles in the *L.* (*V.*) *panamensis/guyanensis* group. In contrast, the *Nhi* 2 locus showed only 2 different patterns in the *L.* (*V.*) *panamensis/guyanensis* group. In the *L.* (*V.*) *panamensis/guyanensis* group (26 stocks), 21 different isoenzyme profiles or zymodemes were recorded (genotypic variability = 0.807). Five stocks from the Zumba Area showed complex patterns in the *Nhi* 2 and *Pgm* loci.

The UPGMA dendrogram designed from the Jaccard's distance matrix separated the zymodemes (distinct isoenzyme profiles) into 4 distinct clusters, which are not linked to either host or geographic origin, except for the cluster 2 (Figure 2). The first cluster included all the strains identified as either *L.* (*V.*) *panamensis* or *L.* (*V.*) *guyanensis,* as well as the *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis* reference stocks. The average genetic distance within the *L.* (*V.*) *panamensis/guyanensis* group (without the reference stocks) was 0.264 with a standard deviation of 0.104. The second cluster is composed of only the 5 stocks from Zumba exhibiting complex *Nhi* 2 and *Pgm* profiles. The third cluster included the *L.* (*V.*) *braziliensis* reference stocks and the two stocks E79 and E72. The fourth cluster included the *L.* (*V.*) *equatorensis* and *L.* (*V.*) *colombiensis* reference stocks and the 2 stocks PL2 and PL21 isolated from sloths. These sloth stocks appeared to be equally distant (genetic distance = 0.7) from both reference stocks. *Leishmania* (*L.*) *infantum* was phytogenetically very distant from all other stocks included in the study, which is consistent with the assignment of this species to another subgenus (*Leishmania*).

The RAPD technique was performed only on a subset of 16 Ecuadorian stocks belonging to the *L.* (*V.*) *panamensis/guyanensis* group (Table 1) and 5 reference stocks belonging to either *L.* (*V.*) *panamensis* species or *L.* (*V.*) *guyanensis.* We found no RAPD fragment specific to either *L.* (*V.*) *panamensis* or *L.* (*V.*) *guyanensis.* Furthermore, the UPGMA analysis did not show any structuring in 2 clear-cut clusters.

We performed the population genetic tests of recombination from isoenzyme data in the *L.* (*V.*) *panamensis/guyanensis* group, which is the largest part of the sample. The 5 stocks with complex *Nhi* 2 and *Pgm* patterns, which pose specific problems of interpretation, were excluded from the analysis, together with the reference stocks. Finally, the 26 Ecuadorian stocks of cluster 1 were used for the population genetic analysis. We used the recombination tests d1, d2, e, and f, which are all related to the linkage disequilibrium phenomenon, but explore different facets of it.[17] The results were as follows: d1: $P = 5.9 \times 10^{-3}$ (for the genotype of the EK649 stock, represented 2 times), $9 \times 10^{-3}$ (for the genotype of the E12 stock, represented 3 times); d2: $P$ not significant; e: $P$ not significant; f: $P = 10^{-4}$.

## DISCUSSION

In Ecuador, the predominant clinical forms are the cutaneous and mucocutaneous leishmaniases with a large spectrum of clinical variation. It has been inferred that clinical variability of the disease was due to the diversity of *Leishmania* species encountered in Ecuador.[1]

Two of the 35 stocks analyzed were identified as *L.* (*V.*) *braziliensis.* These stocks were from a patient from in hospital in Santo Domingo, and from a patient in Vozandes Hospital in Quito (Figure 1). These results confirm previously published data showing the presence of *L.* (*V.*) *braziliensis* in Ecuador.[3] The identification of 2 *L.* (*V.*) *braziliensis* stocks is consistent with the presence of mucocutaneous clinical forms in Ecuador.[20, 21]

Two stocks, PL2 and PL21, isolated from sloths in the site at La Tablada (Figure 1) have specific isoenzyme multilocus genotypes. Their isoenzyme profile is distinct from any known *Leishmania* species. Nevertheless, they are clustered, although remotely, with the *L.* (*V.*) *colombiensis* and *L.* (*V.*) *equatorensis* reference stocks (Figure 2). These 2 sloth stocks have similar genotypes (only 1 allele difference). It is unexpected that these 2 sloth stocks are not identified as *L.* (*V.*) *panamensis,* since Edentata are known to be the major reservoir of this *Leishmania* species.[22, 23] It would be necessary to increase the sample of *Leishmania* stocks from sloths in Ecuador to better understand the epidemiology and taxonomic status of these specific genotypes.

The *L.* (*V.*) *panamensis/guyanensis* group is dominant in the present sample. Indeed, of 35 examined stocks, 26 stocks isolated from humans, a dog, or *Lu. trapidoi* pertain to this group. These results are consistent with the data of Hashigushi and others.[5] Only a few cases of dog infection by the species *L.* (*V.*) *panamensis/guyanensis* have been reported.[24] Until now, the domestic dog has been previously demonstrated to be essentially the reservoir of *L.* (*L.*) *infantum.* One case of canine infection by *L.* (*V.*) *tropica* has been reported.[25] It is also suspected to be a reservoir for the *L.* (*V.*) *peruviana* species.[26] Nevertheless, clinico-epidemiologic data suggest that the dog infection by the species *L.* (*V.*) *panamensis/guyanensis* recorded here is accidental, since this case was the only one in an extensive survey performed on dogs. The 4 stocks isolated from *Lu. trapidoi* were also identified as *L.* (*V.*) *panamensis/guyanensis.* These data are consistent with the notion that *Lu. trapidoi* is the major vector of *L.* (*V.*) *panamensis* in Ecuador.[23]

The 5 stocks from Zumba grouped in cluster 2 (Figure 2) could not be classified by the classical diagnostic markers in the *L.* (*V.*) *panamensis/guyanensis* group. The complex profiles of *Nhi* 2, *Pgm,* and *Mpi* loci for the stock P5 were interpreted as heterozygous patterns between the *L.* (*V.*) *guyanensis* and *L.* (*V.*) *braziliensis* species.[27]

On the MLEE UPGMA dendrogram, it is impossible to distinguish any clear additional subdivision within the cluster 1 composed of the *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis* reference stocks and the Ecuadorian stocks related to them. This lack of clear-cut subdivisions is fully confirmed by the RAPD data obtained from a subset of 16 Ecuadorian stocks and the reference stocks belonging to the *L.* (*V.*) *guyanensis* and *L.* (*V.*) *panamensis* species, which again show no tendency of clear clustering into 2 discrete groups that would correspond to each of these 2 species.

The 6-*Pgd* locus, which has been proposed as a diagnostic locus between these 2 species, shows considerable allelic diversity and does not permit any clear-cut subdivisions within the Ecuadorian sample of *L.* (*V.*) *panamensis/guyanensis* stocks (Figure 3).[9]

The only locus that could suggest a possible discrimination between the 2 species is *Nhi* 2 (Table 2). All *L.* (*V.*) *guyanensis* reference stocks show an allele with a strong enzyme activity, whereas the *L.* (*V.*) *panamensis* reference stocks show a weak or null allele (Figure 4). The Ecuadorian stocks have either one or the other of these 2 clearly distinct profiles. The 4 stocks isolated from *Lu. trapidoi* were also characterized by specific monoclonal antibodies, and were identified as *L.* (*V.*) *guyanensis* (Le Pont F, unpublished data). This species identification corresponds to the one obtained with the *Nhi* 2 locus (Table 2).

Nevertheless, based on only 1 of 15 isoenzyme loci and

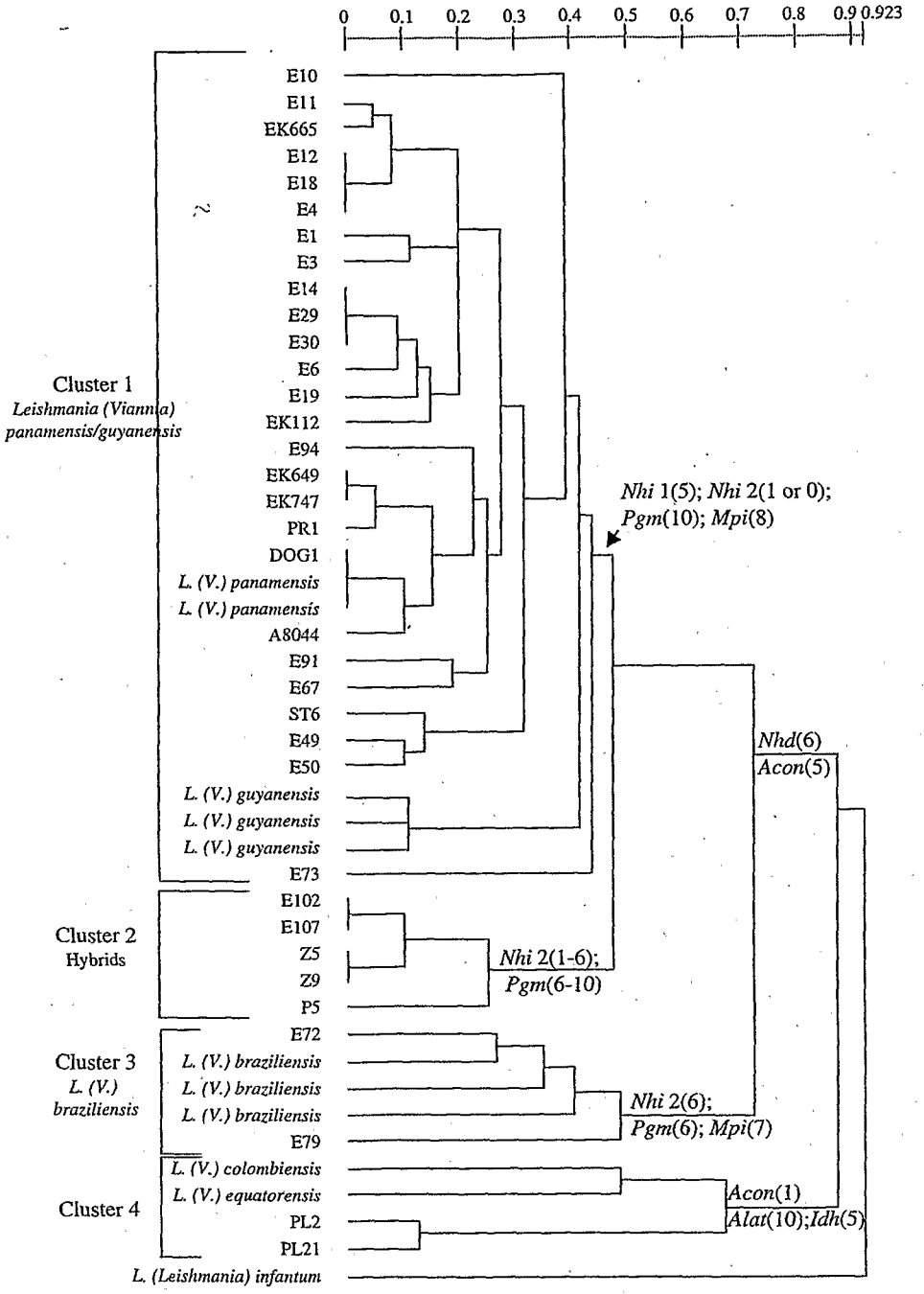


FIGURE 2. Unweighted pair group method with arithmetic averages dendrogram built from Jaccard's genetic distances calculated from multilocus enzyme electrophoresis data. The genotypes that are specific for a given cluster are noted on the dendrogram. For definitions of enzyme loci, see Materials and Methods.

13 RAPD primers, it would be extremely tentative to infer that *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis* correspond to true distinct monophyletic lineages (discrete typing units [DTUs]).[28] If this were true, these two DTUs would be closely related, and the relevance of describing them as separate taxonomic entities would be doubtful. Table 2 summarizes the tentative species attributions that can be drawn from the 6-*Pgd* and *Nhi* 2 loci.

In the *L.* (*V.*) *panamensis/guyanensis* group considered as a whole, the genotype diversity is high (0.807), whereas the phylogenetic variability is limited (mean ± SD genetic distances = 0.264 ± 0.104). Diagnostic characters (Tags)[28] can be proposed for the entire *L.* (*V.*) *panamensis/guyanensis* group (including the reference stocks, without the hybrid stocks) based on the *Nhi, Pgm,* and *Mpi* loci.

The 5 stocks from Zumba with complex isoenzyme patterns could not be classified as either *L.* (*V.*) *panamensis/guyanensis* or *L.* (*V.*) *braziliensis.* Indeed, these stocks showed heterozygous profiles for the three loci (*Nhi* 2, *Pgm,* and *Mpi*) able to discriminate *L.* (*V.*) *braziliensis* from *L.*

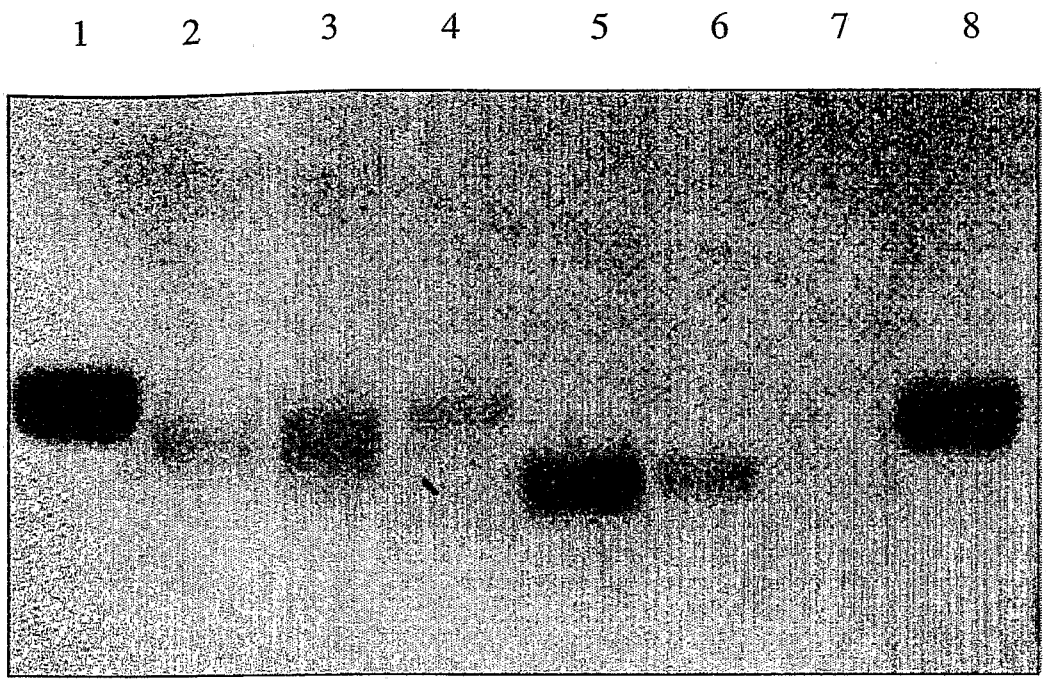


FIGURE 3. Multilocus enzyme electrophoresis profiles obtained with the 6-phosphoglucontate dehydrogenase enzyme system. Lane 1, M2903 (*Leishmania* (*Viannia*) *braziliensis* reference stock); lane 2, E72; lane 3, E94; lane 4, EK649; lane 5, M4039 (*L.* (*V.*) *panamensis* reference stock); lane 6, EK665; lane 7, no profile because of enzyme degradation); lane 8, A8044.

TABLE 2

Tenative attribution to either *Leishmania* (*Viannia*) *panamensis* or *L.* (*V.*) *guyanensis* by 2 isoenzyme loci (*6PGD* and *Nhi* 2) of the *Leishmania* stocks isolated in the present study*

| Code | *6PGD* | *Nhi* 2 |
|---|---|---|
| E73 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *panamensis* |
| E50 | *L.* (*V.*) *panamensis* | *L.* (*V.*) panamensis |
| E49 | *L.* (*V.*) *panamensis* | L. (*V.*) *guyanensis* |
| EK747 | ? | *L.* (*V.*) *guyanensis* |
| A8044 | *L.* (*V.*) *guyanensis* | *L.* (*V.*) *panamensis* |
| E18 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *guyanensis* |
| E3 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *guyanensis* |
| E4 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *guyanensis* |
| E6 | ? | *L.* (*V.*) *guyanensis* |
| E19 | ? | *L.* (*V.*) *guyanensis* |
| E10 | *L.* (*V.*) *guyanensis* | *L.* (*V.*) *panamensis* |
| E11 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *guyanensis* |
| E14 | ? | *L.* (*V.*) *guyanensis* |
| E12 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *guyanensis* |
| E1 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *guyanensis* |
| E30 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *guyanensis* |
| DOG1 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *panamensis* |
| EK112 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *guyanensis* |
| EK665 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *guyanensis* |
| EK649 | ? | *L.* (*V.*) *guyanensis* |
| E94 | ? | *L.* (*V.*) *guyanensis* |
| E91 | ? | *L.* (*V.*) *panamensis* |
| E29 | ? | *L.* (*V.*) *guyanensis* |
| ST6 | ? | *L.* (*V.*) *guyanensis* |
| PR1 | *L.* (*V.*) *guyanensis* | *L.* (*V.*) *guyanensis* |
| E67 | ? | *L.* (*V.*) *panamensis* |
| 755 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *panamensis* |
| LS94 | *L.* (*V.*) *panamensis* | *L.* (*V.*) *panamensis* |
| LEM669 | *L.* (*V.*) *guyanensis* | *L.* (*V.*) *guyanensis* |
| M5378 | *L.* (*V.*) *guyanensis* | *L.* (*V.*) *guyanensis* |
| H166 | *L.* (*V.*) *guyanensis* | *L.* (*V.*) *guyanensis* |

* *6PGD* = 6-phosphogluconate dehydrogenase, *Nhi* 2 = nucleoside hydrolase substrate inosine 2; ? = stocks that do not have the *6PGD* band level of *L.* (*V.*) *guyanensis* reference stocks or the *6PGD* band level of *L.* (*V.*) *panamensis* reference stocks.

(*V.*) *guyanensis/panamensis.* For each stock, the other enzymatic systems presented profiles that were also observed either for the *L.* (*V.*) *braziliensis* reference strains or for *L.* (*V.*) *panamensis/guyanensis* strains. Based on the complete analysis of their complex genotypes and on already published RAPD data, we have proposed that these 5 stocks correspond to hybrid genotypes between *L.* (*V.*) *panamensis/guyanensis* and *L.* (*V.*) *braziliensis.*[27] If we accept as a working hypothesis that the *Nhi* 2 strong allele corresponds to *L.* (*V.*) *guyanensis,* these 5 stocks would be hybrids between this species and *L.* (*V.*) *braziliensis.* There heterozygous patterns corresponding to this locus are 5-banded (the nucleoside hydrolase inosine substrate 2 is a tetrameric enzyme). Conversely, the hybrids postulated by Belli and others[29] in Nicaragua would be hybrids between *L.* (*V.*) *panamensis* and *L.* (*V.*) *braziliensis,* since their heterozygous pattern for the *Nhi* 2 locus is asymmetrical 3-banded (this pattern would correspond to an heterozygous tetrameric enzyme with a null allele).

The d1 and f linkage disequilibrium tests showed highly significant results, which shows that gene flow is severely restricted in this set of stocks. The most parsimonious hypothesis to account for this result is a basically clonal population structure, which has already been inferred for other *Leishmania* species and other major human parasites.[18] Several comments can be made regarding this hypothesis. 1) It is not parsimonious to attribute departures from panmictic expectations to geographic distance and genetic drift. If this were the case, distribution of genotypes would be linked to geographic distance, which is not verified here.[19] 2) The 5 putative hybrid genotypes were excluded from the linkage disequilibrium analysis. Departures from panmictic expectations are not therefore attributable to the presence of genes

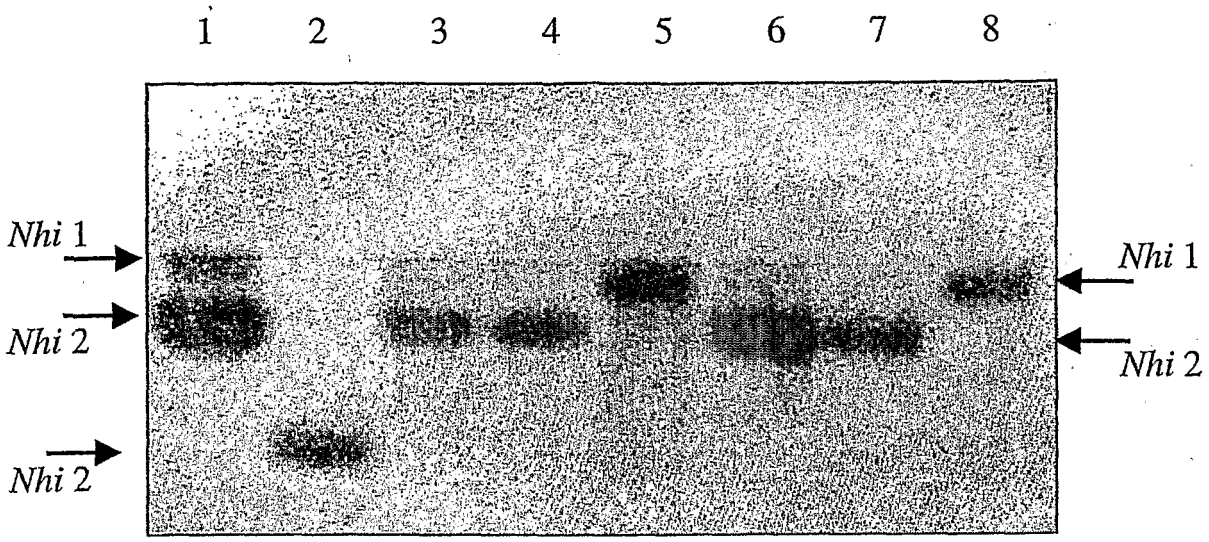


FIGURE 4. Multilocus enzyme electrophoresis profiles obtained with the nucleoside hydrolase substrate inosine (*Nhi*) enzyme system. Lane 1, M5378 (*Leishmania* (*V.*) *guyanensis* reference stock); lane 2, E72; lane 3, E94; lane 4, EK649; lane 5, M4039 (*L.* (*V.*) *panamensis* reference stock); lane 6, EK665; lane 7, EK747; lane 8, A8044.

from another species. 3) The existence of hybrids is not incompatible with the hypothesis of a basically clonal population structure. As a matter of fact, this hypothesis does not imply that sex is totally absent. It states only that recombination is not frequent enough to break the prevalent pattern of clonal population structure.

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