Is *Leishmania* (*Viannia*) *peruviana* a Distinct Species? A MLEE/RAPD Evolutionary Genetics Answer

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ABSTRACT. A set of 38 *Leishmania* stocks from the Andean valleys of Peru was characterized by both Multilocus Enzyme Electrophoresis (MLEE) and Random Amplified Polymorphic DNA (RAPD). Data were analyzed in terms of taxonomy and evolutionary genetics. Synapomorphic MLEE and RAPD characters, clear-cut clustering, and strong agreement between the phylogenies inferred from either MLEE or RAPD supported the view that *Leishmania* (*Viannia*) *peruviana* and *Leishmania* (*Viannia*) *braziliensis* correspond to two closely related, but distinct monophyletic lines (clades) and can therefore be considered as "discrete typing units" (DTUs). The question whether the *L.* (*V.*) *peruviana* DTU deserves species status is dependent upon the desirability of it, in terms of epidemiological and medical relevance. A previous Orthogonal Field Alternating Gel Electrophoresis (OFAGE) analysis of the same *L.* (*V.*) *peruviana* isolates was published by Dujardin et al. (1995b). The data from the different markers (i.e. MLEE, RAPD and OFAGE) were compared by population genetics analysis. RAPD and OFAGE provided divergent results, since RAPD showed a strong linkage disequilibrium whereas OFAGE revealed no apparent departure from panmictic expectation. MLEE showed no linkage disequilibrium. Nevertheless, contrary to OFAGE, this is most probably explainable by the limited variability revealed by this marker in *L.* (*V.*) *peruviana* (statistical type II error). RAPD data were consistent with the hypothesis that the present *L.* (*V.*) *peruviana* sample displays a basically clonal population structure with limited or no genetic exchange. Disagreement between RAPD and OFAGE can be explained either by accumulation of chromosomal rearrangements due to amplification/deletion of repeated sequences, or by pseudo-recombinational events.

Key Words. Clade, clone, discrete typing unit, speciation, statistical type II error.

IN the case of pathogenic micro-organisms, the biological concept of species (Mayr 1942) is generally hardly applicable, and definition of species is only a matter of convenience. In applied microbiology, it would be advisable to describe new species on the basis of both specific epidemiological/medical properties and genetic characters.

In the genus *Leishmania,* about thirty species have already been defined. A large majority of these species was originally described on the basis of clinical, epidemiological, and biological features. Distinctiveness of these species was often confirmed by genetic criteria, although some discrepancies were noted. For example, genetic studies demonstrated that *Leishmania* (*Leishmania*) *pifanoi* and *Leishmania* (*Leishmania*) *garnhami* cannot be differentiated from *Leishmania* (*Leishmania*) *mexicana* and *Leishmania* (*Leishmania*) *amazonensis,* respectively (Barker and Butcher 1983; Kreutzer, Souraty, and Semko 1987; Momen and Grimaldi 1984).

This paper presents the problem of the species status of *Leishmania* (*Viannia*) *peruviana*. Some authors suggest that *L.* (*V.*) *peruviana* may simply be a variant of *Leishmania* (*Viannia*) *braziliensis* and not a valid species (Grimaldi, Tesh, and McMahon-Pratt 1989). These two *Leishmania* (*Viannia*) species are dominant in Peru (Guerra 1988; Lumbreras, and Guerra 1985). *Leishmania* (*V.*) *peruviana* is endemic in the western Andean and inter-Andean valleys, and is responsible for a strictly cutaneous clinical form (Andean leishmaniasis, AL or Uta). *Leishmania* (*V.*) *braziliensis* is encountered mainly in the Amazonian forest, and is the etiological agent of muco-cutaneous leishmaniasis (sylvatic leishmaniasis, SL or Espundia). Until now, characterization of *L.* (*V.*) *peruviana* was not easy, for this species is genetically very close to *L.* (*V.*) *braziliensis*. Only one enzyme system, Mannose Phosphate Isomerase (MPI) and one genomic character (the size of the chromosome bearing the gp63 genes) supposedly distinguish these two species (Arana et al. 1990; Dujardin et al. 1993a; Dujardin et al. 1995b; Guerrini 1993). Confirmation of the distinctiveness of these two species is all the more desirable since they are associated with very distinct clinical forms.

Extensive karyotype analysis by Orthogonal Field Alternating Gel Electrophoresis (OFAGE) has been conducted on Peruvian *Leishmania* stocks by Dujardin et al. (1993b). Sampling was performed in the Andean valleys along a North-South transect covering four biogeographical units (BGUs), defined according to the endemism of butterfly species (Lamas 1982). In order to clarify the taxonomical status of *L.* (*V.*) *peruviana,* we have undertaken an evolutionary genetic analysis by multilocus enzyme electrophoresis (MLEE) and Random Amplified Polymorphic DNA (RAPD) of 38 *Leishmania* stocks isolated from the four Andean BGUs already surveyed by Dujardin et al. (1993b).

MATERIALS AND METHODS

Parasite stocks. Thirty-eight stocks from the Peruvian Andean valleys were analyzed, of which 37 were isolated from humans with Uta, while the last one was isolated from the sandfly *Lutzomyia ayacuchensis* (Table 1). At present, there is no *L.* (*V.*) *peruviana* reference strain officially identified by WHO (P. Desjeux, pers. commun.), although two stocks from the present sample (LC26 and LC106) were often used as *L.* (*V.*) *peruviana* reference stocks (Arana et al. 1990; Dujardin et al. 1993a; Dujardin et al. 1993b; Dujardin et al. 1994; Dujardin et al. 1995b; Guerrini 1993; Inga et al. 1998; Montoya et al. 1993; Victoir et al. 1995).

Twelve Peruvian stocks characterized as *L.* (*V.*) *braziliensis* in a previous work (Bañuls unpubl. data; Dujardin et al. 1995a) were included in the MLEE study in order to give a reliable picture of the genetic diversity of this species. They were from different departments of Peru (Huanuco, Madre de Dios, Junin, Cusco). Six reference stocks were added for species attribution. Table 1 summarizes the origin of all the stocks under study, including the twelve Peruvian *L.* (*V.*) *braziliensis* stocks previously analyzed, and the reference stocks. The fresh isolates were sampled from the four biogeographical units (BGUs) previously described by Lamas (1982) according to the endemic butterfly fauna (see Fig. 1) (Tobie, Von Brandt, and Mehlman 1950). These four BGUs are from North to South: Huancabam-

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Table **1**. Origin and code of the *Leishmania* stocks under study, including Peruvian *Leishmania braziliensis* stocks and the reference stocks used for species attribution.

Stock	Origin
Uta isolates from Peru	**Huancabamba BGU (HB)**
MHOM/PE/90/HB22	Huancabamba, Huancabamba
MHOM/PE/90/HB31	Huancabamba, Huancabamba
MHOM/PE/90/HB39	Huancabamba, Sondorillo
MHOM/PE/90/HB44	Huancabamba, Sondorillo
MHOM/PE/90/HB55	Huancabamba, Sondor
MHOM/PE/90/HB56	Huancabamba, Sondor
MHOM/PE/90/HB67	Huancabamba, Sondor
MHOM/PE/90/HB83	Huancabamba, Canchaque
MHOM/PE/90/HB86	Huancabamba, Faique
MHOM/PE/89/LC900	Huancabamba, Sondorillo
	Surco North BGU (SUN)
MHOM/PE/90/LC443[a]	Bolognesi, Huayllacayan
MHOM/PE/88/LC292[a]	Bolognesi, Huayllacayan
MHOM/PE/90/LC468[a]	Bolognesi, Huayllacayan
MHOM/PE/90/LC447[a]	Bolognesi, Raquia
MHOM/PE/84/LH115[a]	Recuay, Pararin
MHOM/PE/84/LC26[a]	Bolognesi, Huayllacayan
MHOM/PE/89/LH741[a]	Recuay, Pararin
MHOM/PE/88/LC272[a]	Bolognesi, Raymondi
MHOM/PE/90/LC436[a]	Bolognesi, Huayllacayan
MHOM/PE/90/LC446[a]	Bolognesi, Huayllacayan
MHOM/PE/90/LC423[a]	Bolognesi, Huayllacayan
	Surco Center BGU (SUC)
MHOM/PE/84/LH78[a]	Huaylas, Caraz
MHOM/PE/89/LH696[a]	Huaral, Sunvilca
MHOM/PE/89/LH760[a]	Huaral, Atavillosbajo
MHOM/PE/90/LH827[a]	Canta, Lampiane
MHOM/PE/91/LC1015[a]	Canta, Cachaqui
MHOM/PE/85/LC106[b]	Huarochiri, Santa Eulalia
MHOM/PE/76/SL5[b]	Huarochiri, Santa Eulalia
MHOM/PE/90/LH807[a]	Canta, San Buenaventura
MHOM/PE/90/LH937[a]	Canta, Huamantaga
MHOM/PE/90/LH925[a]	Canta, San Buenaventura
MHOM/PE/90/LH911[c]	Huaral, Sunvilca
	Surco South BGU (SUS)
MHOM/PE/90/LCA01	Lucanas, Sancos
MHOM/PE/90/LCA04	Lucanas, Sancos
MHOM/PE/90/LCA08	Lucanas, Sancos
MHOM/PE/90/LCA09	Lucanas, Sancos
MHOM/PE/90/LCA11	Lucanas, Sancos
MHOM/PE/90/LH249	Lucanas, Sancos
Sandfly isolate	**Surco South BGU (SUS)**
IAYA/PE/90/LA36	Parinacochas, Pullo
Peruvian *L. (V.) braziliensis* stocks (used only for the MLEE study)	
MHOM/PE/91/LC1409[d]	Huanuco
MHOM/PE/91/LC1412[d]	Huanuco
MHOM/PE/91/LC1417[d]	Huanuco
MHOM/PE/–/LH310	Madre de Dios
MHOM/PE/–/LH805	Madre de Dios
MHOM/PE/–/LH849	Junin
MHOM/PE/–/LC1569	Cusco
MHOM/PE/–/LC1578	Cusco
MHOM/PE/–/LC2034	Cusco
MHOM/PE/–/LC2044	Cusco
MHOM/PE/–/LC2178	Cusco
MHOM/PE/–/LC2321	Cusco
Reference stocks	
Leishmania (*Viannia*) *braziliensis*: MHOM/BR/75/M2903	Brazil
L. (*V.*) *brazilensis*: MHOM/BO/84/LPZ595	Bolivia
L. (*V.*) *guyanensis*: MHOM/BR/78/M5378	Brazil

Table **1**. Continued.

Stock	Origin
L. (*Leishmania*) *infantum*: MHOM/MA(BE)/67/ITMAP 263	Morocco
L. (*L.*) *amazonensis*: IFLA/BR/67/PH8	Brazil
L. (*L.*) *mexicana*: MNYC/BZ/62/M379	Belize

[a] Stocks used for population genetics analysis and studied by the three techniques, MLEE, RAPD, OFAGE.
[b] Stocks used for population genetics analysis and studied by the two techniques, MLEE, RAPD.
[c] Stocks used for population genetics analysis and studied only by the OFAGE technique.
[d] Dujardin et al. 1995a.

ba (HB) and Surco North, Center and South (SUN, SUC, and SUS, respectively). A non-xerophytic area, HB BGU, is contiguous to the Amazonian forest and close to the Porculla Pass, the lowest point in the Peruvian Andes (2,700 m above sea level), while the 3 Surco BGUs are xerophytic. Twelve of these stocks have been previously analyzed by molecular karyotyping (Dujardin et al. 1993b). Eight additional stocks were analyzed by OFAGE in the present study (Table 1). To ascertain the reliability of comparisons among the three methods (MLEE, RAPD, and karyotyping), analyses were carried out on the same cell harvest for every stock. All parasites were grown in blood-agar medium (Tobie, Von Brandt, and Mehlman 1950).

Multilocus enzyme electrophoresis. Technical conditions for sample preparation, cellulose acetate electrophoresis, and staining procedures were performed according to Ben Abderrazak et al. (1993). The fifteen enzyme systems used were: Aconitase (ACON: EC 4.2.1.3), Alanine aminotransferase

Fig. **1**. Map of Peru showing the localization of the different biogeographical units (BGUs) in the Andean Valleys where *Leishmania* stocks were isolated: HB (Huacabamba); Surco (SU), composed of three subunits SUN (Surco North), SUC (Surco Centre), and SUS (Surco South) (Lumbreras and Guerra 1985).

(ALAT: EC 2.6.1.2), 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), Isocitrate Dehydrogenase (IDH: EC 1.1.1.42), Malate 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 hydrolase, substrate deoxyinosine (NHD: EC 2.4.2.*), Nucleoside hydrolase, substrate inosine (NHI: EC 2.4.2.*), Peptidase 1, substrate L-leucyl-leucine-leucine (PEP1: EC 3.4.11. or 13.*), Peptidase 2, substrate L-leucyl-L-alanine (PEP2: EC 3.4.11. or 13.*), 6 Phosphogluconate Dehydrogenase (6PGD: EC 1.1.1.44), and Phosphoglucomutase (PGM: EC 2.7.5.1).

Random Amplified Polymorphic DNA. DNA was extracted according to the protocol of Sambrook et al. (1989). RAPD fingerprinting, first described by Williams et al. (1990) and Welsh and McClelland (1990), has been applied to the characterization of various parasitic protozoa. Following the results obtained by Tibayrenc et al. (1993) for *Leishmania,* five decamer primers were selected from the Operon technologies kit (Alameda, CA), as follows: primer A1: CAGGCCCTTC; A4: AATCGGGCTG; A7: GAAACGGGTG; A10: GTGA-TCGCAG; and A15: TTCCGAACCC. PCR conditions followed Williams et al. (1990). RAPD polymorphism was analyzed on 1.6% ethidium bromide-stained agarose gels.

OFAGE analysis. Procedures for DNA preparation and chromosomal separation by Orthogonal Field Alternating Gel Electrophoresis (OFAGE) have been described elsewhere (Dujardin et al. 1993a). In the present study, the two chromosomes considered were identified respectively by (i) a probe recognizing the small subunit of *L. (V.) braziliensis* rDNA genes (ssu) (Inga et al. 1998; Van Eys et al. 1992), and (ii) pLb-168, a random genomic probe isolated from *L. (V.) braziliensis* (Dujardin et al. 1993a).

Phenetic analysis. The Jaccard distance (*D* ij) was used for both MLEE and RAPD data, according to the following formula (Jaccard 1908):

$$D \text{ ij} = 1 - [a/ (a + b + c)]$$

a = number of bands that are common to the i and j stocks
b = number of bands present in the i stock and absent in the j stock
c = number of bands present in the j stock and absent in the i stock

Genetic relationships among the stocks were estimated by the Unweighted Pair-Group Method with Arithmetic Averages (UPGMA) (Sneath and Sokal 1973). Agreement between the phenetic relationships depicted by MLEE and RAPD was tested by evaluating the correlation between genetic distances inferred from the two methods for any possible pair of stocks with a non-parametric Mantel test (Mantel 1967; Tibayrenc 1995a). Briefly, this test relies on a Monte Carlo simulation with 10^4 iterations, which randomly permutes the different cells of each distance matrix. Contrary to the classical correlation test, this randomization procedure requires no asssumption about the number of degrees of freedom. In this case, the test was performed on the whole set of stocks, including the reference stocks. Here, its specific goal was to check for agreement between MLEE and RAPD overall phylogenies.

Population genetic analysis. To evaluate the possible impact of genetic exchange on the population under survey, population structure was explored by a set of complementary statistical tests. All of them take as a null hypothesis a situation of free genetic exchange (panmixia). Two categories of population genetic tests are usable. First, segregation tests explore

the random reassortment of alleles occurring at given loci. Second, recombination tests are based on the analysis of linkage disequilibrium, or non-random association of genotypes observed at different loci. Both segregation and recombination tests were used in the case of OFAGE data. Each OFAGE variant was equated to an ''allele,'' and each of the two chromosomes under study was equated to a ''locus.'' In the case of MLEE and RAPD data, the identification of alleles is tentative. Only linkage disequilibrium analysis (recombination tests) was used in this case. Both segregation and recombination tests have been described by Tibayrenc, Kjellberg, and Ayala (1990), while the possible biases brought by geographical distance and natural selection have been discussed by Tibayrenc et al. (1991). In order to decrease the biases due to geographical separation, only the stocks from the center of the area under survey (SUN and SUC BGUs, 2 foci separated by 150 km) attributed to the sole species *L. (V.) peruviana* (see below) were analyzed (i.e. 21 stocks for MLEE and RAPD data and 20 stocks for the karyotype data; see list in Table 1). Linkage disequilibrium analysis included a Mantel test performed on this limited sample of stocks (Mantel 1967). The goal of this test was different from the Mantel test used in phylogenetic analysis. Here, the Mantel test aimed to check for departures from panmictic expectations.

RESULTS

Identification. The fifteen enzyme systems under study made it possible to explore the variability at 16 different putative genetic loci, since the NHI system exhibited the activity of two distinct loci referred to as *Nhi* 1 and *Nhi* 2 (1 = the locus with fastest migration on gels) (Table 2).

By comparing the MLEE profiles of the Peruvian stocks with those of the *L. (V.) braziliensis* reference strains and of the Peruvian *L. (V.) braziliensis* stocks, it was possible to attribute the 38 Andean stocks to a discrete group distinct from *L. (V.) braziliensis* and *Leishmania (Viannia) guyanensis.* From now on, this group will be designated *L. (V.) peruviana.* Only one enzyme system (MPI) distinguished *L. (V.) peruviana* from *L. (V.) braziliensis* (Table 2, Fig. 2). However, with the technique used here, this enzyme system showed an identical profile for *L. (V.) peruviana* stocks and the *L. (V.) guyanensis* reference strain; thus, it cannot be considered as specific of *L. (V.) peruviana.* Nevertheless, seven enzyme systems out of the 15 studied distinguished *L. (V.) peruviana* from *L. (V.) guyanensis* (Table 2).

RAPD profiles confirmed the differences between *L. (V.) peruviana* and *L. (V.) braziliensis* stocks: one A10 fragment (320-bp) was common to all *L. (V.) peruviana* stocks (Fig. 3, arrow), and absent from the reference and Peruvian *L. (V.) braziliensis* stocks and from the other species (Dujardin et al. 1995a; Fig. 3). The absence of this fragment in *L. (V.) braziliensis* has since been verified on about fifty stocks (Bañuls, unpubl. data).

Genotypic and phenetic analysis. The 38 *L. (V.) peruviana* stocks were classified into eight different zymodemes, characterized by distinct isoenzyme multilocus genotypes. Genotype variability in the whole sample is therefore 8/38 = 0.210. Genotype diversity was slightly higher in the two central BGUs, SUN and SUC, than in the rest of the sample (0.286 and 0.235, respectively). Nevertheless, this result was not statistically significant.

RAPD fingerprinting proved to be more discriminative than MLEE, since 17 RAPDemes (composite RAPD profiles), vs eight zymodemes, were identified among the 38 *L. (V.) peruviana* stocks (genotype variability = 0.447 vs 0.210). Not only did RAPD show a higher genotype variability than MLEE, but it also yielded higher genetic distances, as confirmed by mea-

Table **2**. Multilocus enzyme electrophoretic (MCEE) patterns of the *Leishmania* stocks under study. The MLEE patterns were coded by a number according to their speed of migration. The fastest band was designated 1, the second fastest band was designated 2, and so on. The numbers were attributed to each electrophoretic pattern considering the whole *Leishmania* sample characterized by our laboratory.

Code	Acon	Alat	G6pd	Gpi	Got	Idh	Mdh	Me	Mpi	Nhi 1	Nhi 2	Nhd	6pgd	Pgm	Pep 1	Pep 2
HB22	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
HB31	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
HB39	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
HB44	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
HB55	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
HB56	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
HB67	5	8	1-3-5	4	4	11	16	7	8	2	6	6	6	6	4	5
HB83	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
HB86	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
LC900	5	8	5	4	4	11	16	7	8	2	6	6	6	6	4	5
LC443	5	8	5	4	4-6	11	16	7	8	2	6	8	6	6	4	5
LC0292	5	8	5	4	4-6	11	16	7	8	2	6	8	6	6	4	5
LC0468	5	8	5	4	4-6	11	16	7	8	2	6	8	6	6	4	5
LH249	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LH115	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LC26	5	8	5	4	4-6	11	16	7	8	2	6	8	6	6	4	5
LH78	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LC1015	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	8
La36	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LC106	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
SL5	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LC0447	5	8	5	4	4-6	11	16	7	8	2	6	8	6	6	4	5
LCA01	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LCA04	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LCA08	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LCA09	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LCA11	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LH696	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LH760	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LH827	5	8	5	4	4-7	11	16	7	8	2	6	6	6	6	4	5
LH925	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LH741	5	8	5	4	4	11	16	7	8	2	6	8	6	6	4	5
LH807	5	8	5	4	4-6	11	16	7	8	2	6	8	6	6	4	5
LC436	5	8	5	4	4-7	11	16	7	8	2	6	8	6	6	4	5
LH937	5	8	5	4	4-6	11	16	7	8	2	6	8	6	6	4	5
LH423b	5	8	5	4	4-6	11	16	5	8	2	6	8	6	6	4	5
LC446	5	8	5	4	4-7	11	16	7	8	2	6	8	6	6	4	5
LC272	5	8	5	4	4-6	11	16	5	8	2	6	8	6	6	4	5
LC1409	5	8	5	4	4	11	17-18	6	7	4	6	6	6	6	4	5
LC1412	5	8	5	4	4	11	17-18	5	7	4	6	6	6	6	4	5
LC1417	5	8	5	4	4	11	17-18	5	7	4	6	6	6	6	4	5
LH849	5	8	5	4	4-6	10	18	5	7	4	6	6	6	6	4	5
LH310	5	8	5	4	4-6	10	18	7	7	5	6	6	6	6	4	5
LC1569	5	8	5	4	4-6	11	18	5	7	4	6	6	6	6	4	5
LC1578	5	8	5	4	4-6	11	18	5	7	2	6	6	6	6	4	5
LC2034	5	8	5	4	4	11	18	5	7	2	6	6	6	6	4	5
LC2044	5	8	5	4	4-6	11	18	7	7	4	6	6	6	6	4	5
LC2178	5	8	5	4	4-6	11	17-18	5	7	2	6	6	6	6	4	5
LC2321	5	8	5	4	4	11	18	7	7	2	6	6	6	6	4	5
LH805	5	8	5	4	4-6	11	18	7	7	5	6	6	6	6	4	5
M2903	5	8	5	4	4	9	17	5	7	2	6	6	6	6	4	5
Lpz595	5	8	5	4	4	11	17	5	7	5	6	6	6	6	4	5
MON1	4	7	3-6	1	7	5	6-10-13	2	6	5	9	1	5	11	7	6
M379	4	1	7-8	11	6-9	4	5-8-14	1	4	6	7	3	2	4	6	6
PH8	4	4	7-8	8	6-9	4	5-8-14	1	4	6	0	1	2	11	6	6
M5378	5	7	4-5-6	4	4-6	9	16	7	8	5	1	6	5	10	4	4

surement of Jaccard's genetic distance within the *L. (V.) peruviana* group, with an average of 0.137 ± 0.065 and 0.248 ± 0.106 for the MLEE and RAPD distances, respectively. This difference was statistically highly significant ($p = 0$). Nevertheless, in some cases MLEE was more discriminative than RAPD. For example, the LC272 stock shared the same MLEE genotype with only one stock, although it shared the same RAPD genotype with five other stocks. Like the MLEE data,

RAPD showed a tendency for a higher genotype variability in the central area (SUN and SUC BGUs) vs other areas (0.524 and 0.353, respectively). Nevertheless, again, this difference was not statistically significant.

The UPGMA dendrograms computed from the matrices of genetic distances clearly distinguished between *L. (V.) braziliensis* and *L. (V.) peruviana* (Fig. 4, 5). In the MLEE study, 12 additional Peruvian *L. (V.) braziliensis* stocks were added to the

Fig. 2. Profiles obtained for the MPI enzyme system for *Leishmania* stocks. Lane 1: *Leishmania* (*Viannia*) *lainsoni* reference stock (MHOM/BR/81/M6426); Lanes 2, 4, 5, 6: *L.* (*V.*) *braziliensis* stocks; Lane 3: *L.* (*V.*) *peruviana* stocks and *L.* (*V.*) *guyanensis* stocks.

two *L.* (*V.*) *braziliensis* reference stocks. These 14 *L.* (*V.*) *braziliensis* stocks were classified into 13 zymodemes, which all fell into a discrete group, within the *L.* (*V.*) *peruviana* group (Fig. 4).

Moreover, both MLEE and RAPD dendrograms cluster the *L.* (*V.*) *peruviana* stocks according to the BGUs: the stocks isolated in the HB BGU are clearly separated from the stocks isolated from the other BGUs (Fig. 4, 5). This illustrates the fact that some markers are specific for the stocks isolated from a given BGU. These specific markers are noted on the dendrograms. For example, the NHD enzyme system had a specific electrophoretic level for the HB BGU (Fig. 6). Some RAPD

primers (A7, A15, A4) also showed a BGU specificity (Fig. 4, 5, 7). Other MLEE and RAPD markers are specific for whole cluster subdivisions, and are also indicated on the dendrograms. The Mantel test between the MLEE data and RAPD data performed on the set of stocks analyzed with the two methods, including reference stocks, was highly significant (p < 10^{-4}) (Mantel 1967).

Population genetic analysis of MLEE and RAPD data. *MLEE and RAPD data.* Population genetic analysis was applied on 21 *L.* (*V.*) *peruviana* stocks only from the central area (the SUN and SUC BGUs). This was done to avoid the bias due to geographical structuring. The results can be summarized as follows: RAPD analysis gave highly significant results in favor of a departure from panmixia. The probability of observing a given genotype as often as, or more often than, in the sample is significant, $p < 10^{-3}$ (*d1* test) (Tibayrenc, Kjellberg, and Ayala 1990). The probability of observing any genotype as often as, or more often than, the most common genotype in the sample is significant, $p < 10^{-2}$ (*d2* test). The probability of observing as few as, or fewer genotypes than, actually observed is significant, $p < 10^{-2}$ (*e* test); the probability of observing a linkage disequilibrium level as high as, or higher than, actually observed is significant, $p = 10^{-3}$ (*f* test). In contrast with RAPD, MLEE data showed no significant departure from panmictic expectation. Moreover, no significant correlation was observed by the Mantel test between MLEE and RAPD data on the 21 stocks specifically submitted to population genetic analysis (*g* test) (Mantel 1967; Tibayrenc 1995a).

Chromosome analysis and population structure. Previous work had shown a bimodal size distribution of the two chromosomes under survey (recognized respectively by probes for ssu and pLb-168) among *L.* (*V.*) *peruviana* geographical populations (Dujardin et al. 1993b). Parasites originating from the North and South of SUN and SUC were monomorphic and differentiated by the presence of a single size-variant of both chromosomes (North: 1370 kb ssu and 635 kb pLb-168 chro-

Fig. 3. Random amplified polymorphic DNA (RAPD) profiles of *Leishmania* stocks obtained with the A10 primer. Lane 1, 2, 3: *Leishmania* (*Leishmania*) *infantum* (ITMAP 263), *Leishmania* (*Leishmania*) *chagasi* and *Leishmania* (*Leishmania*) *donovani* reference stocks (L101, L13); Lane 4, 5: *Leishmania* (*Leishmania*) *tropica* and *Leishmania* (*Leishmania*) *major* reference stocks (K27, 5ASKH); Lane 6, 7, 8, 9: two *Leishmania* (*Viannia*) *braziliensis* reference stocks (M2903, LPZ595) and two stocks isolated from Peru and characterized by Guerrini (1993) (LH852, LH754); Lane 10, 11, 12: *Leishmania* (*Vianna*) *peruviana* isolates from this study (HB44, LH925, LCA04); Lane 13, 14, 15: *Leishmania* (*Viannia*) *guyanensis* (M5378), *Leishmania* (*Viannia*) *panamensis* (REST417) and *Leishmania* (*Viannia*) *lainsoni* (M6426) reference stocks; Lane 16: *Leishmania* (*Leishmania*) *amazonensis* (PH8) reference stocks; M: Molecular weight standards. The arrow indicates the 320-bp DNA fragment specific for *Leishmania* (*Viannia*) *peruviana*. Values on the right are in base pairs.

Fig. **4**. UPGMA dendrogram of *Leishmania* stocks built from Jaccard's genetic distance matrix calculated from MLEE data (Table 2). On the dendrogram, only one stock is represented for each zymodeme, and the numbers in parentheses indicate the number of stocks that share the same genotype. The genotypes that are specific for a given cluster are noted by arrows on the dendrogram.

mosomes; South: 1150 kb ssu and 690 kb pLb-168 chromosomes). In contrast, parasites from SUN and SUC were highly polymorphic. For comparison with isoenzyme and RAPD data in the populations from Surco-North and Surco-Centre, OFAGE data on eight new isolates were added to the ones already published and they confirmed previous results (Dujardin et al. 1993b). Accordingly, in a total of 20 isolates, eight of the nine theoretical combinations between variants (as defined by the bimodal distribution) of each chromosome were encountered (Fig. 8); stock LH911 (lane b7) presented a doublet of ssu chromosomes, although this pair of chromosomes was very close in size and each belonged to the "smaller" mode of size distribution of this chromosome. Thus, we considered this stock as homozygous for the small ssu chromosome variant. The weaker hybridization intensity of the smaller ssu band in the case of double patterns (Fig. 8B, lanes b3 and b8) was due to a lower copy number of rDNA genes (Inga et al. 1998). Both segregation and recombination tests, performed on the OFAGE "alleles" and "loci" as defined in Materials and Methods, showed no statistical departure from panmictic expectations (see Table 3).

DISCUSSION

Species identification, phylogenetic aspects and taxonomic status of *Leishmania* (*Viannia*) *peruviana*. For a long time, the identity of *Leishmania* (*Viannia*) *peruviana* was based only on geographical (i.e. distribution limited to the Andean valleys) and clinical (i.e. significant difference in severity and absence of mucosal metastasis) characters. No genetic marker was available to distinguish *L.* (*V.*) *peruviana* from *L.* (*V.*) *braziliensis*. However, with the increasing human migrations and the ensuing risk of sympatry of the two species, it has become essential to

distinguish them by reliable genetic markers. Arana et al. (1990) first identified two discriminative enzyme systems (MPI and MDH). This result was partially confirmed by Guerrini (1993) for the MPI system only (see below). OFAGE differences were later encountered between the two species (Dujardin et al. 1993a).

The existence of discriminative MPI profiles between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* was fully confirmed. Nevertheless, in our study, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *peruviana* showed identical MPI profiles. It is therefore necessary to consider additional isoenzyme characters for identifying specifically *L.* (*V.*) *peruviana*, since *L.* (*V.*) *guyanensis* has now been encountered repeatedly in Peru, notably in the Andes (Bañuls, in prep.). The NHI system can be added, since in our study, it showed profiles that were identical between *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis* but different from the *L.* (*V.*) *guyanensis* profile. In contrast to Arana et al. (1990), our results do not consider the MDH system as a reliable marker to distinguish between *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis*. Indeed the study of a broader sample of *L.* (*V.*) *braziliensis* stocks revealed that the MDH system is polymorphic in this species: two MDH profiles can be identified in *L.* (*V.*) *braziliensis*, one of which is identical to the *L.* (*V.*) *peruviana* profile while the other one has a slower migration (Bañuls, unpubl. data).

Out of the five RAPD primers tested, only one 320-bp fragment of the A10 primer distinguished *L.* (*V.*) *peruviana* from *L.* (*V.*) *braziliensis*. Moreover, this RAPD fragment is not observed in any other species including *L.* (*V.*) *guyanensis*. This marker permits a rapid identification of *L.* (*V.*) *peruviana* although only 7 enzyme systems out of 15 for MLEE distinguished between *L.* (*V.*) *peruviana* and *L.* (*V.*) *guyanensis*: all

Fig. 5. UPGMA dendrogram built from Jaccard's genetic distance matrix calculated on the basis of RAPD results (see Fig. 3). On the dendrogram, only one *Leishmania* stock for each RAPD genotype is represented, and the numbers in parentheses indicate the number of stocks that shared the same genotype. The RAPD primers that generate synapomorphic characters are indicated at the origin of the corresponding clusters.

RAPD primers used here permit a clear-cut distinction between these two species (see Fig. 3, A 10 primer).

The species-specific markers identified in the present study (Table 2) can be assumed to be synapomorphic characters if they are shared by all members of a given clade and only by them. This is the case for the 320-bp RAPD fragment generated by the A10 primer. In contrast, the NHI and MPI enzyme systems generated profiles that identify the *L.* (*V.*) *peruviana* spe-

cies when they are considered jointly, but these profiles cannot be regarded as synapomorphic because they are shared by other species.

Clear-cut clustering, statistical agreement between MLEE and RAPD data verified by the Mantel test (Mantel 1967), and the presence of specific characters support the view that *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis* correspond to two closely related, but distinct, monophyletic lines (clades). On the basis

Fig. **6**.   Profiles of *Leishmania* stocks obtained for the NHD enzyme system. Lanes 1, 2, 3: *Leishmania* (*Viannia*) *peruviana* stocks from the North of the Andean Valleys (HB BGU); Lanes 4, 5: *L.* (*V.*) *peruviana* stocks from the South of the Andean Valleys (SU BGU).

of a cladistic analysis, Chouicha et al. (1997) claimed that *L. peruviana* should be included in *L. braziliensis* because of the ancestral position against *L. braziliensis* and *L. peruviana* of two zymodemes, MON*204 and MON*205. However, all this reasoning is based on the inference that these two zymodemes can be assigned *L. braziliensis*. However, Chouicha et al. (1997) concluded this assignment was questionable. Apart from the questionable case of these zymodemes, our results and the results of Chouicha et al. (1997) are totally convergent in that they show the phylogenetic discreteness of *L. braziliensis* on

one hand, and *L. peruviana* on the other hand. Together with karyotype data (Dujardin et al. 1993a; Dujardin et al. 1993b; Dujardin et al. 1995b) and specific epidemiological and medical criteria, this is an additional argument in favor of ranking *L.* (*V.*) *peruviana* as a distinct species.

More generally, the overall phylogenies obtained from the whole set of stocks, including the reference stocks, seem to be robust, since MLEE and RAPD gave convergent results, as shown by both the visual observation of the two dendrograms, and the highly significant result of the Mantel test performed on the whole set of stocks that have been analyzed by both MLEE and RAPD (Mantel 1967; Tibayrenc 1995a). From these analyses and from the described specific genetic markers *L.* (*V.*) *peruviana* appears monophyletic. This genetic distinction linked to the epidemiological and clinical criteria indicates *L.* (*V.*) *peruviana* is a species not just a genetic variant of *L.* (*V.*) *braziliensis*. Obviously, the species concept retained here is not the biological concept of species (Mayr 1942). When the phylogenetic criterion is used, the relevant parameter is not the overall phylogenetic diversity of the putative species, but rather, its clear discreteness by comparison with other evolutionary units.

The stocks under study can be a source of *L.* (*V.*) *peruviana* reference strains in the future. The stocks that could be used as representative of this species are MHOM/PE/90/HB86, MHOM/PE/84/LC26, MHOM/PE/85/LC106, and MHOM/PE/90/LCA08. These stocks are available at the Laboratory of Protozoology, Prince Leopold Institute of Tropical Medicine of Antwerpen Belgium.

Moreover, MLEE and RAPD data made it possible to identify two geographical populations within *L.* (*V.*) *peruviana*. The *Nhd* locus showed an informative geographical distribution pattern with two different profiles: one specific for the *L.* (*V.*) *peruviana* stocks from the North (HB BGU) and one specific for the stocks from the South (SUN, SUC, SUS) (Fig. 6). The *Nhd* profile of

Fig. **7**.   RAPD profiles for *Leishmania* stocks with the A15 primer. Lanes: 1 to 9 and 13: *Leishmania* (*Viannia*) *peruviana* isolates from the HB BGU (HB22, HB31, HB39, HB44, HB55, HB56, HB67, HB83, HB86, LC900) (see Fig. 1). Lanes: 10, 11, 12: *Leishmania* (*Viannia*) *guyanensis* stocks from Peru (unpubl. data); Lanes 14 to 17: *Leishmania* (*Viannia*) *peruviana* isolates from the SUN BGU (LC443, LC292, LC468, LC447); M: Molecular weight standards.

Fig. **8**. Size polymorphism (expressed in kb) of chromosomes for *Leishmania* stocks recognized by probes pLb-168 (A) and pLb-22 (B): Lane b: *L. (V.) braziliensis* M2903 size reference; Lanes 1—3, 5, 7, 8: Surco-Centre (1, LH827; 2, LH937; 3, LC1015; 5, LH741; 7, LH911; Lanes 4, 6: *L. (V.) peruviana* isolates from Surco-North (4, LC292; 6, LC272) and 8, LH760). Note the eight combinations between the two variants (as defined by their bimodal distribution) of each chromosome.

the Peruvian isolates from the North was shared also by all the *L. (V.) braziliensis* stocks (Guerrini 1993). This result confirms the observation by Dujardin et al. (1993b) that the *L. (V.) peruviana* stocks from the HB BGU were closer to *L. (V.) braziliensis* than the *L. (V.) peruviana* stocks from the other BGUs. A similar tendency for the structuring into two sub-populations could be seen in the RAPD genotypes of the *L. (V.) peruviana* stocks. The A4, A7, A15 primers revealed specific fragments that were shared either by all the Northern isolates or by all the Southern isolates (Fig. 7). These data document the geographical structuring of *L. (V.) peruviana* and confirm the results obtained with karyotype data (Dujardin et al. 1993b). This genetic differentiation of the two sub-populations (North, South) within *L. (V.) peruviana* presents an apparent association with a difference in pathogenicity: cutaneous lesions from HB BGU are more severe (larger in average diameter) than those encountered in the South (Davies et al. 1997).

The question of genetic recombination: population genetic analysis. We showed that *L. peruviana* appears to be monophyletic. Now, it is informative to know whether this phylogenetic line corresponds to a sexual or to a clonal species. Although the present proposal that *L. (V.) peruviana* is a valid species is based on epidemiological and phylogenetic criteria, it is important to explore further the biological status of this taxon, and to see whether it undergoes regular mating. To an-

Table **3**. Results of the test on random association of chromosomal size variants of *Leishmania peruviana* stocks as evidenced by Orthogonal Field Alternating Gel Electrophoresis. Numbers in brackets indicate the expected size of the corresponding composite genotype: for instance the genotype composed of the size variants 690 kb and 1369 kb was encountered 10 times and its expected frequency number was 9.2 under the null hypothesis of free reassociation.

	1369 kb	1146 kb	Total
690 kb	10 (9.2)	9 (9.8)	19
634 kb	5 (5.8)	7 (6.2)	12
Total	15	16	31

swer this question, we performed a population genetic analysis from the OFAGE, MLEE, and RAPD data. The three types of genetic markers used in the present study gave apparently conflicting results when applied on a population genetic analysis in SUN, and SUC BGUs. MLEE and OFAGE data showed no apparent departures from panmictic expectations while RAPD results showed a considerable linkage disequilibrium. In the case of MLEE data, a mere statistical type II error (lack of resolution) could be considered. Indeed, the variability of this sample is extremely limited, since only one enzyme system out of 15 showed a notable polymorphism (GOT). This fact reduces proportionally the possibility of obtaining any statistically significant result (Tibayrenc 1995b). In this respect, the highly significant results obtained from RAPD data are especially relevant, since RAPD variability in the present sample remains limited, although higher than for the MLEE results. The hypothesis of a statistical type II error can be retained in the case of OFAGE data. Indeed, putative parental OFAGE genotypes (characterizing populations situated respectively in the North and South of SUN and SUC) and all possible ''offspring'' combinations are present in our sample (Dujardin et al. 1993b). Blaineau et al. (1992) described a similar apparent genetic equilibrium in chromosomal variability in a population of *L. (L.) infantum* from the Pyrenean Valleys in France and they explained it either by random genetic exchange or by a high rate of recurrent chromosomal mutations. In our study, the hypothesis of free genetic exchange is not supported by the RAPD data. Accumulation of chromosomal rearrangements might explain the apparent discrepancy between OFAGE and RAPD results. Indeed, this phenomenon has been extensively described. It is mainly due to amplification/deletion of repeated sequences (Iovannisci and Beverley 1989; Victoir et al. 1995) and a very fast molecular clock (Dujardin, in prep.). However, even if such mechanisms might theoretically generate all the observed combinations, it is still not clear whether they could lead to frequencies that meet panmictic expectations. The discrepancy between OFAGE and RAPD data as reported here is not a constant feature. Indeed, in another Peruvian Andean Valley (Huan-

uco), karyotype analysis and gene markers (MLEE and RAPD) showed convergent results suggesting genetic recombination in Peruvian *Leishmania* (Dujardin et al. 1995a).

A last hypothesis to account for apparent "panmixia" in OFAGE data might be the occurrence of "pseudo-recombination" in *Leishmania* populations. This could happen without sexual exchange, in three steps. First, in a parasite homozygote for RAPD and isoenzymes, rearrangement might occur in one chromosome of a pair of homologues as described elsewhere (Dujardin et al. 1994; Iovannisci and Beverley 1989; Wincker et al. 1996). Secondly, this parasite might become tetraploid (totally or for this chromosome) under stress conditions, as shown by in vitro experiments (Cruz, Titus, and Beverley 1993). Return to diploidy might generate apparent patterns of segregation (or linkage equilibrium if considering 2 chromosomes) of chromosomal variants without affecting the genetic loci characterized by RAPD.

In the present study, RAPD data were sufficient by themselves to reject the hypothesis that the *L. (V.) peruviana* populations from the center of the survey area undergo frequent genetic exchange: they do show a considerable departure from panmictic expectations. This is consistent with the previously proposed hypothesis that *Leishmania* parasites present a basically clonal population structure, with limited or no genetic exchange (Tibayrenc, Kjellberg, and Ayala 1990). To our knowledge, it is the first time that a clonal population structure is documented for *L. (V.) peruviana*. Nevertheless, this result must be verified with additional samplings taken in stricter sympatric conditions.

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UPCOMING MEETING

53rd Annual Meeting of
The Society of Protozoologists

Joint Meeting with the American Society
of Parasitologists
June 24–28, 2000
San Juan, Puerto Rico

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