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Molecular karyotype variation in *Leishmania (Viannia) peruviana*: indication of geographical populations in Peru distributed along a north–south cline

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Forty-one *Leishmania peruviana* isolates were selected along a north–south transect which crossed areas endemic for uta in three different biogeographical regions in the Peruvian Andes. The isolates were analysed by molecular karyotyping and hybridization with three chromosome-derived DNA probes. All the isolates could be distinguished from *L. braziliensis* by their pLb-134 hybridization patterns. However, the patterns with the other probes (pLb-168 and -22) could be used to cluster the Peruvian isolates in discrete groups (karyodemes) which varied in their level of similarity with *L. braziliensis*. The geographical distribution of these karyodemes supports the hypothesis that eco-geographical isolation has contributed to the heterogeneity of *L. peruviana*.

Leishmania (Viannia) peruviana, the causative agent of Andean cutaneous leishmaniasis, known also as uta, is found on the western slopes of the Andes and in the inter-Andean valleys, 1300–2800 metres above sea level (m.a.s.l.), at latitudes of 5–13° south (Herrer, 1962; Lumbreras and Guerra, 1985; Llanos-Cuentas, 1991). Although the small cutaneous lesion that characterizes this disease resembles the onset of the sylvatic leishmaniasis found in the Amazonian basin (Romero *et al.*, 1987), metastatic mucosal lesions do not develop from it as they do from the Amazonian lesions (Walton, 1987; Campos, 1990). The parasites causing the Andean and Amazonian diseases were therefore presumed to differ and were given specific status, as *L. peruviana* and *L. braziliensis*, respectively (Lumbreras and Guerra, 1985; Lainson and Shaw, 1987). Biochemical discrimination of these parasites, by comparison of their mannose phosphate isomerase and malate dehydrogenase isoenzymes (Arana *et al.*, 1990), only became possible a few years ago. Very recently, molecular karyotyping and hybridization with chromosome-derived DNA has also permitted consistent discrimination between *L. peruviana* and *L. braziliensis* (Dujardin *et al.*, 1993).

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Molecular karyotyping and other high resolution techniques indicate that *Leishmania* heterogeneity goes far beyond the species level; isoenzyme analysis has been used to cluster large numbers of isolates collected from widespread endemic areas, of several *Leishmania* spp., into zymodeme groups (Saravia *et al.*, 1985; Maazoun *et al.*, 1986; Moreno *et al.*, 1986; Pratlong *et al.*, 1986; Guerrini, 1993) and other techniques, such as use of monoclonal antibodies and restriction fragment length polymorphism (Pacheco *et al.*, 1986; Grimaldi *et al.*, 1987), have indicated similar heterogeneity.

Environmental and geographical isolation are among the factors directly or indirectly involved in genetic variability (Lainson and Shaw, 1987). The present study is an extensive genetic characterization of *L. peruviana* isolates to see if eco-geographical variation contributes to karyotype variation. The areas where uta is endemic vary ecologically and are often separated by rivers, deserts and other natural barriers. The endemic areas studied here were allocated to one of three different biogeographical units (BGU), based on those used by Lamas (1982) in his studies of butterflies of the region. The factors responsible for the variability of the local butterfly populations may also be responsible for that of the leishmanial populations and their vectors.

Molecular karyotyping by OFAGE (Carle and Olson, 1984), used here to compare different geographical populations of *L. peruviana*, can reveal small genetic differences (Bishop and Miles, 1987; Saravia *et al.*, 1990; Lighthall and Giannini, 1992), and has already proved efficient at demonstrating variations within parasite species (Bishop and Miles, 1987; Pagès *et al.*, 1989; Saravia *et al.*, 1990).

MATERIALS AND METHODS

Parasites

Thirty-nine *Leishmania* isolates from Andean patients, all having cutaneous lesions compatible with uta, and two isolates from *Lutzomyia ayacuchensis* caught in Surco South (see below) were studied by molecular karyotyping (Table 1). All 41 isolates were identified as *L. peruviana* by isoenzyme analysis using the methods of Arana *et al.* (1990). Promastigotes of each isolate, which had been subcultured less than 20 times following isolation, were cultured in blood agar medium (Tobie *et al.*, 1950) and harvested at late log phase. Two strains of *L. (V.) braziliensis* (one from Peru), and one strain each of *L. (L.) mexicana*, *L. (L.) amazonensis* (from Peru) and *L. (L.) major* were used as reference material.

Study Areas

Peruvian isolates originated from six foci belonging to three biogeographical units previously defined by the presence of endemic monotypic species or subspecies of butterflies (Lamas, 1982) (Table 1; Fig. 1). These three BGU, which are limited by large rivers or deserts, are named, from north to south: Huancabamba (HB; 4°–5°48′S), Porculla (PO; 4°–8°48′S) and Surco (SU; 8°54′–15°18′S). The Surco BGU was further divided (G. Lamas, unpubl. obs.) into three subunits, based on the prediction that slight variation in any of the subunits would lead to isolation of a parasite–host cycle. The three subunits were Surco North (SUN), which includes Huayllacayan, the most studied leishmaniasis focus in Peru (Herrer, 1962; Romero *et al.*, 1987; Perez *et al.*, 1991), Surco Center (SUC), which includes two foci, and Surco South (SUS); the subunits are delimited by the Rio Fortaleza and the Ica desert (Fig. 1). Ecologically (Lamas, 1982), HB is a forested area (upper montane forest 1500–3500 m.a.s.l.) contiguous with the Amazonian forest, and the lowest pass in the Peruvian Andes. The other two BGU, PO and SU, are non-forested, xerophytic areas (open lower montane 1000–2700 m.a.s.l.). All the isolates except LH78 (coded 25) were obtained within 25 km of a leishmaniasis focus.

Reagents

All reagents used were purchased from Merck (Darmstadt, Germany) unless specified otherwise.

TABLE 1

Biogeographical origin and designation of the Leishmania *strains*

Designation	*Origin*	*Code number*
REFERENCE STRAINS		
Leishmania mexicana		
MNYC/BZ/62/M379	Belize	1
L. amazonensis		
MHOM/PE/89/LH687	La Convencion, Cuzco, Peru	2
L. major		
MHOM/SU/73/5ASKH	Turkmenskaya, Askhabad	3
L. braziliensis		
MHOM/BR/75/M2903	Carajas, Para, Brazil	4
MHOM/PE/90/LH852	Echarate, La Convencion, Peru	5
UTA ISOLATES FROM PERU		
Huancabamba BGU		
MHOM/PE/90/HB22	Huancabamba, Huancabamba	6
MHOM/PE/90/HB31	Huancabamba, Huancabamba	7
MHOM/PE/90/HB39	Sondorillo, Huancabamba	8
MHOM/PE/90/HB44	Sondorillo, Huancabamba	9
MHOM/PE/90/HB55	Sondor, Huancabamba	10
MHOM/PE/90/HB56	Sondor, Huancabamba	11
MHOM/PE/90/HB67	Sondor, Huancabamba	12
MHOM/PE/90/HB83	Canchaque, Huancabamba	13
MHOM/PE/90/HB86	Faique, Huancabamba	14
MHOM/PE/89/LC900	Sondorillo, Huancabamba	15
Porculla BGU		
MHOM/PE/91/LC1446	Salas, Lambayeque	16
MHOM/PE/91/LC1447	Salas, Lambayeque	17
MHOM/PE/91/LC1448	Salas, Lambayeque	18
Surco North BGU		
MHOM/PE/90/LC443	Huayllacayan, Bolognesi	19
MHOM/PE/88/LC292	Huayllacayan, Bolognesi	20
MHOM/PE/90/LC468	Huayllacayan, Bolognesi	21
MHOM/PE/90/LC447	Raquia, Bolognesi	22
MHOM/PE/84/LH115	Pararin, Recuay	23
MHOM/PE/84/LC26	Huayllacayan, Bolognesi	24
Surco Center BGU		
MHOM/PE/84/LH78	Caraz, Huaylas	25
MHOM/PE/89/LH691	Canta, Canta	26
MHOM/PE/89/LH696	Sunvilca, Huaral	27
MHOM/PE/89/LH760	Atavillosbajo, Canta	28
MHOM/PE/90/LH827	Lampiane, Canta	29
MHOM/PE/90/LH925	Sanbuenaventura, Canta	30
MHOM/PE/91/LC1015	Cachaqui, Canta	31
MHOM/PE/76/SL2*	Santa Eulalia, Huarochiri	32
MHOM/PE/00/LC106	Santa Eulalia, Huarochiri	33
MHOM/PE/76/D8*	Santa Eulalia, Huarochiri	34
MHOM/PE/76/SL5*	Santa Eulalia, Huarochiri	35
MHOM/PE/76/SL3*	Santa Eulalia, Huarochiri	36

TABLE 1
Continued

Designation	*Origin*	*Code number*
UTA ISOLATES FROM PERU *continued*		
Surco South BGU		
MHOM/PE/90/LCA01	Sancos, Lucanas	37
MHOM/PE/90/LCA04	Sancos, Lucanas	38
MHOM/PE/90/LCA05	Sancos, Lucanas	39
MHOM/PE/90/LCA06	Sancos, Lucanas	40
MHOM/PE/90/LCA08	Sancos, Lucanas	41
MHOM/PE/90/LCA09	Sancos, Lucanas	42
MHOM/PE/90/LCA11	Sancos, Lucanas	43
MHOM/PE/90/LH249	Sancos, Lucanas	44
SANDFLY ISOLATES FROM SURCO SOUTH BGU, PERU		
IAYA/PE/90/La36	Pullo, Parinacochas	45
IAYA/PE/90/La78	Pullo, Parinacochas	46

*Donated by Dr. M. Chance.
BGU, Biogeographical unit (Lamas, 1982).

Molecular Karyotyping

Agarose plugs containing leishmanial chromosomes were prepared for OFAGE as described by Van Der Ploeg *et al.* (1984) and stored at 4°C. The electrophoresis equipment and conditions were as described by Dujardin *et al.* (1987). Resolution of the whole karyotype was achieved by three distinct OFAGE runs using 45-, 65- or 115-s pulses.

DNA Hybridization

Chromosomal bands resolved by OFAGE were transferred to nylon filters (Hybond N, Amersham), and then depurinated by acid treatment, denatured and neutralized according to the manufacturer's instructions. Probes were derived from a genomic library of *L. braziliensis* MHOM/BR/75/M2904, constructed by cloning DNA in the PstI site of pUC18. Three recombinants were selected after their strong hybridization with probes made up from chromosomal bands of *L. braziliensis* M2904 and *L. peruviana* SL2 (i.e. bands giving polymorphic hybridization patterns in the karyotypes of the different species in the *L. braziliensis* complex). The recombinants were pLb-134 (2·4 kb), pLb-168 (4·8 kb) and pLb-22 (2·8 kb). In *L. braziliensis* M2903, the probes recognized two chromosomes of 760 and 700 kb (pLb-134), one chromosome of 640 kb (pLb-168) and one chromosome of 1300 kb (pLb-22). Each probe was labelled independently with ^{32}P dCTP by random prime labelling. Hybridization was at 42°C, in a solution containing formamide, according to the manufacturer's (Amersham's) instructions. Last washings after hybridization were performed at high stringency (0·1 × SSC, 65°C). When different probes were to be hybridized successively onto the same filter, the first one was washed out of the filter by an alkali treatment, again according to Amersham's instructions. Elimination of the probe was checked by autoradiography, before subsequent hybridization was performed.

Chromosomal Band Size

The karyotype of the reference strain *L. braziliensis* M2903 (sized by comparison with the karyotype of *Saccharomyces cerevisiae* YPH80; Biolabs) was used to estimate the size of the chromosomal bands produced from the isolates. DNA from the reference strain was loaded in three tracks of the gel (left, centre,

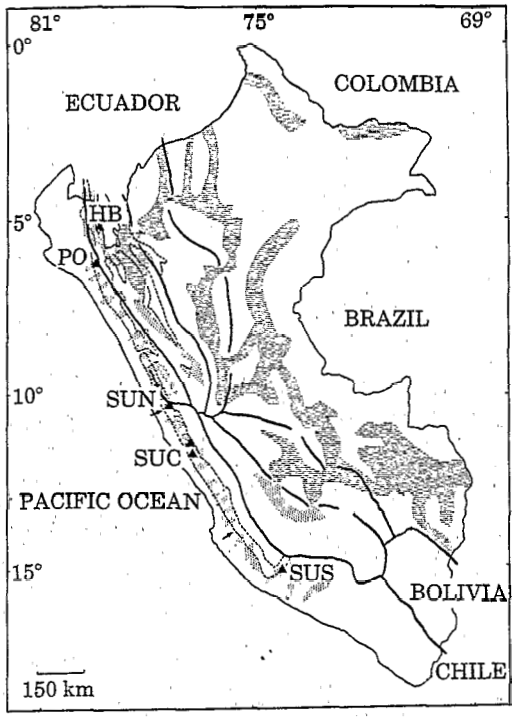

Fig. 1. Map of Peru, showing the origins of the *Leishmania peruviana* isolates (▲) and the main mountain ranges (~). The areas endemic for uta (▩) and sylvatic cutaneous leishmaniasis (□) (Guerra, 1988) are shaded. The limits of the three main biogeographical units studied [Huancabamba (HB); Porculla (PO); and Surco–North (SUN), South (SUS) and Center (SUC)] are also indicated within Peru. The arrows indicate the north–south limits of the three Surco subunits.

right) for each OFAGE run. 'Size-conserved' chromosomes were those with limited size variation (coefficient of variation <5%) between isolates (Giannini *et al.*, 1990; Lighthall and Giannini, 1992). The size distribution of hybridizing chromosomes was compared with a normal distribution by χ^2 tests. When the distribution appeared bi-modal, a cut-off value was chosen dividing the sample into two such that the standard-deviation within each subsample was minimal, and the two means were then compared by a *z*-test.

Principal Component Analysis

Factorial analysis can make a powerful contribution to the numerical processing of genetic

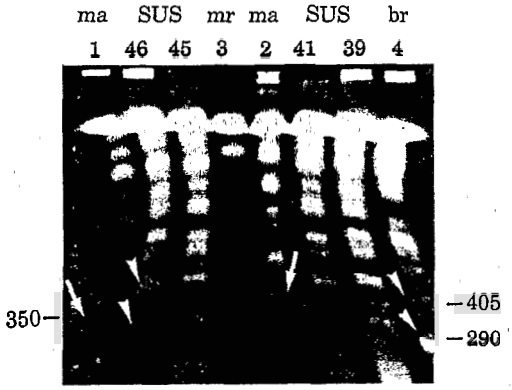

Fig. 2. Identification of the *braziliensis* complex by molecular karyotyping using 45-s pulses. The sizes of the marker bands of the *braziliensis* complex (arrowheads) are given in kb and the 350 kb marker bands of the *mexicana* (ma) and *major* (mr) complexes are also indicated (arrows). SUS, Isolates from Surco South; br, *Leishmania braziliensis* reference strain.

characters in *Leishmania* (Serres and Roux, 1986). In the present study, a normalized-by-columns principal component analysis (PCA) of the sizes of the chromosomes hybridizing with the three probes was performed, using ADE software, version 3.1 (Chessel and Dolédec, 1992).

RESULTS

Karyotype Markers for the *Leishmania braziliensis* Complex are Present in the Andean Isolates

After OFAGE and ethidium bromide staining, 290- and 405-kb size-conserved chromosomal bands were observed in all 41 Andean isolates and the *L. (V.) braziliensis* M2903 reference strain (Fig. 2, arrowhead). These two bands are specific to the *braziliensis* complex (Dujardin *et al.*, 1987, 1993). None of the isolates presented the 350-kb markers typical of the *L. mexicana* (Dujardin *et al.*, 1987, 1993) or *L. major* complexes (Giannini *et al.*, 1990) (Fig. 2, arrow), although parasites in each of these complexes have been isolated from uta-like lesions in Ecuador (Hashiguchi *et al.*, 1991).

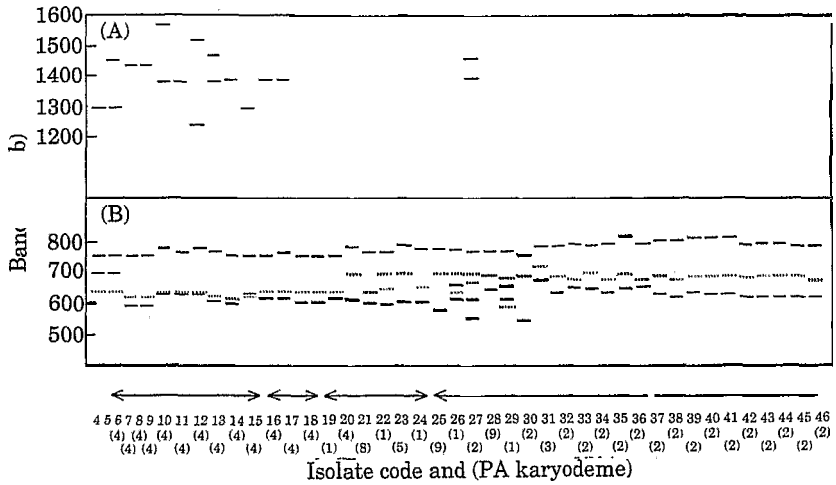

Fig. 3. Diagram of karyotypical patterns following hybridization with probes pLb-22 (A), pLb-134 (B; ——) and pLb-168 (B; ... ...). The isolates are ordered geographically north to south, in terms of their point of origin. Codes 4 and 5 refer to *Leishmania braziliensis* reference strains (see Table 1).

Karyotype Differences between *Leishmania peruviana* and *L. braziliensis*

At least two hybridizing chromosomes were observed in all the Andean isolates when the pLb-134 probe was used [Figs 3(b) and 4]. Their size was variable: 784 ± 37 kb (95% of the size distribution) for the larger one and 622 ± 59 kb for the smaller. Although two hybridizing chromosomes were also observed using this probe and *L. braziliensis* strains LH852 (from the Amazonian jungle of Peru; coded 5) or M2903 (coded 4), these were of 760 and 700 kb and therefore of different sizes [Figs 3(b) and 4]. Comparison with other isolates of *L. braziliensis* showed that the lower pLb-134 hybridizing band produced from *L. peruviana* isolates was always significantly smaller ($P<0{\cdot}05$) than the equivalent one in *L. braziliensis*, which had a mean ± S.D. size of 700 ± 20 kb (Dujardin *et al.*, 1993).

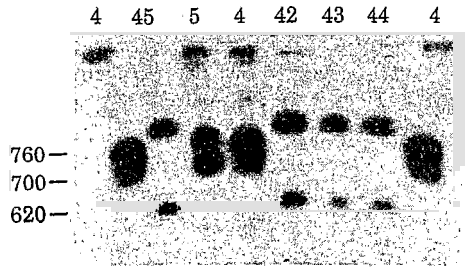

Fig. 4. Karyotypical discrimination of *Leishmania peruviana* isolated in Surco South (coded 42, 43, 44 and 45) from *L. braziliensis* (coded 4 and 5), using probe pLb-134 and 65-s pulses. Band sizes are given in kb.

Karyotype Polymorphism in *Leishmania peruviana*

A single band was usually recognized in the Andean isolates by pLb-22 [Figs 3(a) and 5], and this band had a bimodal size distribution ($P<0{\cdot}05$) [Fig. 7(a)]; most isolates presented one band at 1369 ± 156 [upper size category; Fig. 5(a)] or 1146 ± 78 kb [lower size category; Fig. 5(c)]. Of the seven isolates giving two bands with this probe, three showed bands in both size categories [Fig. 3(a); Fig. 5(b), isolate 28], and four gave two bands within one of these size categories. The upper size category was characteristic of *L. braziliensis* [Fig. 5(c), strains 4 and 5; Dujardin *et al.*, 1993].

Results with the pLb-168 probe were similar; in tests of most isolates the probe hybridized with a single band [Figs 3(b) and 6] which had a bimodal size distribution [$P<0{\cdot}05$; Fig. 7(b)]. This single band was either of 634 ± 29

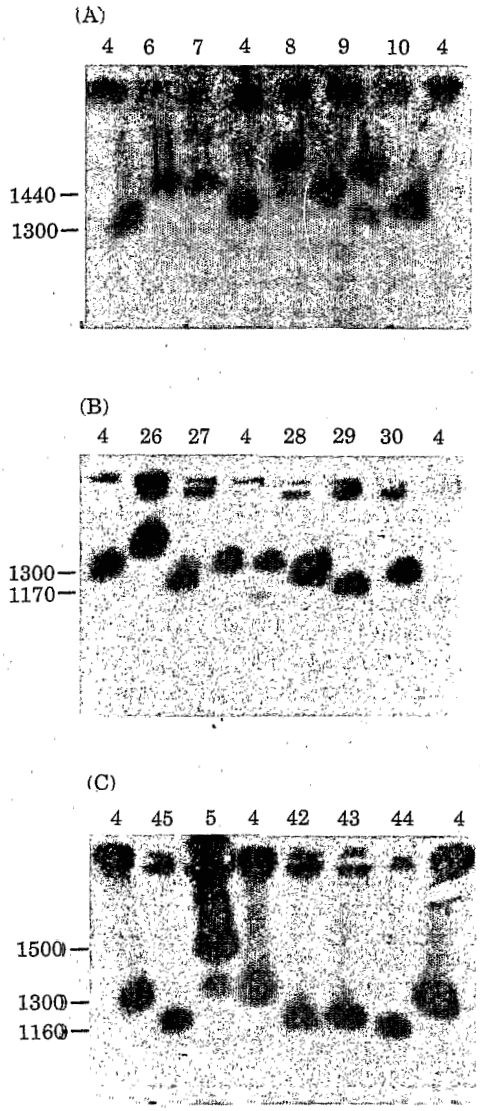

Fig. 5. Karyotype polymorphism within *Leishmania* isolates detected using probe pLb-22 and 115-s pulses. Two strains (coded 4 and 5) are *L. braziliensis* reference strains. All other strains were *L. peruviana* isolates from (A) Huancabamba, (B) Surco Center or (C) Surco South. Band sizes are given in kb.

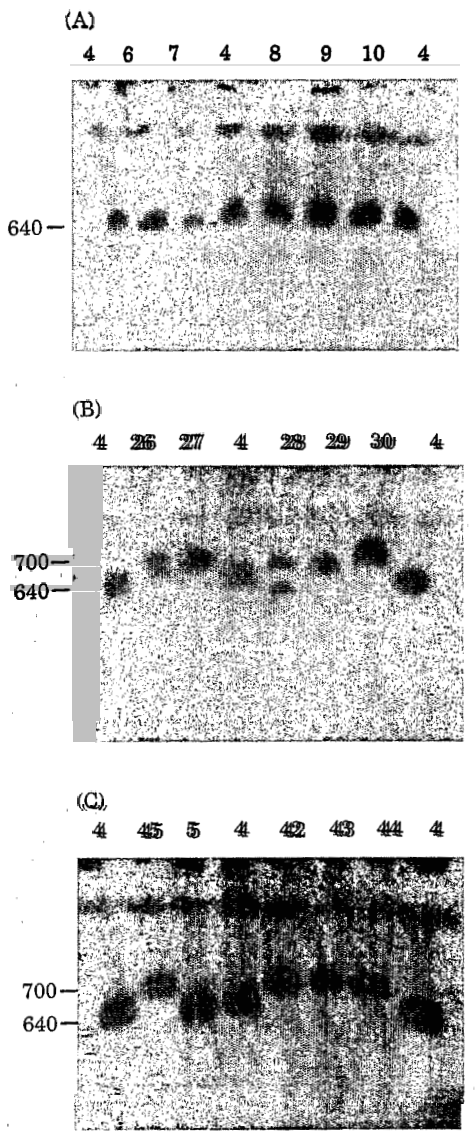

Fig. 6. Karyotype polymorphism for the strains shown in Fig. 5, using probe pLb-168 and 65-s pulses. Two strains (coded 4 and 5) are *Leishmania braziliensis* reference strains. All other strains were *L. peruviana* isolates from (A) Huancabamba, (B) Surco Center or (C) Surco South. Band sizes are given in kb.

[lower size category; Fig. 6(a)] or 690 ± 23 kb [upper size category; Fig. 6(c)]. Of the four isolates with double hybridizing bands, two presented bands in both size categories [e.g. Fig. 6(b), isolate 28], and the two others gave two bands within one of these size categories. The lower size category was characteristic of

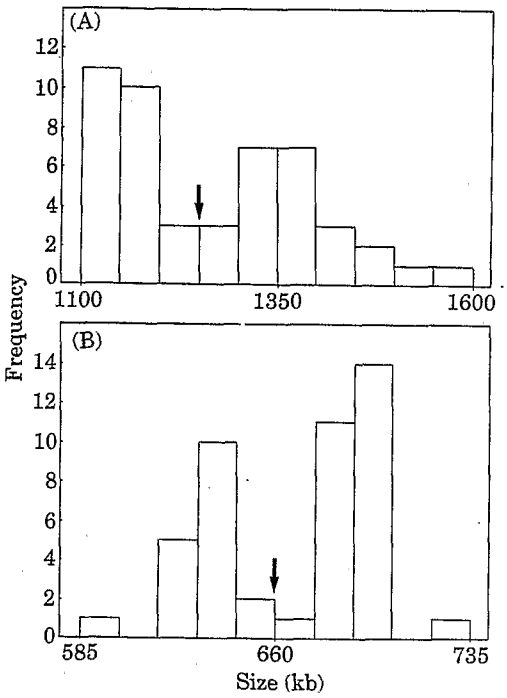

Fig. 7. Frequency distribution according to size of chromosomes hybridizing with (A) pLb-22, divided into 50-kb classes, and (B) pLb-168, divided into 15-kb classes. Each class includes sizes less than the upper limit and greater than or equal to the lower limit. The arrows indicate the cut-off values used to subdivide the populations.

pLb-168-hybridizing chromosomes of *L. braziliensis* [Fig. 6(c), strains 4 and 5; Dujardin *et al.*, 1993].

When the results of the tests with the pLb-22 and pLb-168 probes were combined, seven karyotype populations, or karyodemes, became apparent in the isolates from the Peruvian Andes (PA; Table 2). These karyodemes represented seven of the nine possible combinations of the different characters (Table 2) and were indicative of various degrees of similarity with *L. braziliensis*. The two commonest karyodemes were PA4 (34% of isolates) and PA2 (41%). PA4 was the karyodeme most similar to *L. braziliensis*, in both pLb-22 and -168 hybridization patterns, and PA2 the most different from *L. braziliensis* in both patterns. Intermediate karyodemes were observed, where dissimilarity was limited to the single pLb-168 (PA1) or pLb-22 (PA5) hybridizing band. Finally there were 'mixed' karyodemes, which gave double bands with one or both probes; one of each doublet belonging to the same size category as that of *L. braziliensis*, and one differing from it (PA3, PA6, PA7, PA8 and PA9).

Biogeography of the *Leishmania peruviana* Karyodemes

The PA karyodemes were not randomly distributed along the transect studied (Fig. 3). In northern Peru (HB and PO BGU), for

TABLE 2

Characteristics of the nine possible karyodemes of Leishmania peruviana *based on the size ranges of chromosomes hybridizing with each of two probes**

	Size range of chromosomes hybridizing with pLb-22 (kb)		
	1369 ± 165	*1146 ± 78*	*1369 and 1146*
SIZE RANGE OF CHROMOSOMES HYBRIDIZING WITH PLB-168 (KB)			
690 ± 23	PA1	PA2	PA3
	(5)	(17)	(1)
634 ± 29	PA4	PA5	PA6
	(14)	(1)	(0)
690 and 634	PA7	PA8	PA9
	(0)	(1)	(2)

*The nine karyodemes were designated PA1 to PA9 as shown. Values in parentheses are the number of isolates of each karyodeme (N=41).

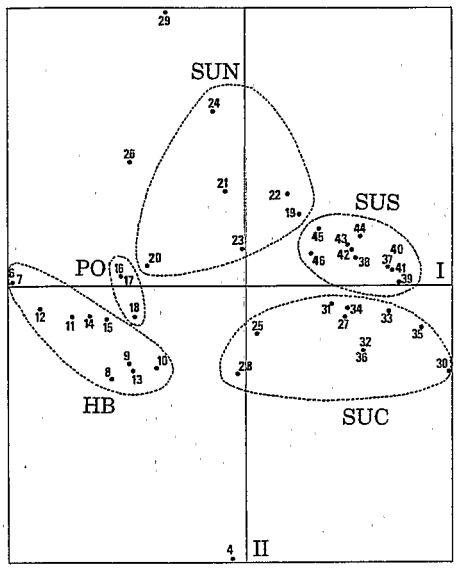

Fig. 8. Principal component analysis for the sizes of chromosomes hybridizing with probes pLb-134, pLb-22 and pLb-168. The main axes are responsible for 57·6% (I) and 23·3% (II) of the total variability.

example, all isolates belonged to PA4. At the other end of the transect, in the SUS BGU, all isolates belonged to PA2. Intermediate or mixed karyodemes were observed only in SUN and SUC, areas where PA2 was dominant (39%) and PA4 uncommon (5%). Further processing of our data confirmed a very strong geographical bias. A χ^2 homogeneity test on the frequencies of the major karyodemes PA2 and PA4 in each BGU revealed a strong deviation from those expected if the karyodemes' distribution were uniform ($P<0{\cdot}005$). The first two axes in the PCA (Fig. 8) accounted for 80·96% of the total variability and grouped all the isolates according to their BGU of origin, except for two isolates (coded 26 and 29) from SUC.

DISCUSSION

Environmental and geographical isolation as promoters of genetic variation (Lainson and Shaw, 1987) form an attractive hypothesis. A formal assessment of the effect of geography on New World *Leishmania* heterogeneity requires the comparison of isolates of a single parasite species collected from geographically isolated areas. *Leishmania peruviana* is an obvious candidate for any such study, as this species is restricted to Andean valleys (Lumbreras and Guerra, 1985; Guerra, 1988) separated by natural barriers that vertebrate or invertebrate hosts may seldom, if ever, cross. Furthermore, the biogeography of the area endemic for uta has already been well documented as part of a study on butterflies (Lamas, 1982). Our working hypothesis was that the geographical factors responsible for the variation in the local butterfly populations were also responsible for the variation in the *L. peruviana* populations (and their vectors).

We have now extended a preliminary study on leishmaniasis in one BGU (Arana *et al.*, 1990; Dujardin *et al.*, 1993) to include 41 isolates from five areas in three BGU (Fig. 1). These areas represent most of the Peruvian territory where uta has been described. All the isolates have been isoenzymatically identified as *L. (V.) peruviana* (Guerrini, 1993).

Karyotype analysis after ethidium bromide staining revealed that all the isolates presented markers typical of the *braziliensis* complex (see Dujardin *et al.*, 1987), confirming previous results on the nature of the complex circulating in the Andes (Lopez *et al.*, 1988). Hybridization patterns with pLb-134 confirmed that the Andean isolates were *L. peruviana*, proved that the parasites causing uta are genetically distinct from *L. braziliensis* (see Arana *et al.*, 1990; Dujardin *et al.*, 1993), and thereby strengthened *L. peruviana* as a separate taxon. A second observation of epidemiological significance was the demonstration, in Surco South, of a *L. peruviana* infection in *Lu. ayacuchensis* (see Caceres *et al.*, 1991, unpubl. obs.); Herrer (1982) and Perez *et al.* (1991) found infections only in *Lu. peruensis* (in Surco North).

The genetic heterogeneity of parasites causing uta (Ebert, 1987; Walton, 1987; Reiner *et al.*, 1989) was confirmed by the heterogeneity observed when pLb-22 and pLb-168 DNA probes were used on OFAGE karyotypes. pLb-22 recognized a chromosome of about 1369 kb and/or one of about 1146 kb.

pLb-168, in contrast, recognized a chromosome of about 690 kb and/or one of about 634 kb. Of the nine possible combinations of these banding patterns (karyodemes, Table 2), seven were observed. Karyodemes PA2 (1146 kb/690 kb) and PA4 (1369 kb/634 kb) were predominant, whereas PA6 and PA7 could not be found, either because they do not occur in the region or are relatively uncommon. Note that our classification should not be considered definitive; analysis of other chromosomes with additional probes will improve the resolution.

The finding of 'mixed' karyodemes (with doublets constituted by chromosomes similar to and others different from those of *L. braziliensis*) raises the question whether this is a result of the 'mosaic' structure (Bastien *et al.*, 1990) of these uncloned populations, of genetic exchange between parasites (Evans *et al.*, 1987; Kelly *et al.*, 1991), or of chromosomal rearrangement (Bishop and Miles, 1987; Iovannisci and Beverley, 1989; Pagès *et al.*, 1989; Bastien *et al.*, 1990; Blaineau *et al.*, 1991, 1992). Preliminary work seems to exclude the 'mosaic' hypothesis for at least some of the parasites belonging to the mixed karyodemes. When LH78 (PA9) was cloned by the microdrop method (Van Meirvenne *et al.*, 1975), it kept the same karyotype as the parental line (data not shown). Furthermore, when the isolates studied here were characterized by isoenzyme analysis (Guerrini, 1993), two different nucleoside hydrolase electromorphs were observed in karyodemes PA4 and PA2; the 'mixed' karyodemes showed one of these two variants, never both.

Association of the karyodemes with the BGU where they were isolated led to three important conclusions. First, the distribution of each karyodeme varied from north to south, and from one BGU to another; there is thus strong geographical structuring of the *L. peruviana* populations. Second, karyodeme similarity with *L. braziliensis* decreases from north to south. Third, although all the Andean isolates were *L. peruviana*, the PA4 karyodeme (from the north) was karyotypically closer to *L. braziliensis* than to *L. peruviana* PA2; PA4 shared two characters (the sizes of the pLb-168 and -22 hybridizing chromosomes) out of three with *L. braziliensis*, and only one out of three (the size of pLb-134 hybridizing chromosome) with PA2. The Peruvian Andean parasites may differ in virulence, host-specificity and clinical manifestations and the PA karyodemes are therefore of epidemiological importance.

How are such genetically different *Leishmania* populations generated? Relatively rapid genetic drift or selection (Dobzhansky, 1955) probably occurs in the Peruvian Andes. The BGU are separated by natural barriers which appear relatively difficult to pass and each has a distinctive ecology (Lamas, 1982). These facts, plus the low rate of inter-valley human migration, must represent near-optimal conditions for genetic drift and selection in the parasites (and their hosts) (Lainson and Shaw, 1987). The possible contribution of selection is strengthened by the existence of different *Lutzomyia* species in a least two of the areas investigated in the present study (Herrer, 1982; Caceres *et al.*, 1991; Perez *et al.*, 1991; A. Caceres, unpubl. obs.). These results show the necessity of multidisciplinary studies of the parasites and their hosts in the future. Intraspecific variability in the vector populations should be analysed, as geographical variation is not uncommon within sandfly species (Lane, 1988).

Parasite reproduction may be the third mechanism involved in the observed genetic variation. A clonal structure has been claimed for leishmanial populations in general (Tibayrenc *et al.*, 1990), but genetic exchange, however rare an event (Kelly *et al.*, 1991), may be an additional cause of variability. In the valleys of Surco North and Center, genetic exchange could have produced the intermediate karyodemes that were found. The situation might be similar to that described in one geographically isolated Pyrrenean valley (Blaineau *et al.*, 1992). Studies on the association equilibrium between these intermediate forms are necessary to test for evidence of genetic exchange and further sympatric sampling is required.

Another interesting speculation concerns the observed karyotypical dissimilarity of *L.*

peruviana and *L. braziliensis*; the further from the Amazonian forest, the less the parasites resemble *L. braziliensis*. The isolates from the Huancabamba BGU, which is the closest to the Amazonian basin and the lowest in altitude of those sampled, were most like *L. braziliensis*. This BGU is possibly where *Leishmania* disseminated from the forest to the coast or vice versa; sampling of isolates from the north of Huancabamba (i.e. Ecuador) and along a transect, similar to the present one, in the Peruvian forest, should permit this hypothesis to be tested.

Some of the genetic heterogeneity in the New World *Leishmania* spp. has now been quantified and the variability within other species of the *braziliensis* complex should now be studied. Preliminary studies on Bolivian, Brazilian and Peruvian isolates of *L. braziliensis* (Dujardin *et al.*, 1993, unpubl. obs.) indicate a lower level of heterogeneity within that species than in *L. peruviana*. Comparison of our results with those of other studies on the *braziliensis* complex (Saravia *et al.*, 1990; Tavares *et al.*, 1992) may be difficult because of the different experimental conditions used. Use of a common reference standard (we propose *L. (V.) braziliensis* M2903) would make future comparisons easier.

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