The gp63 gene locus, a target for genetic characterization of *Leishmania* belonging to subgenus *Viannia*

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SUMMARY

In the present study the gp63 gene locus was used as a target for genetic characterization of *Leishmania* parasites by 2 methods: (i) RFLP analysis with several restriction enzymes (gp63–RFLP), and (ii) intra-genic PCR amplification coupled with restriction analysis (PCR–RFLP). Both methods were applied to a large number of natural isolates belonging to 4 species of the subgenus *Viannia*, namely *L.* (*V.*) *braziliensis*, *L.* (*V.*) *peruviana*, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *lainsoni*: reference stocks of subgenus *Leishmania* were included as outgroups. Multilocus isoenzyme typing (MLEE) was used as a reference. On the one hand gp63–RFLP evidenced an extensive polymorphism and revealed specific markers for subgenus, species and geographical populations: congruence with MLEE was demonstrated statistically. The particular interest of gp63–RFLP was illustrated by infra-specific polymorphism, because of the possible relationship with phenotype diversity. On the other hand intra-genic amplification was less resolutive than gp63–RFLP, but also allowed discrimination of the 2 subgenera (PCR alone) and all the species tested in the subgenus *Viannia* (PCR–RFLP). PCR–RFLP presents an important operational advantage as it allows genetic characterization of minute amounts of parasites, using *Leishmania* specific primers. The polymorphism revealed by gp63–RFLP and PCR–RFLP illustrates the very high genomic and genetic plasticity of gp63 genes.

Key words: *Leishmania*, *Viannia* subgenus, gp63, PCR, taxonomy, gene organization.

INTRODUCTION

Leishmania are parasitic Protozoa belonging to the Trypanosomatidae family. Their important phenotypic diversity (pathology, ecology, etc.) has led to a complex taxonomy with more than 20 described species (Lainson & Shaw, 1987). The majority of these species has been described in Latin America. Among them, parasites of the subgenus *Viannia* are epidemiologically the most important as they exhibit the broadest range of variation in pathology, from benign cutaneous lesions with *L.* (*V.*) *peruviana* (Guerra, 1988) to severe lesions and mucosal metastasis with *L.* (*V.*) *braziliensis* (Walton, 1987).

To support current taxonomy, diverse methods of genetic characterization have been applied, such as isoenzyme electrophoresis (Chance, 1979, 1982), kDNA analysis (Grimaldi & Tesh, 1993), random amplification of polymorphic DNA (Tibayrenc *et al.* 1993) and molecular karyotyping (Dujardin *et al.* 1993*a*, *b*). Among several genetic characters, those corresponding to repetitive genes can be very informative for many operational and biological reasons. First, they are detected more easily than single copy genes. Second, polymorphism can be observed, either through variations in copy number (Iovannisci & Beverley, 1989), size or DNA sequence of non-transcribed spacers (Ramirez & Guevara, 1987; Fernandes *et al.* 1994). Third, these 2 characteristics can lead to the development of resolutive PCR characterization methods, as was done for mini-exon (Hassan *et al.* 1993; Fernandes *et al.* 1994) and rDNA (Cupolillo *et al.* 1995). Finally, repeated genes can eventually be used to analyse the genetic bases (if any) of important phenotypic properties, such as pathogenicity.

In that context, the gp63 gene locus might constitute a complementary target for genetic characterization. Indeed, gp63 is the major surface glycoprotein of the promastigote stage of the parasite, and is considered as an important virulence factor

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(Chakrabarty *et al*. 1996). Furthermore, it is encoded by tandemly repeated genes, from 6 described in *L*. (*L*.) *major* (Button *et al*. 1989) up to 71 in *L*. (*V*.) *braziliensis* (Victoir *et al*. 1995). Preliminary work revealed different levels of genetic polymorphism in subgenus *Viannia*. First, in the same organism, different families of gp63 genes may co-exist (Steinkraus *et al*. 1993; Victoir *et al*. 1995). Secondly, the total copy number of gp63 genes appears to vary between *L*. (*V*.) *braziliensis* and *L*. (*V*.) *peruviana*, being partially responsible for extensive size polymorphism of homologous chromosomes between these 2 species (Dujardin *et al*. 1994; Victoir *et al*. 1995). Thirdly, in a limited number of isolates, gp63–RFLP patterns were shown to differ between *L*. (*V*.) *braziliensis* and *L*. (*V*.) *peruviana*, and between eco-geographical populations of *L*. (*V*.) *peruviana* (Espinoza *et al*. 1995; Victoir *et al*. 1995).

Present work aimed to further evaluate the use of gp63 locus as a target for genetic characterization within the subgenus *Viannia*. First, RFLP analysis of gp63 genes was extended to other stocks and species in total 53 natural isolates of, *L*. (*V*.) *braziliensis* (35), *L*. (*V*.) *peruviana* (12), *L*. (*V*.) *guyanensis* (2) and *L*. (*V*.) *lainsoni* (2) and putative hybrids between *L*. (*V*.) *braziliensis* and *L*. (*V*.) *peruviana* (2) and 3 reference stocks of subgenus *Leishmania* as outgroups. Secondly, for validation of RFLP, results were compared to those obtained on the same sample from allozymes, independent and well-codified characters, which are considered as a reference in genetic characterization (Rioux *et al*. 1986). Thirdly, the gp63 locus was analysed by an additional method, an intra-genic PCR amplification coupled with restriction analysis (PCR–RFLP). Our results evidence a high genomic and genetic variability of the gp63 genes, support current taxonomical classification of New World *Leishmania*, and provide relevant informations at infra-specific level.

MATERIALS AND METHODS

Parasites

Leishmania promastigotes were grown in blood agar medium (Tobie, Von Brand & Mehlman, 1950). The analysed isolates (Table 1) were initially characterized by isoenzyme analysis (13 enzymes; Bañuls (1993) unpublished information; Guerrini, (1993)): 35 *L*. (*V*.) *braziliensis* isolates (2 from Brazil, 7 from Bolivia and 26 from Peru), 17 *L*. (*V*.) *peruviana* from Peru, 3 putative *L*. (*V*.) *braziliensis*/*L*. (*V*.) *peruviana* hybrids, all isolated in Peru (Dujardin *et al*. 1995*a*), 4 *L*. (*V*.) *guyanensis* from Peru, Brazil and French Guyana and 2 *L*. (*V*.) *lainsoni*, both from Peru. *L*. (*L*.) *amazonensis*, *L*. (*L*.) *mexicana*, *L*. (*L*.) *major* and *L*. (*L*.) *infantum* reference strains were used as outgroups. Marked isolates (*) were cloned by the microdrop method (Van Meirvenne, Janssens & Magnus, 1975).

DNA isolation, digestion and electrophoresis

High molecular weight genomic DNA was isolated as described elsewhere (Sambrook, Fritsch & Maniatis, 1989). DNA from amplification products was extracted by an equal volume of phenol/chloroform and ethanol precipitated. Genomic DNA and amplification products were digested to completion (overnight, with an excess of enzymes) with restriction enzymes (*Bgl*I, *Sal*I, *Eco*RI and *Apa*I) according to the manufacturer's instructions (Life Technologies). Electrophoresis was performed in 0·7 % and 1·5 % agarose, for RFLP and PCR–RFLP analysis, respectively. Lambda *Bst*EII DNA ladder and *Φ*X174 *Hae*III digest were used to size DNA molecules. All patterns were verified by performing at least 2 digestions of each DNA.

DNA hybridization

DNA was transferred, after depurination and denaturation with alkali to a Nylon Hybond N+ Amersham membrane, according to the 'pocket blotting' method (Cuny, Veas & Roizès, 1991). We used as probe a 300-bp sequence, pLb-134 Sp from *L*. (*V*.) *braziliensis* M2904 (Dujardin *et al*. 1993*a*, 1994) that is located in the 5′ part of the gp63 gene (corresponding to the N-terminal sequence of the protein), one of the most conserved regions of that gene in *L*. (*V*.) *guyanensis* and other *Leishmania* species (Medina-Acosta, Beverley & Russell, 1993; Steinkraus *et al*. 1993; Dujardin *et al*. 1994). The agarose containing the 300-bp insert was excised after *Pst*I digestion of the plasmid and electrophoresis in 1·0 % Nu-Sieve agarose (FMC), and purified by microcentrifugation through glass-wool. The probe was labelled with [^{32}P]dCTP by random primer labelling (Boehringer). Hybridization and washings (1 × SSC and 0·1 × SSC) were performed at 65 °C according to the manufacturer's instructions (Amersham).

PCR conditions

A PCR assay aiming at the amplification of the central portion of the gp63 genes (Fig. 1) was developed by using as primers 2 sequences found to be more conserved among the *L*. (*V*.) *guyanensis* genes (Steinkraus *et al*. 1993). The following oligonucleotides were used as primers in the PCR experiments: 5′GTCTCCACCGCAGACCTCACGGA (TDM1) and 5′TGATGTAGCTGCCATTCACGAAG (TDM2), respectively situated at positions 410–422 and 1721–1741 in the Lg63c1 cDNA gp63 sequence of *L*. (*V*.) *guyanensis*, according to Steinkraus *et al*. (1993). Amplification reactions were performed in volumes of 50 μl containing: 100–300 ng template DNA, 10 mM Tris–HCl, pH 8·3, 1·5 mM $MgCl_2$, 50 mM KCl, 200 μM each of dATP, dCTP, dGTP and dTTP

Table 1. Origin (Province and District, for isolates of Peru) and code of the stocks under study

International Code	Geographic origin	Type of lesions†	Analysis‡
L. (*V.*) *braziliensis*	*Brazil*		
MHOM/BR/75/M2903,	Para, Carajas	C	1,2
MHOM/BR/75/M2904*	Para, Carajas	C	1
	Bolivia		
MHOM/BO/83/LPZ155*	Beni	C	1,2
MHOM/BO/83/LPZ355*	—	C	1
MHOM/BO/94/CH29BL3	Chapare, Aroma	M	1,2
MHOM/BO/94/CH22B	Chapare, Aroma	M	1,2
MHOM/BO/94/CH25B	Chapare, Aroma	M	1
MHOM/BO/84/CEN002	Santa Cruz, Santa Fe	C	1,2
MHOM/BO/85/CEN007	Santa Cruz, Guarayos	C	1
	Peru		
MHOM/PE/91/LC1409	Huanuco, Huancapallac	C	1,2
MHOM/PE/91/LC1412	Huanuco, Huancapallac	C	1
MHOM/PE/90/LH1013	La Convencion, Quillabamba	C	1,2
MHOM/PE/91/LC1565	Paucar Tambo, Pilcopata	C	1,2
MHOM/PE/91/LC1568	Paucar Tambo, Pilcopata	M	1
MHOM/PE/91/LC1569	Paucar Tambo, Pilcopata	C	1
MHOM/PE/91/LC1578	Paucar Tambo, Pilcopata	C	1
MHOM/PE/91/LC1580	Paucar Tambo, Pilcopata	M	1,2
MHOM/PE/91/LC1584	Paucar Tambo, Pilcopata	C	1
MHOM/PE/91/LC2041	Paucar Tambo, Pilcopata	C	1
MHOM/PE/91/LC2043	Paucar Tambo, Pilcopata	M	1,2
MHOM/PE/91/LC2123	Paucar Tambo, Pilcopata	C	1
MHOM/PE/91/LC2125	Paucar Tambo, Pilcopata	M	1
MHOM/PE/91/LC2141	Paucar Tambo, Pilcopata	M	1
MHOM/PE/91/LC2147	Paucar Tambo, Pilcopata	C	1
MHOM/PE/91/LC2176	Paucar Tambo, Pilcopata	C	1
MHOM/PE/91/LC2289	Paucar Tambo, Pilcopata	C	1,2
MHOM/PE/91/LC2318	Paucar Tambo, Pilcopata	C	1,2
MHOM/PE/91/LC2319	Paucar Tambo, Pilcopata	C	1
MHOM/PE/91/LC2320	Paucar Tambo, Pilcopata	M	1
MHOM/PE/91/LC2321	Paucar Tambo, Pilcopata	C	1,2
MHOM/PE/90/LH825	Padre Abad, Aguaytia	C	1
MHOM/PE/00/LH800	Tambopata, Puerto Maldonado	C	1,2
MHOM/PE/00/LH699	Manu, Haypetue	C	1,2
MHOM/PE/84/LC01	Tahuamanu, Iberia	C	1,2
MHOM/PE/84/LC03	Tambopata, Mazuco	C	1,2
L. (*V.*) *peruviana*	*Northern Peru*		
MHOM/PE/90/HB31	Huancabamba, Huancabamba	C	1,2
MHOM/PE/90/HB44	Huancabamba, Sondorillo	C	1,2
MHOM/PE/89/LC900	Huancabamba, Sondorillo	C	1,2
MHOM/PE/90/HB86	Huancabamba, Faique	C	1
MHOM/PE/90/HB55	Huancabamba, Sondor	C	2
MHOM/PE/90/HB56	Huancabamba, Sondor	C	2
	Central Peru		
MHOM/PE/90/LC443	Bolognesi, Huyallacayan	C	2
MHOM/PE/89/LH741	Recuay, Pararin	C	2
MHOM/PE/90/LC436	Bolognesi, Huyallacayan	C	2
MHOM/PE/84/LC26*	Bolognesi, Huayllacayan	C	1,2
MHOM/PE/90/LC468	Bolognesi, Huayllacayan	C	1,2
MHOM/PE/90/LH827	Canta, Lampiane	C	1
MHOM/PE/90/LH925	Canta, San Buenaventura	C	1,2
MHOM/PE/00/LC106*	Huarochiri, Santa Eulalia	C	1,2
	Southern Peru		
MHOM/PE/90/LCA04	Lucanas, Sancos	C	1,2
MHOM/PE/90/LCA08	Lucanas, Sancos	C	1,2
IAYA/PE/90/La36	Lucanas, —	(Sandfly isolate)	1,2
Putative *L.* (*V.*) *peruviana*/ *L.* (*V.*) *braziliensis* hybrids	*Peru*		
MHOM/PE/91/LC1407	Huanco, Huancapallac	C	1,2
MHOM/PE/91/LC1408	Huanuco, Huancapallac	M	1,2
MHOM/PE/91/LC1099	Ambo, Ambo	C	2

Table 1 *continues on p.* 4.

Table 1. (*cont.*)

International Code	Geographic origin	Type of lesions†	Analysis‡
L. (*V.*) *guyanensis*			
MHOM/PE/89/LH691	Canta, Canta, Peru	C	1,2
IPRN/PE/87/Lp52	Bolognesi, Huayllacayan, Peru	(Sandfly isolate)	1,2
MHOM/GF/85/LEM669	—, French Guyana	C	2
MHOM/BR/78/M5378	Para, Monte Dorado, Brazil	C	2
L. (*V.*) *lainsoni*			
MHOM/PE/91/LH1154	Leoncio Prado, Tingo Maria, Peru	C	1,2
MHOM/PE/91/LC1581	Paucartambo, Pilcopata, Peru	M	1,2
L. (*L.*) *amazonensis*			
MPRO/BR/77/M185	Para, Brazil	C	1,2
MHOM/BR/76/LTB012	Brazil	C	1,2
L. (*L.*) *mexicana*			
MNYC/BZ/62/M379	Belize	C	2
L. (*L.*) *major*			
MHOM/SU/73/5-ASKH	Turkmenskaya, Askhabad, USSR	C	2
L. (*L.*) *infantum*			
MHOM/MA(BE)/67/ ITMAP263	Morocco	V	1

* Cloned stocks.
† Clinical origin of stocks: mucosal (M), cutaneous (C) or visceral (V) lesions.
‡ Analysis performed: gp63–RFLP (1), PCR–RFLP (2).

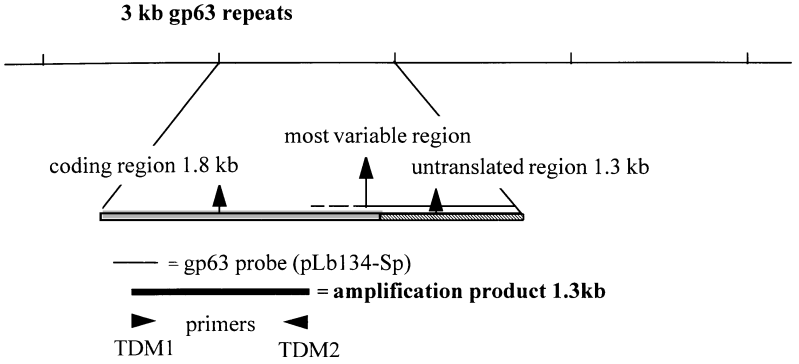

Fig. 1. Gp63 gene repeat: localization of (i) the sites of priming of TDM1 and TDM2 generating a 1·3 kb amplification product, and (ii) the target for the gp63 probe (pLb-134 Sp) used for identification of PCR products and RFLP analysis (from data of Steinkraus *et al.* (1993)).

(Boehringer) and 2·5 U *Taq* DNA polymerase (Boehringer). The primers were added at a final concentration of 20 pmol/50 μl assay. Samples were amplified in a MJ Research PCT-100 Programmable Thermal Controller with heated lid, according to hot start conditions as follows: (i) initial denaturation of 2 min at 94 °C; (ii) whereafter samples were held at 80 °C while the *Taq* DNA polymerase was added diluted in 2·5 μl of the required 10 × reaction buffer (Boehringer); (iii) denaturation, 30 sec at 94 °C; (iv) annealing, 60 sec at 56 °C; and (v) extension, 1 min 30 sec at 72 °C. Thirty-four PCR cycles were run. This was followed by a final extension cycle of 5 min at 72 °C. Contaminations were monitored by a negative control at every PCR run. Reaction tubes were held at 4 °C prior to analysis.

Multilocus enzyme electrophoresis (*MLEE*)

Cellulose acetate electrophoresis was used. Technical conditions for sample preparation, electrophoresis and staining procedures were performed according to methods described by Ben Abderrazak *et al.* (1993). Thirteen enzyme systems were used: Aconitase (ACON: EC 4.2.1.3), alanine aminotransferase (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 phos-

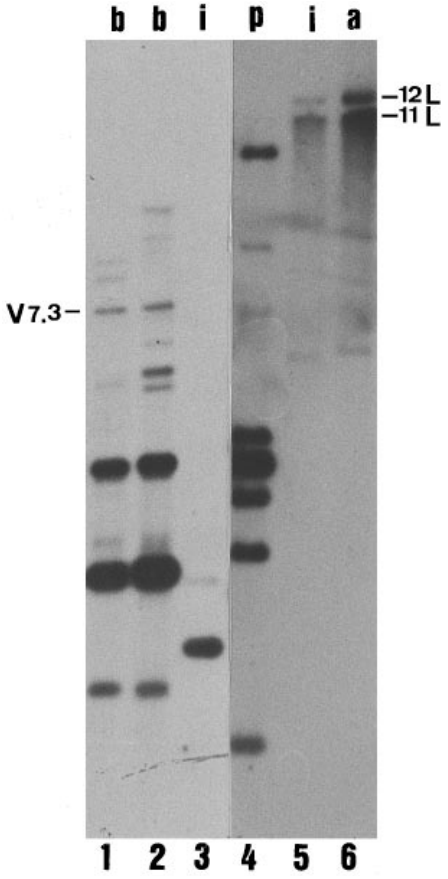

Fig. 2. Hybridization of pLb-134 Sp on *Bgl*I (1–3) and *Apa*I (4–6) digestion products of total DNA. Stocks: *Leishmania* (*V.*) *braziliensis* (**b**) (1, LPZ 155; 2 LC2320), *L.* (*L.*) *infantum* (**i**) (3, 5: ITMAP 263), *L.* (*V.*) *peruviana* (**p**) (4, LCA04) and *L.* (*L.*) *amazonensis* (**a**) (6, LTB012). V and L: fragments specific of subgenera *Viannia* and *Leishmania*, respectively. (All marked sizes are in kb.)

phate isomerase (MPI: EC 5.3.1.8), nucleoside hydrolase, substrate deoxyinosine (Nhd: EC 3.2.2.*), nucleoside hydrolase, substrate inosine (Nhi: EC 3.2.2.*), 6-phosphogluconate dehydrogenase (6PGD: EC 1.1.1.44), and phosphoglucomutase (PGM: EC 2.7.5.1).

Phenetic analysis

Jaccard distance was used for both MLEE and RFLP data, according to the following formula (Jaccard, 1908): $D_{ij} = 1-[a/(a+b+c)]$ where 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 and c = number of bands present in the j stock and absent in the i stock. Phenetic relationships among the stocks were estimated by the Unweighted Pair-Group Method with Arithmetic Averages (UPGMA, Sneath & Sokal, 1973). According to Tibayrenc (1995), agreement between the phenetic relationships depicted by RFLP and MLEE was tested by evaluating the correlation between the genetic distances inferred from the 2 methods for any possible pair of stocks, with a non-parametric Mantel test (Mantel, 1967).

RESULTS

RFLP analysis of the gp63 gene locus

The RFLP pattern of gp63 genes (gp63–RFLP) was mainly analysed in *Leishmania* of subgenus *Viannia*: (53 stocks): 35 *L.* (*V.*) *braziliensis*, 12 *L.* (*V.*) *peruviana*, 2 *L.* (*V.*) *guyanensis*, 2 *L.* (*V.*) *lainsoni*, and 2 putative hybrids between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana*. Reference strains of the subgenus *Leishmania* (1 *L.* (*L.*) *infantum* and 2 *L.* (*L.*) *amazonensis*) were also analysed. Genomic DNA was cleaved with 3 different restriction enzymes (*Bgl*I, *Sal*I and *Apa*I) and hybridized with plb-134Sp DNA probe.

Relatively complex and polymorphic patterns were obtained, a feature typical for a repetitive sequence. Each pattern evidenced specific bands that corresponded to particular taxonomic categories. Thus some characters were specific for subgenera *Viannia* (7·3 kb *Bgl*I and 2·9 kb *Sal*I band) and *Leishmania* (11 and 12 kb *Apa*I band), respectively (see Fig. 2). In addition, restriction patterns were relatively more complex in subgenus *Viannia* than in subgenus *Leishmania* (specially with *Apa*I and *Bgl*I, see Fig. 2).

Within subgenus *Viannia*, each species tested presented specific characters. All stocks of *L.* (*V.*) *braziliensis* presented a 15·8 kb *Sal*I band (see Fig. 3A–C) which distinguished them from *L.* (*V.*) *peruviana* and *L.* (*V.*) *guyanensis*. All *L.* (*V.*) *peruviana* stocks showed common bands (e.g. 8·7 kb with *Sal*I, see Fig. 3A; 1·0 kb with *Bgl*I, not shown; 1·2 kb with *Apa*I, not shown) with most of the *L.* (*V.*) *braziliensis* stocks, but they lacked the 15·8 kb *Sal*I fragment which was present in *L.* (*V.*) *braziliensis* (see Fig. 3A). *L.* (*V.*) *guyanensis* was characterized by a 0·5 kb *Bgl*I band (not shown), a 4·6 kb *Sal*I band (see Fig. 3B, C) and 1·4 and 5·1 kb *Apa*I bands (see Fig. 3D). *L.* (*V.*) *lainsoni* shared a 15·8 kb *Sal*I band with *L.* (*V.*) *braziliensis* but showed also specific *Sal*I fragments (6·9 kb and 1·9 kb, see Fig. 3B, C) and *Apa*I fragments (4·3 and 4·7 kb, see Fig. 3D). Putative hybrids between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* presented the 15·8 kb *Sal*I fragment encountered in *L.* (*V.*) *braziliensis* (Fig. 3A) and also some fragments found only in *L.* (*V.*) *peruviana*: a 13·6 kb *Sal*I fragment (see Fig. 3A) and 13·8 and 6·8 kb *Bgl*I fragments (not shown).

At infra-specific level, polymorphism was also observed. Within *L.* (*V.*) *peruviana* we confirmed with 5 additional *L.* (*V.*) *peruviana* stocks earlier findings (Victoir *et al*. 1995) of *Sal*I patterns specific

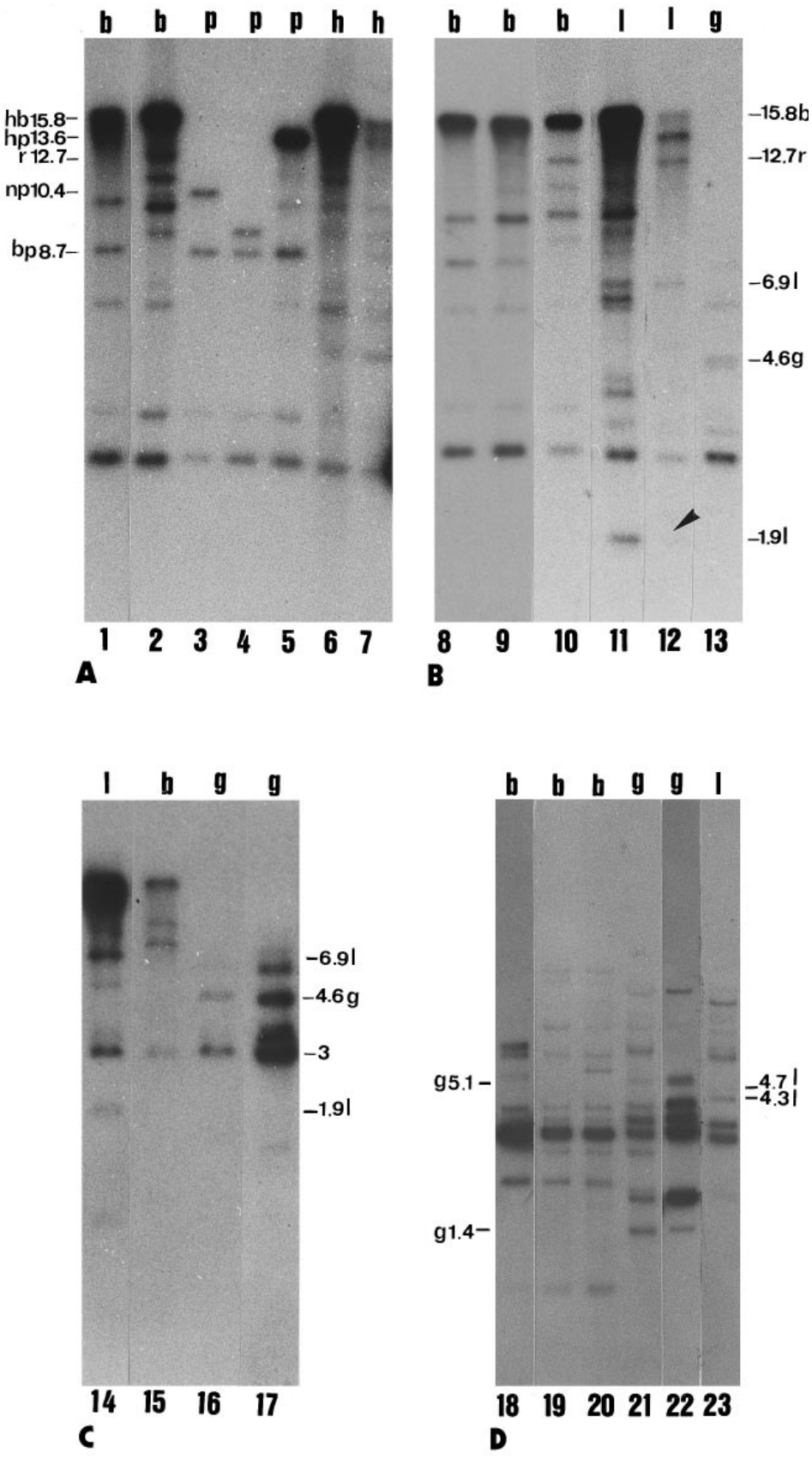

Fig. 3. Hybridization of pLb-134 Sp on *Sal*I (A, B, C) and *Apa*I (D) restriction products of total DNA. Stocks: *Leishmania* (*V.*) *braziliensis* (**b**) (reference strain, maintained long-term *in vitro*; 2, 10, 18, LPZ155; natural populations: 1, 8, LC1850; 9, LC1565; 15, LH 825; 19, LH800; 20, LH699), *L.* (*V.*) *peruviana* (**p**) (3, Northern Peru HB31; 4, Central Peru LC26; 5, Southern Peru LCA04), *L.* (*V*). *braziliensis*/*L.* (*V.*) *peruviana* putative hybrids (**h**) (6, LC1408; 7, LC1407), *L.* (*V.*) *guayanensis* (**g**) (13, 16, 22, Lp52; 17, 21, LH691), *L.* (*V.*) *lainsoni* (**l**), (11,

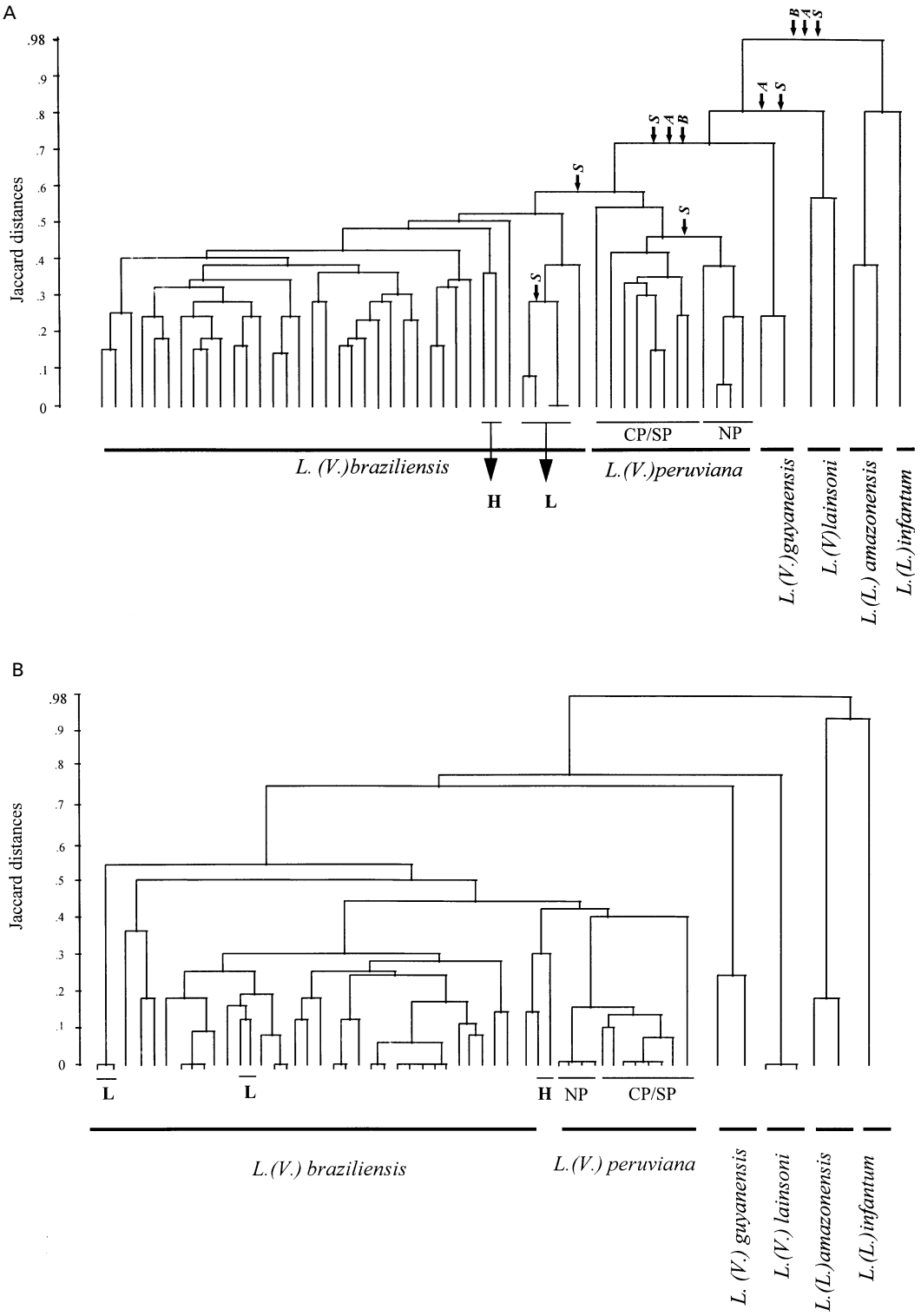

Fig. 4. UPGMA dendrogram built up from the matrix of Jaccard distances estimated from data of (A) gp63–RFLP with *Bgl*I (*B*), *Sal*I (*S*) and *Apa*I (*A*); enzymes responsible for main subdivisions are indicated by arrows on the horizontal branch separating respective groups. (B) MLEE-analysis with 13 different enzymes. (SP = Southern Peru, CP = Central Peru, NP = Northern Peru), (L) = stocks maintained long-term *in vitro*, (H) = *Leishmania* (*V.*) *braziliensis*/*L.* (*V.*) *peruviana* hybrids.

LC1581, 12, 14, 23, LH1154). Fragments common to *L.* (*V.*) *braziliensis* and *L.* (*V.*) *lainsoni* (bl), fragments encountered in *L.* (*V.*) *braziliensis* stocks maintained long-term *in vitro* (r); fragments specific of *L.* (*V.*) *guyanensis* (g), *L.* (*V.*) *lainsoni* (l); fragment common between *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis* (bp), fragment common between *L.* (*V.*) *braziliensis*/*L.* (*V.*) *peruviana* putative hybrids and *L.* (*V.*) *braziliensis* on one hand (hb) and *L.* (*V.*) *peruviana* on the other hand (hp). (All marked sizes are in kb.)

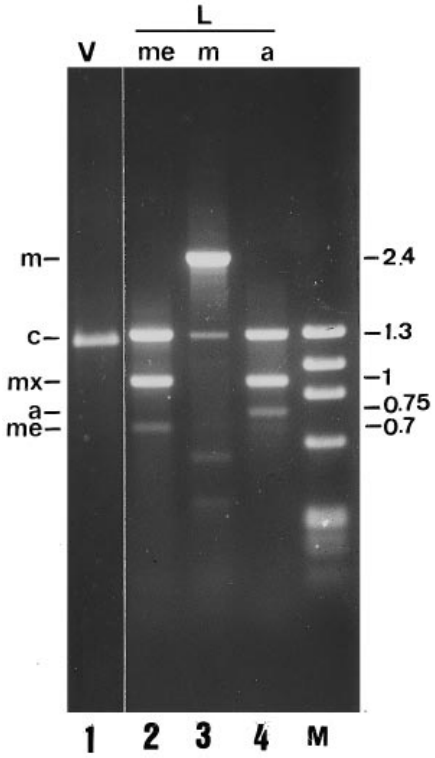

Fig. 5. Gp63 gene PCR amplification (uncut) of *Leishmania* and *Viannia* strains with a common 1·3 kb band (c); *L.* (*V.*) *braziliensis* (V) (1, LC2043); *L.* (*L.*) *mexicana* (me) (2, M379) with a specific band (me); *L.* (*L.*) *amazonensis* (a) (4, LV78) with a specific band (a); *L.* (*L.*) *major* (m) (3, 5-ASKH) with a specific band (m); specific band of the *L. mexicana* complex (mx). MW = molecular weight marker. (All marked sizes are in kb.)

to the geographical origin of the stocks: a fragment of 10·4 kb was present in all the stocks from Northern Peru but absent in stocks from Central and Southern Peru (see Fig. 3A). Within *L.* (*V.*) *braziliensis*, despite the observed polymorphism, no specific banding pattern related to the geographical or clinical origin of the different isolates could be identified. However, some specific characters, such as the 12·7 kb *Sal*I fragment, were constantly observed in strains maintained for long-term *in vitro*, and were generally absent in recently isolated stocks (see Fig. 3A, B).

Phenetic analysis confirmed the results mentioned above and allowed analysis of the relationships between the different groups of parasites (see Fig. 4A, enzymes responsible for major groupings are indicated on the dendrogram). Outgroups belonging to subgenus *Leishmania* clearly clustered separately. Within subgenus *Viannia*, the 4 species studied were individualized as independent clusters: *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* were the more closely related species, *L.* (*V.*) *guyanensis*, constituted a separate cluster while *L.* (*V.*) *lainsoni* was the most remote from any of the 3 other species. Putative hybrids between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* clustered within *L.* (*V.*) *braziliensis*. This is probably induced by the unique 15·8 kb *Sal*I fragment which is a distinctive feature of *L.* (*V.*) *braziliensis* compared to *L.* (*V.*) *peruviana*. Within *L.* (*V.*) *peruviana*, 2 clusters were observed, 1 containing all stocks from Northern Peru and 1 grouping together the stocks from Central and Southern Peru. Within *L.* (*V.*) *braziliensis*, stocks maintained long-term *in vitro* clustered separately.

Comparison of gp63–RFLP and Multi Locus Enzyme Electrophoresis (MLEE) data

Genetic polymorphism within subgenus *Viannia* as evidenced by gp63–RFLP was compared with MLEE analysis, a reference method for genetic characterization. Gp63–RFLP revealed a higher polymorphism than isoenzyme analysis of 13 different enzymes did (see Fig. 4B), since every stock analysed showed a distinct gp63–RFLP genotype, whereas several of these stocks showed the same isoenzyme profile. Nevertheless, a general congruence was observed between the 2 sets of data. First, as shown by Mantel test, a highly significant correlation ($P < 10^{-4}$) was observed between the genetic distances calculated from either gp63–RFLP or MLEE, for all species studied as well as for *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis* separately. Secondly, hierarchical relationships among species and infra-specific populations were the same for both gp63–RFLP and MLEE i.e. proximity of *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana*, divergence of *L.* (*V.*) *lainsoni*, and geographical structuring of *L.* (*V.*) *peruviana* populations. Two exceptions could be noted: (i) isoenzyme data branched hybrids in an intermediate position between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana*, whereas they clustered within *L.* (*V.*) *braziliensis* in gp63–RFLP analysis; (ii) the 'long-term culture' isolates were scattered by MLEE, while they clustered by gp63–RFLP.

PCR analysis of the gp63 genes

Intragenic PCR amplification of gp63 genes was applied to 41 stocks of subgenus *Viannia* (same species as those studied by gp63–RFLP), and 1 stock of each of the following subgenus *Leishmania* species: *L.* (*L.*) *major*, *L.* (*L.*) *mexicana* and *L.* (*L.*) *amazonensis*. During all PCR assays no contamination could be detected in negative controls.

All the stocks of subgenus *Viannia* gave a single 1·3 kb amplification band; see Fig. 5): this was the expected length between the annealing sites of the 2 primers on the known *L.* (*V.*) *guyanensis* gp63 cDNA sequence (Steinkraus *et al*. (1993) see Fig. 1). Stocks of the subgenus *Leishmania* also generated the 1·3 kb amplicon, but were differentiated from subgenus *Viannia* by the presence of additional bands: a fragment of 2·4 kb in *L.* (*L.*) *major*, 2 fragments of

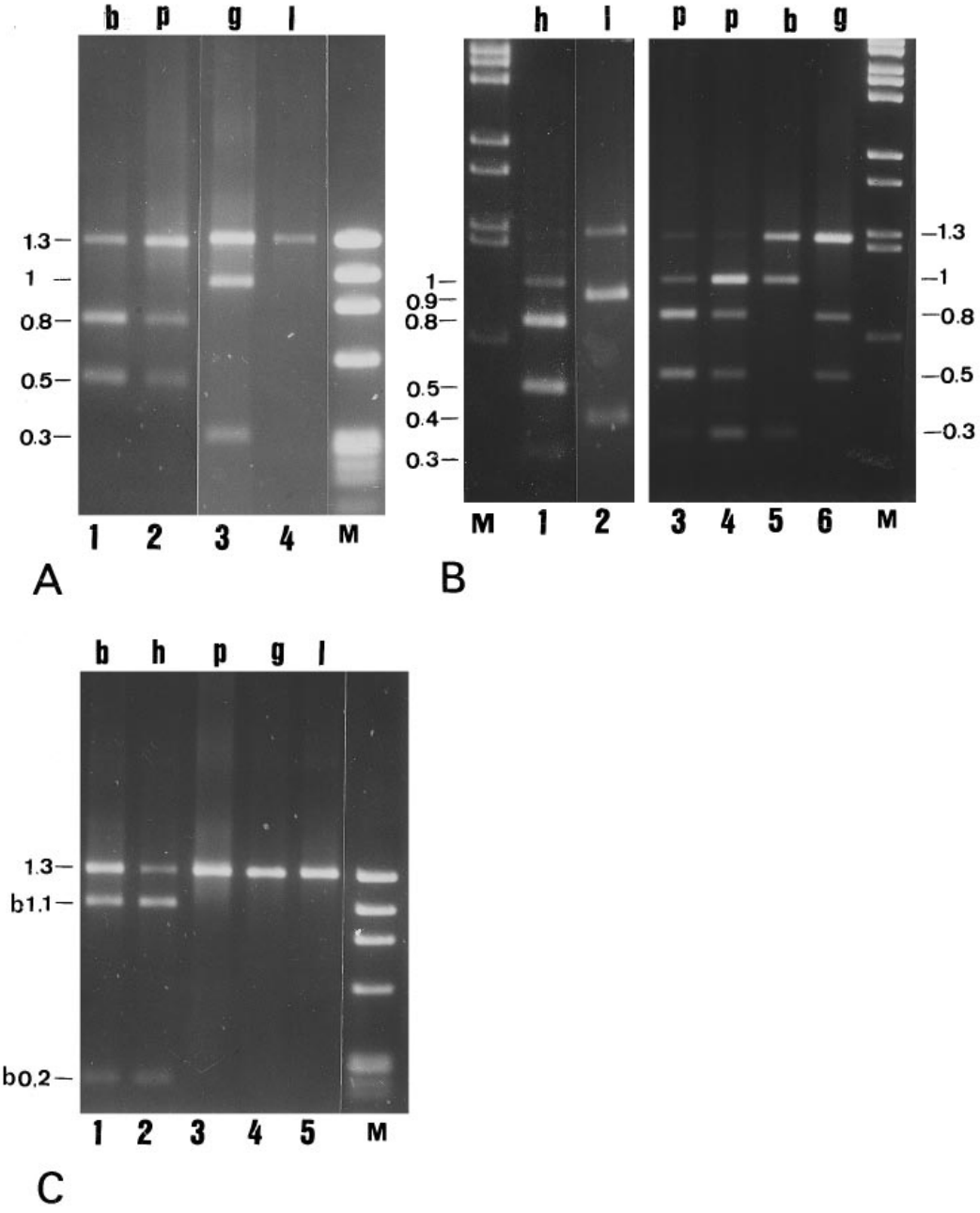

Fig. 6. Restriction of gp63 gene amplification products of *Viannia* strains with *Bgl*I (A), *Eco*RI (B) and *Sal*I (C). *L.* (*V.*) *braziliensis* (b) (A1, LC2289; B5, LC2043; C1, CEN 2), *L.* (*V.*) *peruviana* (p) (A2, LCA08; B3, La36; B4, LC106; C3, HB44), putative *L.* (*V.*) *peruviana*/*L.* (*V.*) *braziliensis* hybrids (h) (B1 and C2, LC1407), *L.* (*V.*) *guyanensis* (g) (A3, LEM699; B6, LH691; C4, Lp52) *L.* (*V.*) *lainsoni* (I) (A4, LC1581; B2, LH1154; C5, LH1154). MW = molecular weight marker. (All marked sizes are in kb.)

respectively 1·0 and 0·7 kb in *L.* (*L.*) *mexicana* and, 2 fragments of respectively 1·0 and 0·75 kb in *L.* (*L.*) *amazonensis* (see Fig. 5). All amplification patterns were reproducible, and all products were checked for their gp63 identity by Southern blot hybridization with the pLb 134-Sp probe (300 bp fragment of *L.* (*V.*) *braziliensis* gp63 gene expected to be present in the amplicon, see Fig. 1). All fragments were found to hybridize under normal stringency (1 × SSC), but signal disappeared at high stringency (0·1 × SSC), except for the 1·3 kb band (not shown). This suggests that only the latter band would correspond to a gp63 gene fragment. The additional bands, however, were displayed reproducibly and may still be characteristic for the species.

PCR–RFLP analysis of the gp63 genes

In order to evaluate the possibility of discriminating species within subgenus *Viannia*, the 1·3 kb amplicons were cleaved (PCR–RFLP) with *Apa*I, *Sal*I, *Bgl*I and *Eco*RI. Reproducibility of PCR–RFLP patterns was checked with several independently obtained amplification products. In most

Table 2. Summary of the restriction patterns (in kb) obtained in subgenus *Viannia* after digestion of the 1·3 kb gp63 amplification product

	L. (*V.*) *braziliensis*	*L.* (*V.*) *peruviana*	*L.* (*V.*) *braziliensis* /*L.* (*V.*) *peruviana* hybrids	*L.* (*V.*) *guyanensis*	*L.* (*V.*) *lainsoni*
Enzyme					
*Bgl*I					
			1·3		
	1·3/0·8/0·5	1·3/0·8/0·5	**1·3**/0·8/0·5	1·3/1/0·3	1·3
*Eco*RI					
		1·3/1/0·3	1·3/1/0·3		
	1·3/1/0·3	**1·3**/1/0·8/0·5/0·3	1·3/1/0·3/0·8/0·5	1·3/0·8/0·5	1·3/0·9/0·4
		1·3/0·8/0·5			
*Sal*I					
	1·3/1·1/0·2	1·3	1·3	1·3	1·3
			1·3/1·1/0·2		
*Apa*I					
	1·3	1·3	1·3	1·3/1/0·3	1·3

1·3 = Not always present.

cases, an uncut 1·3 kb band was found together with cleaved fragments following restriction. This is compatible with sequence data from *L.* (*V.*) *guyanensis* (Steinkraus *et al*. 1993), which indicates that some gp63 sequences lack the restriction sites here analysed. However, in order to exclude incomplete digestion, the uncut 1·3 kb band was excised, purified, reamplified and reincubated with the same restriction enzyme. Each species could be distinguished by one or more of the PCR–RFLP patterns (Fig. 6 and Table 2).

Two cases deserve particular attention. First, *peruviana* was the only species that (i) did not present specific bands (the absence of bands specific to the 3 other species allowed its identification), and (ii) presented infra-specific polymorphism (*Eco*RI, Fig. 6B) which, however, was not correlated with eco-geography. Secondly, some hybrids between *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis* showed a typical *L.* (*V.*) *peruviana* 5 band restriction pattern with *Eco*RI (Fig. 6B) together with the typical *L.* (*V.*) *braziliensis Sal*I pattern (Fig. 6C).

DISCUSSION

In the present work, genetic characterization of *Leishmania* was performed by using the gp63 gene locus as a target. Two methods were applied on a large sample of parasites: (i) restriction analysis of genomic DNA and hybridization with a probe containing a conserved part of the gp63 gene (gp63–RFLP), and (ii) an original method, consisting in the PCR amplification of the gp63 intragenic portion, coupled with restriction analysis of amplification products (PCR–RFLP). The validity and specificity of both methods was illustrated.

Gp63–RFLP was found to be the method of greatest resolution revealing relationships between *Leishmania* that were congruent with those obtained by other molecular methods. Congruence was demonstrated statistically by the strong correlation ($P < 10^{-4}$, Mantel) observed between genetic distances calculated on the same sample from gp63–RFLP and MLEE data. In particular, clusters defined by specific markers corresponded to previously described entities: subgenera *Viannia* and *Leishmania* (Lainson & Shaw, 1987), and within subgenus *Viannia*, (i) the very similar *L.* (*V.*) *braziliensis*, *L.* (*V.*) *peruviana* (Guerrini, 1993; Bañuls, 1993) and putative hybrids (Dujardin *et al*. 1995), (ii) *L.* (*L.*) *guyanensis* and (iii) the more remote *L.* (*V.*) *lainsoni* (Fernandes *et al*. 1994; Eresh *et al*. 1995).

In addition to these taxonomic applications, gp63–RFLP was most informative at the infra-specific level, particularly because of the possible relationships with phenotypic diversity. Previously described clustering within *L.* (*V.*) *peruviana* (Espinoza *et al*. 1995; Victoir *et al*. 1995) was not affected by the extension of our sample to other isolates and species. This strengthens the significance of such a structuring, that can be correlated with variation in (i) clinical symptoms (Davies *et al*. 1997), (ii) *in vitro* growth rate (Dujardin, 1995) and (iii) vector species (Davies *et al*. 1993; Dujardin *et al*. 1993*b*; Villaseca *et al*. 1993). This relationship between gp63–RFLP patterns and phenotype could be indirect, but considering the possible role of gp63 in virulence (Chakrabarty *et al*. 1996) and survival in the invertebrate vector (Medina-Acosta *et al*. 1993), the possibility of a direct association should be further analysed. Within *L.* (*V.*) *braziliensis*, extensive polymorphism in gp63–RFLP patterns was also observed but, in contrast to *L.* (*V.*) *peruviana*, infra-specific clusters could not be associated with geographical or clinical origin (cu-

taneous or mucosal) of corresponding isolates. The only significant cluster corresponded to stocks maintained long-term *in vitro*. Clustering was generated by the presence of specific bands and is likely due to specific rearrangements during long-term *in vitro* maintenance, as shown previously (Victoir *et al*. 1995). This phenomenon should also be further analysed specifically to know if it could be related with the decrease in virulence that has been described in long-term cultures (Shaw, 1997). However, this relative instability did not concern bands responsible for clustering at higher levels.

This report confirms the validity of RFLP for *Leishmania* identification purposes (Beverley, Ismach & McMahon-Pratt, 1987; Van Eys *et al*. 1989; Mendoza-Leon, Havercroft & Barker, 1995). According to Beverley, Ismach & McMahon Pratt (1987), polymorphism could be associated with virtually any gene and might distinguish major lineages of *Leishmania*. However, the level of discrimination also depends on the locus considered (Pogue *et al*. 1996), and the selective pressure upon it. In that context, the advantage of the gp63 locus is the access with the same probe to different levels of discrimination: from subgenus to stocks. This characteristic likely reflects different selective pressures existing on gp63 characters specific of these respective levels.

PCR analysis of the gp63 loci, although less discriminative than gp63–RFLP, allowed alone or in combination with restriction enzymes (PCR–RFLP), the same taxonomical applications. This is particularly interesting for *L*. (*V*.) *braziliensis*/*L*. (*V*.) *peruviana* as this is the first specific PCR allowing the discrimination of these 2 species, genetically very similar (Bañuls, 1993; Guerrini, 1993) but clinically different (Guerra, 1988). Infra-specific polymorphism was evidenced by PCR–RFLP mainly within *L*. (*V*.) *peruviana*, but in contrast with gp63–RFLP, it did not evidence any eco-geographical structuring. This is probably due to the fact that corresponding RFLP restriction sites are located within the intergenic regions of the gp63 complex. Analysis of polymorphism at infra-species level might thus be developed in the future by designing a PCR assay for the study of the 3′ end of the gene together with intergenic regions, both shown in *L*. (*V*.) *guyanensis* to be more variable than the central region here analysed (Steinkraus *et al*. 1993).

Although it showed a lower discrimination power than gp63–RFLP, PCR–RFLP, however, represents an operationally essential improvement. Indeed, this method permits genetic characterization on very small amounts of parasites, and theoretically *in situ* (biopsy, vector), since this method relies on *Leishmania*-specific primers (in contrast with random primers). Up to now, 2 other nuclear sequences (intergenic sequences of rDNA (Cupolillo *et al*. 1995) and mini-exon genes (Hassan *et al*. 1993; Fernandes *et al*. 1994) were shown to offer similar advantages. As the respective sequences could be under different selective pressures (intergenic *versus* intra-genic sequences), their respective discriminatory power should be compared on the same and large sample of parasites. Even with a similar level of discrimination, the description of new PCR–RFLP loci is not redundant and is essential for any multilocus analysis. A particular field of interest would be population genetics without cultivation (possible selection bias): indeed, as the 3 genes here mentioned are situated on different chromosomes (Dujardin *et al*. 1994, 1995: Wincker *et al*. 1996), they should allow recombination tests.

The genetic polymorphism of the gp63 locus here evidenced is most probably due to 2 phenomena, (i) changes in genomic organization of gp63 genes e.g. reflected by their difference in copy number as reported at the level of subgenus, species and individual stocks (Button *et al*. 1989; Webb, Button & McMaster, 1991; Medina-Acosta *et al*. 1993; Roberts *et al*. 1993; Steinkraus *et al*. 1993; Victoir *et al*. 1995), and (ii) genetic heterogeneity of the genes themselves and their flanking regions: from 1 gene family in *L*. (*L*.) *major* (Button *et al*. 1989) to at least 4 in *L*. (*V*.) *guyanensis* (Steinkraus *et al*. 1993). PCR–RFLP data might give additional information on the distribution of gp63-gene families among species of subgenus *Viannia*. Indeed, we observed species-specific fragments in *L*. (*V*.) *braziliensis*, *L*. (*V*.) *guyanensis* and *L*. (*V*.) *lainsoni* that suggest a species-specific distribution of gp63 gene families. *L*. (*V*.) *peruviana*, however, constitutes a particular case in this context. Indeed, no specific fragments were noted for this species: only the absence of some fragments (like the 1·1/0·2 kb *Sal*I bands) distinguished that species from others. Considering together the lower copy number of gp63 genes in *L*. (*V*.) *peruviana* (Victoir *et al*. 1995), and the hypothesis of a recent evolutionary divergence of *L*. (*V*.) *peruviana* from *L*. (*V*.) *braziliensis* (Dujardin *et al*. 1993*b*, 1995), the absence of these fragments might be compatible with a deletion (partial or total) affecting a specific gene family in *L*. (*V*.) *peruviana*, with possible functional consequences. Indeed, differential expression of specific genes (in species containing different gene families) through the life-cycle of the parasite has been associated with functional differences at the protein level (Medina-Acosta *et al*. 1993). Comparison of physical maps of gp63 gene loci in both species, and analyses at transcription level might allow confirmation of this hypothesis.

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