MOLECULAR DIVERSITY IN THE *LEISHMANIA* SUBGENUS *VIANNIA*

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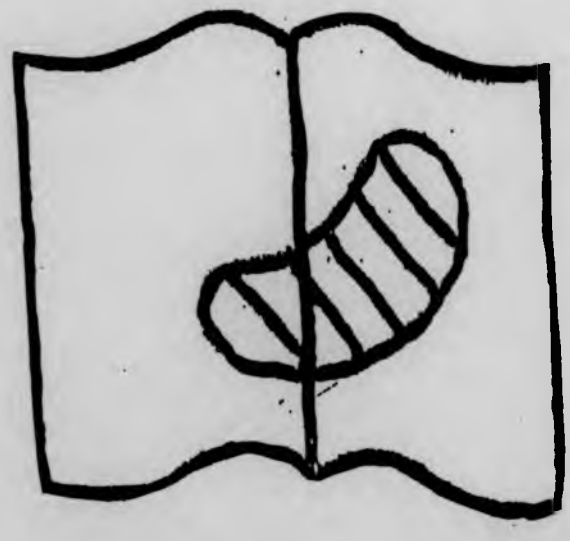

NUMEROUS ORIGINALS
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ABSTRACT

The aim of this work was to provide further insight into the relationships between species of the *Leishmania* subgenus *Viannia* by examining inter- and intra-specific genetic diversity.

Diversity among *Viannia* strains and stocks was investigated using biochemical and molecular techniques. Isoenzyme and microsatellite analyses were found to be the most discriminatory and generated results which could be interpreted genotypically. These techniques were used to study diversity in *Viannia* populations consisting of i) Brazilian *L. V. braziliensis* stocks, ii) Nicaraguan stocks belonging to different *Viannia* species complexes, including putative species-complex hybrids, and iii) uncharacterized stocks from a new epidemic focus in Huánuco, Peru.

IEA identified all stocks to at least the species level. Microsatellite analyses using Genescan® / Genotyper® and direct sequencing were found to be more discriminatory than IEA for all populations. The application of Genescan® / Genotyper® to the identification of alleles at these microsatellite loci has not been described previously. Phylogenetic analysis was carried out for each population using enzyme and microsatellite sequence data: phylogenies constructed from multilocus enzyme data were most accurate.

Putative species heterozygotes between *L. V. braziliensis* / *L. V. panamensis* and *L. V. braziliensis* / *L. V. peruviana* were identified in the Nicaraguan and Huánuco populations, respectively, using IEA. Microsatellite analysis identified heterozygous stocks in all 3 populations. This approach also supported the hybrid status of the Nicaraguan and Huánuco stocks. Population genetic analysis of stocks from Huánuco provided statistical evidence for a limited degree of genetic recombination between stocks in this population. Results indicated, however, that clonal expansion was the predominant mode of replication.

To explore the possibility of the occurrence of genetic recombination between species, genetic transformation experiments were initiated using putative parental stocks from the Nicaraguan population

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This thesis is dedicated to the memory of my grandparents, in particular to that of "Big Grandad", Stan Henderson, who died while I was writing-up.

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ε-ACA	ε-amino-caproic acid
ADP	adenosine diphosphate
ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
a.s.l.	above sea level
ble	bleomycin gene
CAE	cellulose acetate electrophoresis
CL	cutaneous leishmaniasis
CMIR	cell-mediated immune response
DAT	direct agglutination test
DCL	diffuse cutaneous leishmaniasis
ddH_2O	deionised distilled water
DGGE	denaturing gradient gel electrophoresis
DHFR	dihydrofolate reductase
diH	di-hydrogen
diNa	di-sodium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EBr	ethidium bromide
EC	Enzyme Commission
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ES	esterase
EST	expressed sequence tag
ETS	external transcribed spacer
FCS	foetal calf serum
F1,6 diP	fructose-1, 6-diphosphate
F6P	fructose-6-phosphate
*gp*63	63 kDa glycoprotein gene
G6PD	glucose-6-phosphate dehydrogenase
G1P + G1,6diP	glucose-1-phosphate + glucose-1,6-diphosphate
GPI	glucose phosphate isomerase
HCl	hydrochloric acid
HIV	human immunodeficiency virus
*hsp*70	70 kDa heat shock protein gene
ICD	isocitrate dehydrogenase
IEA	isoenzyme analysis
IEF	isoelectric focusing
IFAT	immunofluorescence antibody test
IRT	intergenic repeat typing
ITS	internal transcribed spacer (ribosomal RNA gene)
kb(p)	kilobase (pair)
KCL	potassium chloride
kDa	kiloDalton
kDNA	kinetoplast DNA

LB	Luria-Bertani
LDH	lactate dehydrogenase
LSU	large subunit (ribosomal)
MCL	mucocutaneous leishmaniasis
MDH	malate dehydrogenase
ME	malic enzyme
MEM	minimal essential medium
$MgCl_2$	magnesium chloride
$MnCl_2$	manganese chloride
M6P	mannose-6-phosphate
MPI	mannose phosphate isomerase
MTT	3-(4,5-dimethyl triazolyl,1-2)-2,5-diphenyl tetrazolium bromide
4-MUB	4-methylumbilliferyl butyrate
mw	molecular weight
NaCl	sodium chloride
NAD	nicotinamide-adenine dinucleotide
NADH	nicotinamide-adenine dinucleotide, reduced form
NADP	nicotinamide-adenine dinucleotide phosphate
NaOH	sodium hydroxide
neo	neomycin phosphotransferase II gene
NHd	nucleoside hydrolase - deoxyinosine substrate
NHi	nucleoside hydrolase - inosine substrate
NTS	non-transcribed spacer
NW	New World
NWL	New World leishmaniasis
OTU	operational taxonomic unit
OW	Old World
OWL	Old World leishmaniasis
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBSS	proline-balanced salt solution
PCR	polymerase chain reaction
PEP	phosphoenol pyruvate
PEPD	proline immunopeptidase
6PG	6-phosphogluconate
6PGD	6-phosphogluconate dehydrogenase
PGI	phosphate glucose isomerase
PGM	phosphoglucomutase
PKDL	post kala-azar dermal leishmaniasis
pI	isoelectric point
PK	pyruvate kinase
PMS	phenazine methosulphate
PO_4	phosphate
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SL	spliced leader

SSCP	single-stranded conformational polymorphism
SOD	superoxide dismutase
SSU	small subunit (ribosomal)
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TE	tris-EDTA
Tm	melting temperature
TS	thymidylate synthetase
TSGE	thin-layer starch gel electrophoresis
UV	ultraviolet
VL	visceral leishmaniasis
WHO	World Health Organization
UMS	universal minicircle sequence

CHAPTER 1 - INTRODUCTION

1.1 Biology and life cycle of *Leishmania*

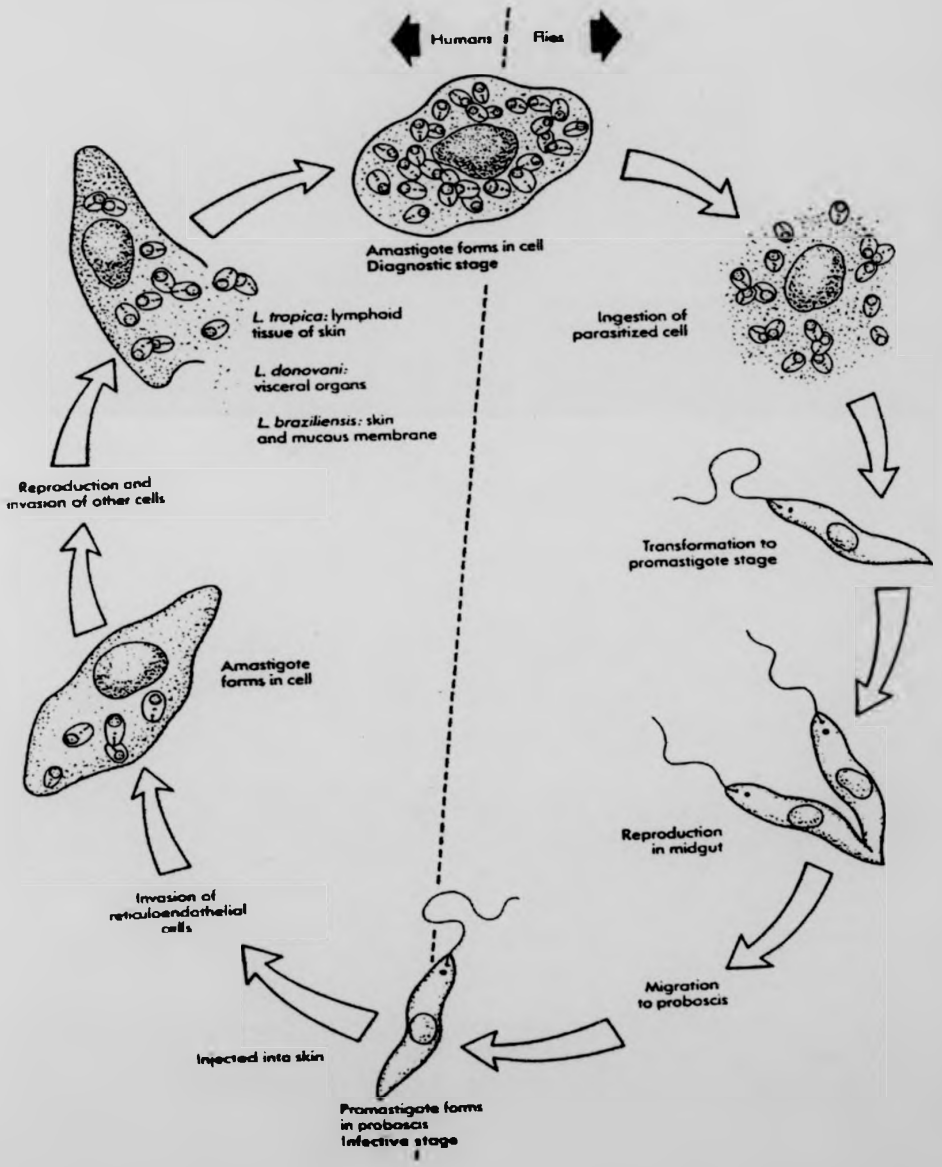
Organisms within the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) are digenetic unicellular parasites which have life cycles alternating between various mammalian hosts and blood-sucking phlebotomine sand fly vectors (Diptera: Psychodidae, Phlebotominae). *Figure 1.1* illustrates the life cycle of the parasite. Promastigotes are transmitted to the mammalian host during the bite of an infected female sand fly. The parasite enters macrophage cells of the host by complement receptor-mediated phagocytosis and transforms into the non-flagellated amastigote stage. The parasite resides inside the phagolysosome by evading the lysosomal degradative pathway and begins to multiply. Multiplication continues until the cell ruptures, liberating amastigotes which are taken up by other macrophages. The cycle is completed when parasites are taken up from an infected host during the blood meal of a sand fly. Amastigotes are released in the fly's stomach and transform into elongated promastigotes. These flagellated forms attach to the hindgut or midgut epithelium (dependent on *Leishmania* species), where they begin to divide rapidly. Promastigotes migrate through the lumen of the midgut into the foregut and pharynx; some transform into highly infective, elongated metacyclic forms. Parasites form a "plug" in the pharynx which is thought to be regurgitated when the fly is probing for blood, thus transmitting the parasite into the next mammalian host (Killick-Kendrick, 1990). Multiplication of the parasite occurs by binary fission in both host and vector: there is, as yet, no known sexual cycle.

1.2 Basic molecular biology of *Leishmania*

Leishmania are kinetoplastid organisms and, as such, are distinguishable from other protozoa by the presence of kinetoplast DNA (kDNA, see section 1.10.1.3), an unusual form of mitochondrial DNA found in the kinetoplast, a structure located near the basal body of the flagellum. Kinetoplastid organisms exhibit many unusual molecular phenomena: a lack of introns, multicistronic transcription, discontinuous transcription and *trans*-splicing, and RNA editing (Borst, 1986; Van der Ploeg, 1986). These phenomena have been extensively studied to provide an insight into the mechanisms of gene expression in these organisms. Several aspects of kinetoplastid biochemistry are also unusual: they carry out glycolysis from glucose to glycerol-3-phosphate

Figure 1.1*: The life cycle of *Leishmania

(adapted from Blaxter, 1987)



in a unique membrane-bound structure termed a glycosome (Opperdoes, 1987), are unable to synthesize purines *de novo*, and have several unique enzymes e.g. trypanothione reductase and pathways e.g. glycolipid biosynthesis. These features have been identified as potential targets for rational drug design since the chemotherapy of diseases caused by these organisms is lacking or unsatisfactory.

1.3 Classification of the *Leishmaniae*

Parasite morphology within the genus *Leishmania* is very similar hence early classifications were based mostly on clinical and geographical differences (Lainson & Shaw, 1987). The application of new molecular techniques (section 1.10) has revealed complex genetic relationships within the genus, adding to the controversial taxonomic status of the group (reviewed by Cupolillo *et al.*, 2000) and to doubts about the reproductive mechanisms involved (see Gibson & Stevens, 1999).

By definition, a species comprises a reproductively isolated population sharing a common gene pool. In organisms such as *Leishmania*, which are considered to exhibit essentially clonal reproduction (Tibayrenc *et al.*, 1990), this definition is hard to demonstrate and other criteria have to be applied. Classifications of *Leishmania* have included criteria such as behaviour in the vector, hamster model and *in vitro* culture, species of sand fly vector and biochemical characteristics such as DNA and isoenzyme analysis (Lainson & Shaw, 1987). Some workers have preferred to use subspecific names when describing different populations in the same area (Lainson & Shaw, 1987). Subspecies are defined as "...local, geographically-isolated populations which show some taxonomic differences from other geographically separated populations of the same species..." (Anonymous, 1985). Members of the subgenus *Viannia* were initially designated as subspecies of *L. braziliensis*, i.e. *L. b. panamensis*, *L. b. guyanensis* and *L. b. peruviana*. Whilst *L. V. braziliensis* and *L. V. peruviana* are very similar in many biological, biochemical and serological characteristics, *L. V. panamensis* and *L. V. guyanensis*, although sharing several features, are less similar. To clarify such discrepancies another phrase, that of the "species complex", was coined (Lainson & Shaw, 1972). Although not recognized as formal taxonomic units, species complexes have now been used in both subgenera to group similar *Leishmania* whose species status may be questionable (see *figure 1.3)*. The classification of *Leishmania* remains controversial despite the introduction of modern techniques and many *Leishmania* researchers disagree over what constitutes a

species. For example, there is very little to differentiate between *L. L. infantum* (OW) and *L. L. chagasi* (NW) and many workers believe that they are the same species (Grimaldi *et al.*, 1987; Rioux *et al.*, 1990). To date there are 30 named species of *Leishmania* (Shaw, 1994): their current classification is shown in *figure 1.3*.

The genus is divided into two subgenera based on the location of parasite development within the sand fly vector. Species in which development is restricted to the midgut and foregut (suprapylarian) belong to the subgenus *Leishmania*, whilst those species in which an additional hindgut developmental phase occurs (peripylarian) belong to the subgenus *Viannia*.

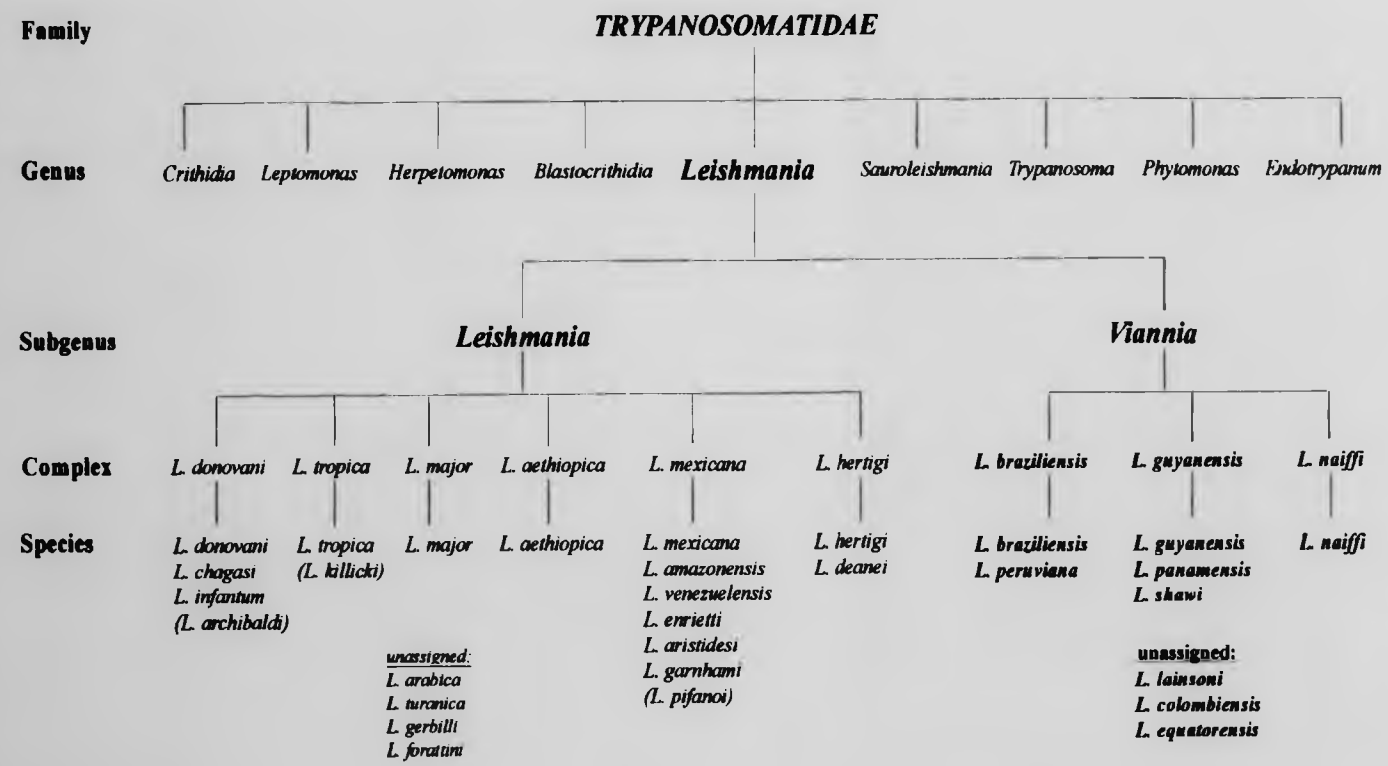
1.3.1 Subgenus *Leishmania*

The subgenus *Leishmania* (Saf'janova, 1982) contains 21 species, 13 of which are known to infect humans; 10 occur in the Old World (OW), and 11 occur in the New World (NW). Important members of this subgenus include: (OW) *L. L. donovani*, *L. L. infantum*, *L. L. tropica*, *L. L. major*, *L. L. aethiopica*; (NW) *L. L. chagasi*, *L. L. amazonensis and L. L. mexicana.*

1.3.2 Subgenus *Viannia*

The subgenus *Viannia* (Lainson & Shaw, 1987), also known by its former name, the *L. braziliensis* complex, contains 7 named species, which are found only in the New World. [Two additional species, *L. (V) equatorensis* (Grimaldi *et al.*, 1992) *and L. (V) colombiensis* (Kreutzer *et al.*, 1991), were originally classified as outlying *Viannia* species but it has recently been proposed that they be reclassified as "paraleishmania" (Cupolillo *et al.*, 2000)]. All of the 7 "true" *Viannia* species infect man causing cutaneous and/or mucocutaneous forms of leishmaniasis. The subgenus is divided into 3 species-complexes: the *L. V. braziliensis* complex (2 species), the *L. V. guyanensis* complex (3 species) and the *L. V. naiffi* complex (1 species). *L. V. lainsoni* remains unassigned at present (Shaw, 1994). *L. V. braziliensis* is the most geographically widespread species, frequently existing sympatrically with other *Leishmania* species. This is unfortunate since *L. V. braziliensis* is also the most important member of this subgenus in terms of clinical disease severity because it is largely responsible for the highly disfiguring and potentially fatal mucocutaneous leishmaniasis (MCL). The species of this subgenus form the basis for this thesis and are discussed further in chapters 3-6.

Figure 1.3: **Classification of the *Trypanosomatidae*** (WHO, 1990; Rioux *et al.*, 1990; Lainson & Shaw, 1994)



1.4 Vectors and reservoir hosts of *Leishmania*

1.4.1 Vectors of *Leishmania*

Leishmania are transmitted by phlebotomine sand flies of the genera *Lutzomyia* (*Lu.*, NW) and *Phlebotomus* (*P.*, OW). Vector species are listed for the different OW and NW *Leishmania* species in *tables 1.4.1* and *1.4.2* respectively. The main criteria for incriminating a particular sand fly species as a *Leishmania* vector are anthropophilic behaviour and repeated identification and isolation of the same parasite from both human cases and the sand fly species in question (Killick-Kendrick, 1990).

Most vector species have definite habitat requirements and exhibit different behavioural characteristics: they are consequently limited in geographical range according to the flora and fauna of, and conditions in, a particular local habitat. Some vector species have distinct host preferences: *Lu. wellcomei*, for example, a vector of *L. V. braziliensis* in Brazil, is highly anthropophilic. Other vector species have a specific ecological niche outside which they are only rarely found. *Lu. umbratilis*, for example, the principal vector of *L. V. guyanensis*, has an arboreal ecology and lives in the canopy of primary forest. Human infections with *L. V. guyanensis* occur primarily in people who have had close association with the forest e.g. workers in deforestation projects. Destruction of this habitat can reduce the incidence of disease caused by *L. V. guyanensis* infection by two related mechanisms. Firstly, deforestation eliminates the reservoir host (sloths) of both parasite and vector as sloths are unable to adapt to other habitats (Shaw, 1994). Secondly, deforestation removes the vector from the transmission cycle by eliminating both its breeding and resting sites (the trunks of large native trees) and its primary food-source, the sloth.

Parasite species may be transmitted by several vector species however, most vector species are usually only responsible for, or capable of, transmitting a single parasite species e.g. *P. papatasi* and *L. L. major*. Some vector species however, e.g. *P. argentipes* and *Lu. whitmani*, can support infection by, and development of, various *Leishmania* species (Pimenta *et al.*, 1994; Rangel *et al.*, 1996). *Lu. whitmani* is a vector of three NW *Leishmania species*: *L. V. braziliensis; L. V. guyanensis* and *L. V. shawi*. *Lu. whitmani* transmits *L. V. guyanensis* and *L. V. shawi* in the primary forest of Amazonian Brazil: the vector in this region is poorly anthropophilic and transmission occurs in a sylvatic habitat. *Lu. whitmani* transmits *L. V. braziliensis* in S. and NE Brazil: the vector in this region is highly anthropophilic and transmission

occurs in a peridomestic / intra-domicilliary habitat. Considering the parasite specificity exhibited by other vector species, it was thought surprising that a single vector species was capable of transmitting different parasites from such disparate habitats. It has recently been proposed that *Lu. whitmani* comprises a species complex where geographically isolated populations have evolved acquiring sufficient differences to be considered separate species (Rangel *et al.*, 1996; Ready *et al.*, 1997, 1998). It is thought that *Lu. whitmani* adapted from a sylvatic niche to that of the peridomestic / intradomicilliary setting (Rangel *et al.*, 1996). Another recent paper reports the coexistence of two *Leishmania* species (one *L. Viannia* sp. and one *L. L. mexicana* complex sp.) in a naturally infected sand fly species, *Lu. ovallesi* (Barrios *et al.*, 1994), a finding that is important to the proposed theory of genetic exchange occurring between *Leishmania* species (see section 1.12).

Considerable sand fly - *Leishmania* species specificity exists in nature. Vector competence is thought to be mediated largely by the ability of *Leishmania* promastigotes to attach to the vector's midgut (Pimenta *et al.*, 1994). The molecule responsible for parasite attachment is lipophosphoglycan, LPG, a surface molecule found almost exclusively on "procyclic" promastigotes. Structural polymorphisms observed in the terminal sugar residues of LPG between *Leishmania* species, in conjunction with variation in vector receptors, are thought to mediate vector-parasite compatibilities (Pimenta *et al.*, 1994; Sacks *et al.* 1995).

1.4.2 Reservoir hosts of *Leishmania*

The reservoir hosts of most *Leishmania* species are non-human mammals which may be domestic, peridomestic or sylvatic. Reservoir hosts may be further subdivided into those which are responsible for maintaining the transmission cycle (primary hosts) and those hosts, in which *Leishmania* infection is also found, thought to be incapable of maintaining the cycle (secondary hosts). Man, generally considered an accidental host of *Leishmania*, can be the only reservoir of infection for some species in a given locale (see below). Reservoir hosts are listed for the different OW and NW *Leishmania* species in *tables 1.4.1* and *1.4.2* respectively.

Reservoir hosts may or may not show signs of infection: sylvatic mammals show few, if any, signs of infection whereas domestic animals such as dogs and equines often have lesions or signs of visceral infection (Marsden, 1994a,b). Additionally, the

localization of *Leishmania* in the reservoir may not correspond to that seen in human disease. For example *L. V. guyanensis*, which causes cutaneous disease in humans, is found in the viscera of the primary host, the two-toed sloth *Choloepus hofmanni* (Lainson *et. al*, 1981). Proving that an infected mammal is a reservoir of a human parasite demands extensive ecological studies including showing that the animal is in close contact with the vector population and sufficiently long-lived to permit transmission of the same parasite species / strain.

1.4.2.1 Domestic and peridomestic reservoir hosts

It is well known that the primary domestic/peridomestic reservoir of VL caused by infection with *L. L. infantum /L. L. chagasi* is the dog (*Canis familiaris*) (Rees & Kager, 1987; Lainson *et al.*, 1994). For other *Leishmania* species however, including *L. V. peruviana* and *L. V. braziliensis*, the dogs' status as a reservoir, although highly suspected, remains to be proven conclusively (Yoshida *et al.*, 1990; Lainson *et al.*, 1994; Davies *et al.*, 1995; Llanos-Cuentas *et al.*, 1999; Reithinger & Davies, 1999). Other domesticated animals e.g. cats (*Felis domesticus*), equines (horses - *Equus caballus* and donkeys - *E. asinus*) may also be infected (Aguilar *et al.*, 1989; Yoshida *et al.*, 1990; Bonfante-Garrido *et al.*, 1991; Passos *et al.*, 1996). Rodents may act as peridomestic or sylvatic reservoirs. The common rat, *Rattus rattus*, has been found to be infected with various *Leishmania* species in both OW and NW (WHO, 1990).

1.4.2.2 Sylvatic reservoir hosts

Known sylvatic reservoirs of *Leishmania* include many species of rodents (including *Heteromys, Nyctomys, Ototylomys, Sigmodon, Akodon, Oryzomys, Proechimys, Rattus, Rhombomys, Meriones, Psammomys, Gerbillus, Arvicanthis*). In addition, many larger mammals are known or implicated as reservoirs: foxes (*Cerdocyon, Vulpes*), the hyrax, jackals, badgers (*Meles*), serval (*Felis serval*), genet (*Genetta*), mongoose (*Helogale*), sloths (edentates: *Choloepus* and *Bradypus* spp.), anteaters (*Tamandua*), armadillos (edentates: *Dasypus* spp.), opossums (marsupials: *Didelphis*), procyonids and monkeys (WHO, 1990).

Table 1.4.1: **Reservoir Host and Vector Species of OW *Leishmania* (WHO, 1990; Desjeux, 1992a)**

Clinical type	Species	Reservoir hosts	Vector species include
OWCL	Sympatric *L. L. major & L. L. tropica*	Dogs (Eg); Rodents (Iq, Pk, FU); Dogs & rodents (Af, In*, Ir, Is, Mo, SA*); Man (SA)	*P. sergenti* (Af, In, Ir, Iq, Is, Mo, SA, FU); *P. papatasi* (Af, In, Ir*, Iq, Is, Mo, Pk, SA, FU); *P. salehi* (In*, Pk); *P. caucasicus* (FU)
	L. L. major	Rodents (Al, Eg, Et*, Ga, Jo*, Ke, Se*, Su, Tu*); Dogs (Se*)	*P. papatasi* (Al, Eg, Jo*, Su, Tu), *P. duboscqi* (Et*, Ga, Ke*, Se*, Su); *P. chabaudi* (Tu), *P. sergenti* (Tu
	L. L. tropica	Man (Af, Gr, In, Ir, Iq, Ke, Pk, SA); Hyrax (Na)	*P. saevus* (Ke); *P. rossi* (Na); *P. sergenti* (Af, Gr, In, Ir, Iq, Ke, Pk, SA)
	L. L. aethiopica	Hyrax (Et*, Ke)	*P. longipes* (Et*); *P. pedifer* (Et*, Ke*)
	L. L. infantum	Dogs (Sp*); Rodents (Sp*)	*P. ariasi* (Fr*, Sp); *P. perfiliewi* (It); *P. perniciosus* (Sp)
OWVL	*L. L. donovani ss*	Man (Ch, In, SA, Su)	*P.chinensis** (Ch); *P. argentipes** (In); e.g. *P. orientalis* (SA, Su)
	L. L. donovani sl	Dogs (Iq*); Jackal (Iq)	inc. *P. alexandri* (Iq)
	Sympatric *L. L. donovani sl & L. L. infantum*	Dogs (Et, It*, Ke); Fox (It*), Mongoose (Ke); Genet (Ke, Su); Serval (Su); Jackal (Su); Rodents (It*, Su)	*P. orientalis* (Et), *P. perniciosus* (It); *P. martini** (Ke); *P. alexandri* (Pk)
	L. L. infantum	Dogs (Al*, Fr*, Ga, Ch*, Eg*, Gr*, Ir, Is*, Jo, Mo, Po, SA, Se*, Sp*, Tu*, FU*) Fox (Fr*, Po*); Jackal (Ir, FU*); Badger (FU*), Rodents	*P. perniciosus* (Al*, Fr*, Po, Sp, Tu); *P. ariasi* (Fr*, Mo, Po, Sp); *P. alexandri* (Ch*); *P. langeroni* (Eg), *P. balcanicus* (Gr*); *P. neglectus* (Gr*); *P. syriacus* (Is, Jo); *P. longiductus* (FU*); *P. halepensis* (Ir), *P. arabicus* (SA); *P. langeroni* (Eg)

Key: Af: Afghanistan; Al: Algeria; Ch: China; Eg: Egypt; Et: Ethiopia; Fr: France; Ga: Gambia; Gr: Greece; In: India; Ir: Iran; Iq: Iraq; Is: Israel; It: Italy; Jo: Jordan; Ke: Kenya; Mo: Morocco; Na: Namibia; Pk: Pakistan; Po: Portugal; SA: Saudi Arabia; Se: Senegal; Sp: Spain; Su: Sudan; Tu: Tunisia; FU: Former USSR. *: proven vector species or reservoir host.

Table 1.4.2: **Reservoir Host and Vector Species of NW *Leishmania* (WHO, 1990; Desjeux, 1992a)**

Clinical type	Species	Reservoir hosts	Vector species include *Lu.: Lutzomyia; Ps.: Psychopydogus.*
NWCL	*L. V. braziliensis*	dogs (Ar, Br*, Pe*); sloths (Br, Pa*); equines (Ar, Br); rodents (Be*, Bo, Br*) opposums (Br*)	*Ps. wellcomei* (Br*); *Lu. whitmani ss* (Br*); *Lu. intermedia* (Ar, Br); *Lu. carrerai* (Bo*, Br); *Ps. complexus* (Br*); *Lu. yucumensis* (Bo*); *Lu. amazonensis* (Br); *Lu. ayrozai* (Br); *Lu. migonei* (Br); *Lu. paraensis* (Br); *Lu. pessoai* (Br); *Lu. spinicrassa* (Co*); *Lu. gomezi* (Ve); *Lu. ovallesi* (Ve); *Lu. panamensis* (Ve)
	L. V. peruviana	Dogs (Pe*)	*Lu. ayacuchensis* (Pe); *Lu. peruensis* (Pe); *Lu. verrucarum* (Pe); Lu.
	L. V. guyanensis	1°: Sloths (Br*, FG*, Su); Anteater (Br*, FG); 2°: Opposum (Br*, FG*); Rodents (Br, FG)	*Lu. umbratilis* (Br*, Co*, FG*, Gy,Su); *Lu. anduzei* (Br*); *Lu. whitmani* (Br);
	L. V. panamensis	Dogs (CR); Rodents (CR*); Sloths (CR*)	*Lu. trapidoi* (Co*, CR*, Ec, Ni, Pa*); *Lu. ylephiletor* (CR*, Pa, Ni, Pa*); *Lu. gomezi* (Ec, Pa*); *Lu. hartmanni* (Ec); *Lu. panamensis* (Pa*)
	L. V. shawi	Monkeys (Br*); Sloths (Br*); Coatimundi (Br*)	*Lu. whitmani sl* (Br*)
	L. V. lainsoni	Rodents (Co, Br*)	*Lu. ubiquitalis* (Br*)
	L. V. naiffi	Armadillo (Br*)	*Ps. squamiventris* (Br*); *Lu. paraensis* (Br*); *Lu. ayrozai* (Br*)
	Other *Viannia* species		*L. V. colombiensis: Lu. hartmanni* (Co); *Lu. gomezi* (Pa); *Lu. panamensis* (Pa)
	L.L. amazonensis	Rodents (Br*, FG*, Ve); Marsupials (Br*); Primates (*Sciuris*) (Ec)	*Lu. flaviscutellata* (Br*, Co*, Ec, FG*, Su, Ve); *Lu. olmeca nociva* (Br); *Lu. olmeca olmeca* (Be*)
	L. L. mexicana	Rodents (Be, Gu, Me)	*Lu. olmeca olmeca* (Be, CR, Gu, Ho, Me*); *Lu. ylephiletor* (Gu)
	L. L. chagasi	Dogs (Ho)	*Lu. longipalpis* (Ho)
NWVL	*L. L. chagasi*	Dogs (Bo*, Br*, Co*, ES, Gu, Ho, Me, Ni, Pa,Ve); Fox (Br*); Opossum (Br, Co)	*Lu. longipalpis* (Ar, Bo*, Br*, Co*, CR, ES, Gu, Ho, Me, Ni, Pa, Pr, Ve); *Lu. evansi.* (Br*)

Key: Ar: Argentina; Be: Belize; Bo: Bolivia; Br: Brazil; Co: Colombia; CR: Costa Rica; Ec: Ecuador; ES: El Salvador; FG: French Guiana; Gy: Guyana, Ho: Honduras; Me: Mexico; Ni: Nicaragua, Pa: Panama; Pe: Peru; Pr: Paraguay; Su: Suriname; Ve: Venezuela. *: proven vector species or reservoir host.

1.4.3 Transmission cycles

Leishmania may be transmitted to humans via a zoonotic or anthroponotic cycle. Which cycle operates depends on the species of parasite, vector and reservoir hosts present in a given ecological niche. Leishmaniasis is generally considered a zoonosis, with man being an accidental host. Anthroponotic transmission of *L. L. donovani sensu stricto* occurs in India (vector *P. argentipes*) and in foci in the Sudan, Saudi Arabia and China (WHO, 1990). Anthroponotic transmission has also been documented for other *Leishmania* species including *L. L. tropica* (WHO, 1990). For this type of transmission cycle, active detection and treatment of cases is essential for control of the disease (see section 1.8). In the NW, despite rare reports to the contrary (WHO, 1990), leishmaniasis is thought to be entirely zoonotic.

Some species of *Leishmania* may exist in either a zoonotic or anthroponotic cycle depending on local conditions. CL due to *L. L. tropica*, for example, is an anthroponosis in parts of Kenya (vector *P. saevus*) whereas in other areas the same disease is strictly a zoonosis involving animal reservoirs such as dogs or rodents. VL due to infection with *L. L. donovani* can also be maintained in a zoonotic or anthroponotic cycle however, in this case, two different forms of this species, *sensu stricto* (anthroponotic) and *sensu lato* (zoonotic), are responsible (WHO, 1990). *Leishmania* species with different types of transmission cycle can overlap in a given area if ecological conditions permit. For example, *L. L. donovani ss.* and *L. L. infantum*, both of which cause VL, occur sympatrically in Saudi Arabia: *L. L. donovani* is maintained in a human-vector cycle whereas *L. L. infantum* is maintained in a dog-vector cycle with man as a secondary host.

The maintenance of *Leishmania*, for a given geographical area and ecological niche, is usually dependent on a small number of vector and reservoir host species. For example, *L. V. guyanensis* is transmitted by *Lu. umbratilis*, *Lu anduzei* and *Lu. whitmani* with sloths, anteaters, opossums and rodents acting as reservoirs. In Kenya, transmission of *L. L. aethiopica* involves just one vector (*P. pedifer*) and reservoir host (hyrax) species. Some *Leishmania* species however, notably *L. V. braziliensis*, seem capable of infecting a wide range of both vector and reservoir host species. *L. V. braziliensis* occurs in almost all central and South American countries and is the most widespread NW cutaneous species. Transmission of *L. V. braziliensis* involves at least 14 vector species (see *table 1.4.2*) and, although the definitive host is not

known, this species has been found in many mammals including dogs, sloths, foxes, equines, rodents and opossums (WHO, 1990). *L. V. braziliensis* appears capable of adapting to different ecological situations using the local fauna as host and vector species. In Huánuco Department, Peru, for example, *L. V. braziliensis* exists sympatrically with *L. V. peruviana* (Dujardin *et al.*, 1995b). The principal vector species (thought to be *Lu. tejadai*) of *L. V. peruviana* in this area has also been strongly implicated in the transmission of *L. V. braziliensis*, which has been recently introduced causing both CL and MCL (Clive Davies, personal comm.).

1.5 Leishmaniasis

Leishmaniasis, the term given to disease caused by *Leishmania* infection, is a collective term covering a wide range of clinical forms. Leishmaniasis is endemic throughout most of tropical and sub-tropical America (New World leishmaniasis - NWL), and across Africa, India, central Asia, Russia, parts of eastern Asia, the Mediterranean basin including several southern European countries (Old World leishmaniasis - OWL). Three hundred and fifty million people are at risk of acquiring *Leishmania* infection. The worldwide prevalence of leishmaniasis is 12 million cases with an annual incidence estimated to be in the region of 1.5-2 million clinical cases, although this is likely to be a gross underestimate as many cases go unreported (WHO, 1990; Liew & O'Donnell, 1993).

Infection with *Leishmania* can result in a spectrum of disease depending upon both parasite species involved and the efficiency of the host's immune response to the parasite (Pearson & Wilson, 1989). The main clinical forms of disease are summarized in *table 1.5.1* for the major parasite species involved.

1.5.1 Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) may present in a number of different clinical forms depending upon the infecting species of *Leishmania* and the genetic background and immune status of the patient. There is, unfortunately, no absolute separation of clinical forms for the different species however, empirical diagnoses have previously been made based on presentation of the lesion(s) coupled with knowledge of *Leishmania* species present in the geographical region in which the infection was acquired (Lainson & Shaw, 1987).

Table 1.5.1: **Clinical manifestations of *Leishmania* infection**

Clinical type	Clinical Presentation	Major parasite species involved
Cutaneous (CL)	Single to multiple lesions which may be wet or dry, crusty or nodular, dependent on infecting species. Secondary infection frequently occurs.	*L. L. mexicana* , *L. L. amazonensis* *L. V. braziliensis*, *L. V. guyanensis* *L. V. peruviana*, *L. V. panamensis* *L. L. major*, *L. L. aethiopica*, *L. L. tropica*, *L. L. infantum* (adults)
Diffuse cutaneous (DCL)	Many diffuse lesions, papular, non-ulcerating.	*L. L. amazonensis*, *L. L. aethiopica*
Mucocutaneous (MCL)	Metastatic spread of parasite from CL ulcer, an early sign is nasal septum perforation. Gross disfiguration occurs later if untreated.	Usually *L. V. braziliensis* ; rarely: *L. V. panamensis* , *L. V. guyanensis*, *L. L. amazonensis*, *L. L. aethiopica*, *L. L. donovani s.l.*
Visceral (VL)	General involvement of the reticulo-endothelial system (spleen, liver, etc.), often with fever, diarrhoea, weight-loss and anaemia	*L. L. donovani* , *L. L. infantum* , *L. L. chagasi*
Post kala-azar dermal (PKDL)	Nodular lesions and depigmented scarring	*L. L. donovani*

The "classical" presentations of the major species, which cause CL, along with their known geographical range, are shown in *table 1.5.2.* Lesions ordinarily start as a papule at the site of the bite, which grows, crusts and then ulcerates. The incubation period, from time of bite to evolution of the lesion, ranges from 2 weeks up to two years. Histopathological observation shows intense chronic granulomatous inflammation. Secondary bacterial and fungal infections at the site of the ulcer are often observed. Healing of CL lesions usually, but not always, signifies a life-long immunity against reinfection with the same species.

1.5.2 Mucocutaneous leishmaniasis

The presentation of mucocutaneous leishmaniasis (MCL) ranges with time from simple nasal perforation to highly disfiguring leprosy-like facial tissue destruction. MCL presents months to years after a primary cutaneous episode (Marsden, 1986). Early lesions commonly involve the nasal septum; the disease then spreads through the palate and may also involve other tissues including the lips, pharynx and trachea. As the disease progresses, devastating consequences including malnutrition and respiratory distress ensue as the result of throat and nasal obstruction. Death, usually due to suffocation caused by obstructed airways or pneumonia, is inevitable without treatment, and this often fails.

***Table 1.5.2*: Characteristic lesion type and geographical range of the major parasite species causing cutaneous leishmaniasis.**

Species	Lesion characteristics	Known Geographical Range
L. L. tropica (OW)	Classical "oriental sore". Painless dry ulcer, which heals spontaneously, often leading to disfiguring scars. Incubation period 2-8 months.	Greece, Turkey, Algeria, Tunisia, Egypt, Ethiopia, Kenya, Morocco Namibia, India, Pakistan, Afghanistan, Kuwait, Lebanon, Israel, Jordan, Iraq, Iran, Saudi Arabia, Syria, Turkmenia, Uzbekistan, Yemen
L. L. major (OW)	Painless wet ulcer with multiple lesions and secondary infection common. Slow to heal, leaving large scars. Incubation period <4 months.	Turkey, Algeria, Burkina Faso, Cameroon, Chad, Cote d'Ivoire, Djibouti, Egypt, Ethiopia, Gambia, Guinea, Kenya, Mali, Mauritania, Morocco, Niger, Nigeria, Senegal, Somalia, Sudan, Tunisia, Afghanistan, China, India, Pakistan, Iran, Iraq, Israel, Jordan, Kuwait, Lebanon, Libya, Pakistan, Saudi Arabia, Syria, Turkmenia, Uzbekistan, Yemen.
L. L. aethiopica (OW)	Simple lesions with late or absent ulceration. Slow healing. Also MCL and DCL.	Ethiopia, Kenya
L. L. infantum (OW)	Simple CL in adults only	France, Greece, Italy, Malta, Portugal, Romania, Spain, Turkey, Azerbaijan Khazakhstan, China, Afghanistan, China, Iran, Iraq, Israel, Jordan, Lebanon, Libya, Pakistan, Saudi Arabia, Syria, U.A.E, Algeria, Burkina Faso, Cameroon, Central African Republic, Chad, Cote d'Ivoire, Egypt, Ethiopia, Gambia, Guinea, Kenya, Morocco, Niger, Nigeria, Senegal, Sudan, Tunisia
L. L. mexicana (NW)	"Chiclero's ulcer". Single benign, self-limiting papule, nodule or ulcer, usually on face and ears. Incubation period <6 months.	Colombia, Belize, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Panama, Trinidad & Tobago, Texas (USA).
L. L. amazonensis (NW)	Also DCL	The Amazon Basin, Bolivia, Brazil, Colombia, Ecuador, Peru, Venezuela, French Guyana, Panama, Paraguay, Suriname.
L. V. braziliensis (NW)	Single or multiple lesions, generally more severe and long lasting. Lymphatic spread common. Spontaneous healing very rare. MCL	Argentina, Bolivia, Brazil, Colombia, Ecuador, Paraguay, Peru, Venezuela, Belize, Costa Rica, French Guyana, Guatemala, Honduras, Mexico, Panama, Nicaragua, Suriname.
L. V. peruviana (NW)	"Uta". One or a few lesions, usually self-limiting, no metastasis. Mostly in children.	Peruvian Andes (Peru, Ecuador)
L. V. guyanensis (NW)	"Pian-bois" or "bush yaws". Multiple ulcerative lesions, frequent metastasis. Spontaneous healing very rare, frequent relapses.	Brazil, Colombia, Peru, Guyana, Surinam, French Guyana.
L. V. panamensis (NW)	"Ulcera de Bejuco". Single or few shallow lesions. Frequent metastasis. Also MCL.	Colombia, Ecuador, Venezuela, Costa Rica, Honduras, Panama, Nicaragua.

It is thought that MCL is due to the reactivation of parasites which metastasized to the oronasal and pharyngeal mucosa via the blood from the site of a primary lesion (Costa *et al.*, 1986; Martinez *et al.*, 1992, Almeida *et al.*, 1996). Metastasis may occur from an active lesion or after a latent period from a deep visceral site (Almeida *et al.*, 1996). Between 8 and 16% of MCL cases have no evidence of a previous clinical infection: it is thought that the initial infection was subclinical in these cases (Marsden *et al.*, 1986; Jones *et al.*, 1987). Early-onset MCL is associated with primary lesions located on the face: it has been suggested that parasites spread directly via the lymphatic system in these cases (Weigle *et al.*, 1986). Predisposing factors for MCL are not clear but may include parasite factors, local trauma or immunosuppression (Marsden *et al.*, 1986; Saravia *et al.*, 1990).

MCL is most commonly associated with *L. V. braziliensis*. In Três Braços, Brazil, during an epidemic involving *L. V. braziliensis*, MCL occurred in around 5% of infected individuals (Marsden, 1994a, b). In Colombia, *L. V. panamensis* commonly causes MCL (Saravia *et al.*, 1985) and cases involving *L. V. guyanensis* and *L. L. amazonensis* have also been reported (Sampaio *et al.*, 1985; Santrich *et al.*, 1990). There have also been sporadic reports of MCL due to *L. L. donovani*, *L. L. infantum* and *L. L. major* (Al-Gindan *et al.*, 1983; Alvar *et al.*, 1990; Ghalib *et al.*, 1992).

1.5.3 Visceral leishmaniasis

Visceral leishmaniasis (VL) is caused by species of the viscerotropic *L. L. donovani* complex: *L. L. donovani; L. L. infantum* (OW) and *L. L. chagasi* (NW). These parasites are not restricted to the skin or mucous membranes but migrate to the spleen, bone marrow and lymph nodes. Common symptoms include abdominal swelling, fever, malaise, weight loss, and diarrhoea accompanied by anaemia and hepatosplenomegaly. Enlargement of the liver is due to hyperplasia of Küpffer cells which are packed with amastigotes: extensive hyperplasia eventually impairs the functioning of the organ. The failure of the host to mount an effective immune response leads ultimately to death, usually as a result of secondary infection, if VL is left untreated. There have been sporadic reports of visceralization of other *Leishmania* species including *L. L. amazonensis* (Barral *et al.*, 1991) and *L. V. braziliensis* (Hernandez *et al.*, 1993; Almeida *et al.*, 1996), but in these cases, the patient was usually immunocompromised.

1.5.4 Other Clinical Presentations

1.5.4.1 Diffuse cutaneous leishmaniasis (DCL)

DCL is a rare, anergic, non-ulcerative form of CL seen in individuals infected with *L. L. amazonensis* (NW) and *L. L. aethiopica* (OW) who have a defective cellular immune response. Such individuals lack a T-cell proliferative response and present a negative intradermal skin test, delayed-type hypersensitivity (DTH), reaction to leishmanial antigens (Bryceson, 1970a; Convit *et al.*, 1972; Barral *et al.*, 1991). The condition starts as a single, non-ulcerative lesion around which satellite lesions develop. This is followed by parasite metastasis to distant areas of the skin with resulting lesions all over the body. DCL progresses slowly and persists for a prolonged period, often life and can easily be confused with those of lepromatous leprosy. DCL lesions do not heal spontaneously and respond poorly to treatment (Bryceson, 1970b; Convit *et al.*, 1972). The characteristic histopathological picture of DCL lesions is of a massive infiltration of vacuolated macrophages filled with amastigotes and a striking lack of lymphocytic infiltration (Bryceson, 1969).

1.5.4.2 Post kala-azar dermal leishmaniasis (PKDL)

PKDL is a condition that generally occurs 6 months to several years following treatment for VL, although in some cases there may be no previous history of leishmaniasis (Rees & Kager, 1987). As visceral infection clears, parasites in the skin become activated, resulting in chronic, non-ulcerative, macular, papular or nodular lesions. Lesions may appear all over the body and face, a presentation which, like DCL, may be confused with leprosy. Lesions appear up to 2 years after treatment; most heal within a year or so leaving depigmented scars, but some may persist for as long as 20 years (Jeronimo & Pearson, 1989). PKDL is most common in the Indian subcontinent and is rare in the New World (WHO, 1990).

1.5.4.3 HIV and *Leishmania* co-infection

There have been many reports of VL associated with HIV infection (Clauvel *et al.*, 1986; Berenguer *et al.*, 1989; Altes *et al.*, 1991). Leishmaniasis is now considered a serious opportunistic complication of HIV infection, especially in Mediterranean countries where up to 75% of VL cases are seen in HIV-infected individuals (Desjeux, 1992b). The clinical presentation of VL in these cases is often unusual with several reports noting the presence of cutaneous lesions (Sanz *et al.*, 1991;

Postigo *et al.*, 1997; Ara *et al.*, 1998). The condition is associated with a high relapse rate and is often refractory to therapy (Alvar *et al.*, 1989). CL has also been associated with HIV infection, although to a lesser extent than VL (Machado *et al.*, 1992). CL in HIV-infected individuals is often aggressive with dissemination and mucosal involvement common (Coura *et al.*, 1987; Machado *et al.*, 1992; Echevarria *et al.*, 1993). It has also been reported that *L. V. braziliensis* infection can present as VL in HIV-infected individuals (Hernandez *et al.*, 1993); presumably the reduction in CD4 count resulting from the progression of HIV infection permits the parasite to visceralize.

1.6 Pathogenesis and immunology

Our understanding of the host response to leishmanial infection stems mostly from studies in experimental models using genetically-defined strains of mice: the most studied *Leishmania* species in this respect are *L. L. major* and *L. L. donovani* (for an overview, see Leiby *et al.*, 1994). Unfortunately, the diseases caused by these two species in experimental systems are not the same as those in humans; hence it is not usually possible to transpose results directly from experimental models to human disease. More recently, the hamster model has been used to study *Leishmania* from the subgenus *Viannia*, particularly with regard to their metastatic capabilities (Martinez *et al.*, 1991; Travi *et al.*, 1996). Despite their limitations, experimental models have provided a growing insight into the immune response and mechanisms of pathogenesis involved in leishmaniasis.

The clinical outcome of a *Leishmania* infection is largely dependent on the response of the host's immune system which itself is largely determined by the host's genetic composition (see section 1.6.1). Other factors are also thought to be important including parasite species, site of inoculation, infecting dose, the presence of acquired immunosuppression (discussed in section 1.6.5) and the sex of the host (Bogdan *et al.*, 1996). The following sections deal mainly with cutaneous and mucocutaneous disease since these forms can result from infection with *Leishmania Viannia* parasites (the subjects of the thesis); visceral disease will be mentioned only briefly.

1.6.1 Genetic regulation of *Leishmania* infection

A number of host genetic factors controlling resistance or susceptibility to *Leishmania* infection have been identified in the mouse model including the *lsh* gene

(Bradley, 1977) and the major histocompatibility complex (MHC)-linked *H-11* gene (Blackwell *et al.*, 1985). Relatively little is known about genetic control of infection in humans: it is highly likely however, that genetically controlled factors play a major role considering the wide spectrum of clinical disease observed. An association between MCL and host HLA-DR type has been shown in Brazilian patients; populations of African ancestry were found to be far more likely to develop MCL than those of indigenous roots (Walton & Valverde, 1979; Petzl-Erler *et al.*, 1991). MCL is known to be accompanied by high circulating levels of tumour necrosis factor alpha (TNFα): recent research has shown that an increased risk of developing MCL is associated with the presence of a specific polymorphism in the promoter region of TNF genes (Cabrera *et al.*, 1995).

1.6.2 Parasite factors involved in disease expression

The importance of parasite determinants on the outcome of *Leishmania* infection has been demonstrated effectively by the ability of various species to cause distinct forms of disease within genetically defined mice. For example, BALB/c mice develop progressive lesions when infected with *L. L. mexicana*, but not with *L. V. braziliensis* (Childs *et al.*, 1984). Similarly in human leishmaniasis, certain disease forms are associated with particular species or species complexes. For example, VL is primarily associated with species of the *L. L. donovani* complex, and MCL is caused by species of the subgenus *Viannia*, notably *L. V. braziliensis*. Parasite factors can be expressed as differences in infectivity, pathogenicity and virulence. The factors involved in parasite virulence and pathogenesis are relatively unknown (Weigle & Saravia, 1996): most work to date has been concerned with infectivity. Factors involved in infectivity include lipophosphoglycan (LPG) (Sacks & da Silva, 1987) and possibly gp63, the major surface glycoprotein (Kweider *et al.*, 1987). The structure and composition of LPG varies amongst *Leishmania species* and between life cycle stages within a species: LPG structure is of paramount importance in the relationship between species, host and vector (Pimenta *et al*, 1994; see section 1.4).

1.6.3 Establishment of infection

Parasites injected during sand fly feeding must be taken up and begin replicating inside a macrophage cell to establish an infection. There is an intense inflammatory response to the sand fly bite and invading parasites: the site becomes infiltrated with large numbers of neutrophils (minutes) and inflammatory macrophages (hours). The

majority of injected promastigotes are killed by lytic serum components and oxidative metabolites produced during the respiratory burst from these inflammatory cells. Some promastigotes are able to survive this initial extracellular attack and are actively phagocytosed by dermal macrophages and Langerhans cells. The surviving parasites are thought to be metacyclic forms which have an LPG-thickened glycocalyx which can withstand complement-mediated lysis (Sacks *et al.*, 1995). It has recently been shown that components of sand fly saliva also interfere with extracellular parasite killing (Titus & Ribeiro, 1988; Hall & Titus, 1995).

Once inside the phagocytic cell, the parasite transforms into the amastigote stage and rapidly begins to divide. The parasite resides inside the phagolysosome and is able to resist destruction by the antimicrobial arsenal of the cell (i.e. oxygen and nitrogen metabolites, low pH and cationic proteins). There are several suggested mechanisms for intracellular survival including the modulation of cytokine production (Barral-Netto *et al.*, 1992; Proudfoot *et al.*, 1995), down-regulation of the macrophage response to external cytokines (e.g. Jardim *et al.*, 1995) and selection of immunologically quiescent macrophage subpopulations e.g. Langerhans cells, Kupffer cells (Locksley *et al.*, 1988; Leiby *et al.* 1994).

The vigorous response to infection recruits additional inflammatory macrophages to the site which become new host cells for amastigotes released from ruptured cells. Infection of inflammatory macrophages can result in localization, which ensures a continuous supply of new host cells to the site (dermatotropic species, e.g. *L. L. major*), or transportation to regional lymph nodes and visceral organs (viscerotropic species, e.g. *L. L. donovani*). The infection is thus established: what the immune system does next is crucial to the outcome of infection.

1.6.4 Immune response of the host

The humoral response is not thought to perform a crucial role in leishmaniasis (Bogdan *et al.*, 1996). The resistance to clinical disease and recovery from infection are dependent on protective T cell responses, particularly through the actions of cytokines produced by $CD4^+$ T cells (Liew *et al.*, 1982; Russo *et al.*, 1993). There are two types of $CD4^+$ T cell response, Th1 and Th2, resulting in the production of two different cytokine profiles. The Th1 or Th2 T cell response correlates with resistant or susceptible phenotype in genetically defined mice (Scott, 1989). The Th1

response is characterized by the production of gamma-interferon (IFNγ), interleukin (IL)-2 and IL-12. These cytokines control parasite multiplication non-specifically by activating NK and γ/δ T cells and increase the expression of MHC class II molecules to augment antigen presentation. Production of IFNγ early in the course of infection favours the production of a Th1 cell response and protection against disease (Scott, 1991). The Th2 response is characterized by the production of IL-4, IL-5, IL-6 and IL-10, cytokines which are involved in the regulation of humoral immune responses. The Th2 response is correlated with disease severity in humans (Ghalib *et al.*, 1993); IL-4, for example, which has a role in the inhibition of IFNγ-mediated macrophage activation, has been shown to predominate in MCL (Pirmez *et al.* 1993). The cytokines secreted as a result of each Th cell response positively select for the continued expansion of their Th cell population via feedback loops. For example, the Th2 cytokine IL-10 is responsible for decreased antigen presentation by macrophages and for the inhibition of cytokine production by Th1 cells and macrophages (Fiorentino *et al.*, 1991).

The general consensus is that T cells conferring protective immunity primarily belong to the $CD4^+$ subset however, there is recent evidence to suggest that cytotoxic $CD8^+$ T cells also play an important role (Barral-Netto *et al.*, 1995). $CD8^+$ cells have been shown to be important in conferring protective immunity against *Leishmania* infection in BALB/c mice lacking $CD4^+$ T cells (Hill *et al.*, 1989) and increased numbers of *Leishmania*-specific $CD8^+$ T cells are associated with clinical cure in human CL (Smith *et al.*, 1991).

1.6.5 Infection and clinical disease

1.6.5.1 Asymptomatic infection

The occurrence of inapparent infections has been increasingly recognized in recent years (Weigle *et al.*, 1993; Davies *et al.*, 1995). In a Venezuelan study of individuals living in an endemic area, approximately 12% of individuals without lesions or scars demonstrated a positive delayed type hypersensitivity (DTH) response to leishmanial antigen, i.e. showed evidence of a prior infection (Ramirez & Guevara, 1997). Asymptomatic infections are, presumably, associated with a strong, appropriate immune response such that the host is able to control and kill invading parasites before an infection can become established. The presence of asymptomatic infection

is revealed in patients who have never had leishmaniasis but who develop the disease after becoming immunosuppressed (see section 1.5.4.3). The clinical manifestation in these patients is often more severe than in immunocompetent individuals.

1.6.5.2 CL and MCL

Uncomplicated CL in humans is characterized by a strong cell-mediated immune response which is detectable *in vivo* as a strong DTH response to leishmanial antigens (Ridley *et al.*, 1980). Chronic CL and MCL are also characterized by profound DTH responses (Saravia *et al.*, 1989), again detectable *in vivo* as an elevated antigen-specific DTH response. In MCL, there is evidence of previous CL in the majority of patients (Marsden, 1986; Jones *et al.*, 1987). The immunopathology seen in MCL results from chronic DTH, an overactive cellular response to leishmanial antigens. Simple, self-healing CL is associated with a Th1 type response whereas a Th2 type response is observed in chronic, non-healing CL and MCL (Pirmez *et al.*, 1993; Melby *et al.*, 1994).

1.6.5.3 DCL

DCL presents the other end of the immunological spectrum to MCL in that it is characterized by antigen-specific anergy. Patients with DCL are DTH negative and their T cells respond poorly to leishmanial antigens (Bryceson, 1970a; Convit *et al.*, 1972; Barral *et al.*, 1991). Successful treatment of DCL results in the recovery of the cellular immune response (Convit *et al.* 1993).

1.6.5.4 VL

Pathological changes in VL are dominated by the specific suppression of cell-mediated immunity that permits the dissemination and expansion of the parasite population. There is also a marked humoral response in the form of a proliferative polyclonal or monoclonal B-cell response to parasite and non-specific antigens. VL is characterized by a negative DTH skin response during infection and hyper-gammaglobulinaemia. VL is associated with a depressed Th1 immune response: there are decreased numbers of CD4+ T cells, an impairment of gamma-interferon (IFNγ) production (Carvalho *et al.*, 1985) and high levels of the Th2 cytokines, IL-4 and IL-10 are observed (Ho *et al.*, 1992).

1.6.6 Resolution of disease

Healing of CL, characterized by re-epithelialization accompanied by a clear reduction in inflammatory cells, normally results in a characteristic scar. CL may heal spontaneously or be cured by therapeutic intervention. Spontaneous healing is associated with less virulent *Leishmania* species such as *L. L. major*, *L. L. tropica*, and *L. L. mexicana* (Herwaldt *et al.*, 1992; Convit *et al.*, 1993). Clinical disease caused by infection with the more pathogenic and virulent species, e.g. *L. V. braziliensis*, heals spontaneously only rarely (Marsden *et al.*, 1986). The resolution of clinical disease has several outcomes dependent on whether or not the patient has acquired a protective immune response and whether they harbour persistent parasites (Weigle & Saravia, 1996). Only those individuals who are parasite-free and have acquired immunity will resist subsequent infection.

1.6.6.1 Acquired immunity

The majority of new *Leishmania* cases in endemic areas (NW) are seen in children or young adolescents (Weigle *et al.*, 1993; Davies *et al.*, 1995) and most people who present with CL endure only one episode of the disease (Weigle & Saravia, 1996). These observations suggest that immunity against disease is acquired following infection, and that this immunity develops with time. Resolution of infection with some species, notably *L. L. major*, is associated with lifelong immunity to reinfection, an observation which has been exploited by using this species for vaccination purposes (Modabber, 1990). Clinical resolution does not, however, always result in the acquisition of protective immunity against reinfection or recurrent disease as demonstrated by the high frequency of relapse in some individuals. Immune status following NWL is often more difficult to assess than in OWL due to several factors: there are commonly several species and strains of *Leishmania* circulating in an epidemic area; reinfection and/or recurrent disease are often observed and metastasis is a common feature of many NW species (Weigle & Saravia, 1996). Experiments in the hamster model have shown that protective immunity elicited against *Leishmania Viannia* species is only partial and against homologous strains (Osorio *et al.*, 1998).

Cell-mediated immune responses are more important in acquiring immunity to *Leishmania* infection than humoral antibody responses. Acquired immunity against infection can be transferred by T cells but not B cells (Scott *et al.*, 1986) and resistant mice rendered T cell-deficient by thymectomy and irradiation are virtually unable to

control *L. L. major* infection (Preston *et al.*, 1972). In mice, the immune response to infection with some species of OW *Leishmania* can be shown to be lost progressively with time (Neal *et al.*, 1990). It has been suggested that the continued presence of antigen is necessary for maintaining T cell memory (Moll *et al.*, 1995). This has implications for vaccine design, inferring that a live attenuated vaccine, which allows persistence of the parasite and therefore antigen, may be the best approach.

1.6.6.2 Parasite persistence and metastasis

Persistence refers to the continued presence of parasites after clinical cure. Metastasis is the dissemination of parasites to body sites other than the inoculation / lesion site. The capacity for metastasis and parasite persistence are closely related: those parasites which are known to persist e.g. *L. V. braziliensis* and other species of the subgenus *Viannia*, are also capable of metastasizing (Almeida *et al.*, 1996) and are highly associated with recurrent disease.

Presumably, persistent parasites evade the host immune response by the choice of host cell and by interfering with its activity. Mononuclear phagocytic cells, including macrophages, are most likely the only host cells able to support the replication of *Leishmania*. These cells are also the effectors of dissemination which may occur via the lymphatic or vascular circulatory systems. Sites to which parasite metastasis occur are also potential sites of parasite persistence. Dermatotropic *Leishmania* have been observed in lymph nodes; it is thought that Langerhans cells which acquire *Leishmania* in the skin migrate to these sites in response to inflammatory cytokines (Moll *et al.*, 1993). Macrophages and blood monocytes are responsible for disseminating parasites via the blood system: *Leishmania* have been demonstrated in the peripheral blood (Martinez *et al.*, 1992), spleen (Magill *et al.*, 1993; Almeida *et al.*, 1996) and bone marrow (Barral *et al.*, 1991). *Leishmania* have also been isolated from the scars of healed CL lesions (Aebischer, 1994; Osorio *et al.*, 1998) and parasite DNA has been demonstrated in blood samples from and scars of patients cured many years previously (Guevara *et al.*, 1993; Schubach *et al.*, 1998).

Individuals harbouring persistent parasites may remain asymptomatic however the continued presence of parasites render the host disposed to relapse or parasite metastasis (Saravia *et al.*, 1990; Aebischer, 1994; Osorio *et al.*, 1998). *L. V. braziliensis* and other members of the *Viannia* subgenus are often associated with

relapsing infection (Saravia *et al.*, 1990); *L. L. donovani* and *L. L. tropica* are also implicated (Aebischer, 1994). *Leishmania* are able to persist, after spontaneous resolution or successful chemotherapeutic treatment, despite the presence of an intact cellular immune response of the host. This is evident from reports of individuals with a previous history of leishmaniasis who develop the disease after moving to a non-endemic area (Guevara *et al.*, 1994), and from the increasing number of reports of leishmaniasis occurring in patients, many of whom were unaware of their infection, who subsequently become immunosuppressed by concomitant HIV infection (Coura *et al.*, 1987; Altes *et al.*, 1991; Machado *et al.*, 1992; Echevarria *et al.*, 1993).

1.6.6.3 Recurrent infection

Recurrent leishmaniasis may occur as a result of reinfection or due to the reactivation of persistent parasites. It is difficult to discriminate between reinfection or reactivation unless it is possible to separate the parasites causing primary and secondary infections. It would be relatively easy, for example, to distinguish an infection caused by *L. L. mexicana* from a previous infection caused by *L. V. braziliensis* since these two species can easily be separated by a number of phenotyping and genotyping methods. This is not the case, however, for the *Viannia* species *L. V. peruviana* and *L. V. braziliensis* which are very difficult to separate. It has been noted that relapsing lesions usually occur close to the site of the original infection (Saravia *et al.*, 1990), are smaller and contain fewer parasites than primary lesions (Gutierrez *et al.*, 1991), and usually occur within a year of healing of the previous lesion (Saravia *et al.*, 1990; Davies *et al.*, 1995).

In a Colombian study of 24 patients with recurrent disease caused by *Leishmania Viannia* species, genotypic and phenotypic analyses of parasites recovered from primary and secondary lesions showed that 50% of infections were due to reactivated parasites and that 50% were reinfections (Saravia *et al.* 1990). Population-based studies in Colombia and Peru have shown that the frequency of recurrent lesions in individuals with evidence of past CL were 2.0 and 2.9 per 100 person years respectively (Weigle *et al.*, 1993; Davies *et al.*, 1995). The frequency of reactivation is determined by several factors including *Leishmania* species, immune status of the host and any drug therapy given (Davies *et al.*, 1995). Reactivation of persistent *Leishmania* may be precipitated by local trauma (Rab *et al.*, 1992; Travi *et al.*, 1996) and is also observed in patients whose cellular immune system becomes suppressed

as a result of i) concomitant infections e.g. tuberculosis (Escobar *et al.*, 1996) and HIV (e.g. Machado *et al.*, 1992; Berhe *et al.*, 1995), ii) treatment for cancer or transplantation (Shaw *et al.*, 1976; Golino *et al.* 1992) and iii) coincidental conditions e.g. malnutrition (Walton & Valverde, 1979; Badaro *et al.*, 1996) and diabetes (Rocha *et al.*, 1980).

1.7 Diagnosis of *Leishmania* infection

1.7.1 Clinical diagnosis

Leishmaniasis may be suspected in individuals living in, or with a history of having traveled to, known endemic areas who present with cutaneous or mucosal lesions or with signs and symptoms of visceral infection (see section 1.5). *Leishmania* infection may present in a wide spectrum of clinical forms and symptoms often overlap with those of other diseases. For example, differential diagnoses of NWCL include tropical ulcer, eczema, impetigo, sporotrichosis, blastomycosis, syphilis, leprosy, yaws and cutaneous tuberculosis. Other diagnoses must be ruled out. A definitive diagnosis of *Leishmania* infection requires demonstration of the parasite by microscopical and/or culture techniques. This parasitological diagnosis may be combined with the use of serological or molecular-based tests, or both.

1.7.2 Parasitological diagnosis

A parasitological diagnosis of leishmaniasis involves demonstrating amastigotes in clinical material by preparing smears or touch preparations, or by isolation of parasites from this material using *in vitro* or *in vivo* cultivation methods. For CL and MCL, clinical material is usually obtained by punch biopsy, slit-skin or needle aspiration of the lesion. Samples must be taken from the inflamed, swollen edge of the lesion as this region contains most parasites. The lesion and surrounding area must be meticulously prepared by cleaning with 70% ethanol and, if secondary infection is evident, with 20% by volume hydrogen peroxide. For VL, splenic, liver, bone marrow or lymph node aspirates are taken. Whichever method of sampling is used, smears or touch preparations are then made, air dried and stained with Giemsa. Amastigotes may be found singularly or in groups inside or around macrophages. Amastigotes are 2-3μm in diameter, contain a characteristic kinetoplast and appear identical microscopically for all species of *Leishmania*. In some instances it is important to identify the parasite species and so isolation must also be attempted.

This is usually when infection with *L. V. braziliensis* is suspected: identification is important owing to *L. V. braziliensis*' potential for causing subsequent MCL. Isolation is usually attempted in any case, since microscopy often fails to reveal parasites, especially in MCL and VL cases where amastigotes numbers are usually insignificant.

For parasite isolation, material is inoculated into medium (*in vitro* culture) and / or into animals, usually hamsters (*in vivo* culture). Cutaneous parasites of the subgenus *Viannia* are very fastidious and require complex media including blood or foetal calf serum. Antibiotics must also be included in the medium to prevent parasites being overgrown with contaminating bacteria. The *in vitro* isolation procedure is very demanding and some laboratories, mostly in the NW, also routinely inoculate hamsters for *in vivo* culture of *Viannia* parasites. There are many limitations to isolation procedures: cultivation is usually slow (weeks), *in vitro* culture is highly prone to contamination and not all strains are cultivable.

1.7.3 Serological diagnosis

Serological assays provide a means of diagnosing leishmaniasis by characterizing and measuring the specific immune responses produced as the result of infection. Serological assays include skin testing (Leishmanin or Montenegro test), which detects the cell-mediated immune reaction (CMIR) to the parasite and other assays, i.e. the indirect immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and the direct agglutination test (DAT) which detect the humoral antibody response to infection.

1.7.3.1 Skin testing

The skin test (Leishmanin or Montenegro) demonstrates past or present exposure to leishmanial antigen (not species-specific) by testing the immune response of an individual to an intradermal injection of cultured promastigotes. The test is read, by measuring the presence and size of any resulting induration, after 48-72 hours. The skin test becomes positive 1-3 months after natural infection and remains positive for life. Hence a positive skin test from an individual living in an endemic area does not necessarily indicate an active infection. Conversely, some individuals (especially those progressing to DCL) present false negative results (Marsden, 1994b). Cross-reactions have been reported with leprosy, tuberculosis and some fungal infections.

Skin tests are used to aid the diagnosis of CL and MCL, but are of little use in the diagnosis of VL because the test is normally negative during infection, becoming positive only after the development of cellular responses, which occur after recovery.

1.7.3.2 Antibody detection methods

Antibody detection methods, including IFAT, ELISA and DAT, are of most use in the diagnosis of VL. No single test is without problems and which test is used depends on the laboratory. Wide variations in test specificities and sensitivities occur depending on the area's disease prevalence, species of parasite involved and immune status of the individual. A major problem for all of these assays is antigen production which requires bulk culture of *L. L. donovani* promastigotes and rigorous standardization of the prepared antigen against reference sera (both positive and negative, of known titre).

IFAT is the quickest of the three assays to perform and detects active infection earlier than DAT or ELISA. The disadvantages of the IFAT technique are that interpretation can be difficult and cross-reactivity with malaria and trypanosomiasis. IFAT is, however, a useful assay for monitoring relapses since the antibody response which it detects becomes negative 6-9 months after cure.

The ELISA (Hommel *et al.*, 1978) is performed in antigen-coated microtitre plates and can be carried out for serum, plasma or blood spots. Dot-ELISA (Pappas *et al.*, 1983), a modified protocol of ELISA, employs small quantities of parasite antigen dotted onto nitrocellulose paper and is suitable for whole blood, serum or plasma samples. In both of these assays, antibody is detected following the addition of sample using enzyme-conjugated anti-IgG and then substrate. The dot-ELISA has been found to be more sensitive than the standard ELISA (Sumam *et al.*, 1993). In addition, it is considerably cheaper since it does not require a spectrophotometer. Both techniques are very useful for epidemiological applications. Cross-reactivity with *Trypanosoma cruzi* has been reported for both formats however, the assays remain valuable for the diagnosis of OWVL since *T. cruzi* infection is unlikely in OW areas (Sumam *et al.*, 1993).

The main advantages of DAT (Harith *et al.*, 1986) are its simplicity, ease of interpretation and the stability and cost of equipment and reagents. The test can be performed on serum, plasma, blood spots and whole blood. The assay has been

shown to be highly specific however it has shown low sensitivity in some studies (Zijlstra *et al.*, 1992).

Many alternative serological approaches are being developed and tested to circumvent the limitations of the current diagnostic methods. These include the detection of specific IgE which has recently been shown to be an indicator of active VL (Atta *et al.*, 1998), and an ELISA based on the gene B protein (GBP) from *L. L. major* for diagnosis of OWCL (Jensen *et al.*, 1996). The most promising advances however, have come from the use of defined recombinant antigens, in particular, rK39 (Badaro *et al.*, 1996). rK39 is an amastigote antigen specific to organisms of the *L. L. donovani* complex. A recent study of Sudanese VL found that an ELISA using rK39 as antigen (rK39-ELISA) was more sensitive than DAT in detecting infection; the rK39-ELISA was positive 6 months earlier than DAT in 40% of cases (Zijlstra *et al.*, 1998). Another recent study in Turkey found the rK39-ELISA to be more sensitive than a combination of microscopy and culture methods for the diagnosis of VL (Ozensoy *et al.*, 1998). In addition, the rK39-ELISA also identified 6/83 cases of parasitologically-proven CL from the region. The rK39-ELISA has also been shown to be very useful as a rapid screening assay for canine leishmaniasis (Ozensoy *et al.*, 1998).

1.7.4 Molecular diagnostic methods

The potential of molecular-based assays offering rapid diagnosis of *Leishmania* infection with exquisite sensitivity and specificity has long been recognized. Despite more than a decade of research, however, molecular assays remain, for reasons some of which are outlined below, tools of the research or reference laboratory. Molecular techniques are discussed further in section 1.10.

Early molecular assays focused on the development of DNA probes capable of detecting *Leishmania*-specific kinetoplast DNA (kDNA) sequences (Wirth & Pratt, 1982; Barker & Butcher, 1983; Lopes & Wirth, 1986). Molecular probes based on repetitive genomic DNA sequences including the mini-exon gene and ribosomal RNA (rRNA) spacer region have also been developed (Uliana *et al.*, 1991; Guevara *et al.*, 1992; Van Eys *et al.*, 1992; Hassan *et al.*, 1993; Guizani *et al.*, 1994). Although molecular probes have been shown to be capable of detecting and typing *Leishmania*, the hybridization technique suffers from low sensitivity and hence probes are not

generally useful for diagnostic purposes (Degrave *et al.*, 1994a). In addition, early assays required the use of radioactivity which precluded their use in many laboratories. The problems of low sensitivity and the use of radioactive detection methods have been addressed for the probe Lmet2 (Howard *et al.*, 1991) which is specific for the *L. L. donovani* complex. Initially used in an assay involving radioactivity (Howard *et al.*, 1991), Lmet2 was subsequently incorporated into a non-radioactive chemiluminescent system (Wilson *et al.*, 1992) and a more sensitive colorimetric PCR assay (Qiao *et al.*, 1995).

With the advent of the polymerase chain reaction (PCR) and the availability of more sequence data, highly promising systems have been developed for both detection and subsequent typing of *Leishmania* parasites. Assays have been developed for amplification of *Leishmania* genus-, subgenus-, complex- and species-specific sequences from kinetoplast or genomic targets including, for example, kDNA minicircles, mini-exon, gp63 and ribosomal RNA genes (de Bruijn. & Barker, 1992; Van Eys *et al.*, 1992; Uliana *et al.*, 1991; Guevara *et al.*, 1992; Hassan *et al.*, 1993; Guizani *et al.*, 1994). PCR technology has eliminated the former problems of lack of sensitivity, permitting rapid detection of parasites in clinical samples. The exceptional sensitivity of this approach also permits the use of less invasive sampling methods. Despite the advantages of speed, sensitivity and specificity, PCR-based assays suffer several major disadvantages. PCR requires dedicated facilities, trained staff and a reliable power supply. The technique is expensive both in terms of equipment and reagents (although possibly less expensive than *in vitro* culture methods which are necessary for conventional typing methods, see section 1.7.2). Cost-aside, perhaps the biggest drawback to PCR-based assays is the false positive result. Changes in PCR conditions (for example reagent or sample quality, or even the ambient temperature of the laboratory) may result in lower sensitivity and specificity. False-positive results may occur due to contamination from other samples or "amplicons" in the laboratory. PCR-based assays can also be over-sensitive for the purpose of leishmaniasis diagnosis: there have been several recent reports of *Leishmania* DNA being detected from "cured" cases or inapparent infections (Guevara *et al.*, 1993, 1994; Davies *et al.*, 1995; Schubach *et al.*, 1998). False negatives have also been reported: these may be due to the presence of PCR inhibitors in clinical samples (e.g. haemaglobin in blood) or due to the lack of parasites for example in a badly taken or unsuitable clinical sample (Degrave *et al.*, 1994a). Many

of these drawbacks can be overcome by standardizing protocols, separating the preparation and analysis of samples and by employing positive and negative controls. False positives can also be eliminated using probe hybridization detection on the PCR products, however, this significantly increases the time and cost of the assay.

Considering the important potential advantages of PCR-based diagnostic methods for leishmaniasis, research is continuing into the development and evaluation of PCR-based assays involving alternative target sequences and novel methodologies such as nested PCR, multiplex PCR and random amplified polymorphic DNA (RAPD) (Hassan *et al.*, 1993; Lopez *et al.*, 1993; Piarroux *et al.*, 1994; Degrave *et al.*, 1994b; Rodriguez *et al.*, 1994; Cupolillo *et al.*, 1995; Noyes *et al.*, 1996; Harris *et al.*, 1998).

1.8 Treatment of leishmaniasis

1.8.1 Antileishmanial drugs

The mainstays of chemotherapy for all forms of leishmaniasis (except DCL) are pentavalent antimonial (SbV) compounds. Two similar compounds are available: meglumine antimoniate (Glucantime®: Rhone-Poulenc, France) and sodium stibogluconate (Pentostam®: Wellcome Foundation, U.K.). Glucantime® contains 8.5% SbV and is used in French-, Spanish- and Portuguese-speaking countries; Pentostam® contains 10% SbV and is used in English-speaking countries. Both Glucantime® and Pentostam® are highly toxic: common minor side-effects include anorexia, nausea, vomiting, malaise, myalgia, and lethargy. Cardiac arrhythmias and renal damage are serious side effects of SbV treatment and indications for stopping therapy. Treatment failure and clinically resistant isolates are common. For recent reviews on chemotherapy of the leishmaniases, see Berman (1997) and Croft *et al.* (1997).

Second-line drugs for leishmaniasis include the anti-fungal polyene antibiotic, amphotericin B (Fungizone), the aromatic diamidine, pentamidine isothiocyanate and the broad-spectrum antiparasitic aminoglycoside paromomycin (aminosidine). Amphotericin B is currently recommended as a second line of treatment for VL and MCL (WHO, 1990). The use of early amphotericin B formulations was restrained due to a low therapeutic index, acute toxicity and infusion-associated side effects. New formulations however, notably liposomal amphotericin B (e.g. AmBisome), have been shown to be effective in the treatment of VL, with lower toxicity than earlier

formulations (Coukell & Brogdan, 1998). Liposomal formulations have been used for the treatment of both CL and MCL (Llanos-Cuentas *et al.*, 1991; Marsden 1994b) however their efficacy has not been proven conclusively (Yardley & Croft, 1997). In some areas, pentamidine administered in a low-dose / short course regimen has been proven to be highly efficacious in the treatment of CL, and can be used as an alternative first-line drug or for SbV treatment failure (Berman, 1997). The pentamidine regimen for VL, however, consists of a long course at high-dosages; at these levels, toxicity is thought to be greater than for SbV regimens hence pentamidine is used only when SbV treatment failure is common and where pentamidine resistance has not been reported (Berman, 1997). Paromomycin has some efficacy in the treatment of VL, but appears more efficacious when combined with short-course SbV regimens (Chunge *et al.*, 1990; Seaman *et al.*, 1993). This combination has also been used successfully in the treatment of OW DCL (Teklemariam *et al.*, 1994). Other drugs are being tested in trials including ketoconazole and itraconazole. Immunotherapeutic approaches are also being tried using recombinant human interferon-gamma (IFNγ) and interleukin-12 (IL-12) (for review, see Berman, 1997).

1.8.2 Treatment of CL

There is no established treatment for CL that applies to all species and all geographical areas. The "standard regimen" for the treatment of CL (WHO, 1990) is intramuscular (im) or intravenous (iv) SbV (20mg/kg/day X20 days). This regimen is not always applicable for reasons of toxicity and cost i.e. in areas or for species where lesions have a tendency to heal spontaneously. For example the relatively benign, self-healing lesions caused by *L. L. major* do not usually require treatment (WHO, 1990). A low-dose SbV regimen (im or iv, 5mg/kg/day X20 days), which produces fewer side effects without an increase in relapse rate, is gaining widespread use for NWCL (Oliveira-Neto *et al.*, 1997a). Many other therapeutic approaches are under continuous evaluation with varying degrees of success. These include: intralesional injection with SbV; topical ointments including paromomycin-methyl benzochloride (MBCl) and glyceryl trinitrate (GTN); and new drugs including itraconazole (Berman, 1996; Dogra & Saxena, 1996; Oliveira-Neto *et al.*, 1997b; Zeina *et al.*, 1997).

OW regimens include intralesional SbV (repeated 1-2X at 1-2 day intervals) for simple lesions (*L. L. major*, if necessary, and *L. L. tropica*) or im SbV 10-20mg/

kg/day until 1 week after parasitological cure for more severe lesions (*L. L. tropica*). *L. L. aethiopica* is unresponsive to SbV therapy; for CL either leave untreated (not oronasal lesions) or use pentamidine (3-4mg/kg 1-2X/week for one month). For DCL, pentamidine is used (3-4mg/kg once/week until 4 months after parasitological cure) or, alternatively, amphotericin B (10mg/kg/day up to 8 weeks), administered by slow iv infusion (WHO, 1990; Berman, 1997). Physical treatments are also employed, mostly for simple CL (i.e. excluding infections with *L. L. aethiopica*, *L. L. amazonensis* and *L. V. braziliensis*), including localized heating and freezing, curettage and radiation (Grenz rays - long wavelength X-rays) (WHO, 1990).

For NWCL, CL resulting from *L. V. braziliensis* infection is treated with SbV (im or iv; 5-20mg/kg/day for a minimum of 4 weeks). In South American countries, if species typing is not available, all cases of CL should be treated in areas where *L. V. braziliensis* is known to occur owing to the possible development of MCL. In Central America, MCL is very rare (Weigle & Saravia, 1996) and lesions resulting from *L. V. braziliensis* infection often self-heal, hence simple CL is commonly left untreated. For lesions caused by other *Leishmania Viannia* species, which regimen is used depends on the area and clinical disease presentation therein. For *L. L. amazonensis* infections, treatment is as for *L. V. braziliensis*, to prevent the development of DCL associated with infection by this species (Berman, 1997). *L. L. mexicana* lesions do not generally require treatment except if they occur on the ear (Chiclero's ulcer), where they tend to be persistent (WHO, 1990).

1.8.3 Treatment of MCL

Marsden (1994b) recommends SbV for MCL (iv, 20mg/kg/day for at least 30 days often up to 90+ days). Parasitological cure is very difficult to monitor due the paucity of parasites in the lesion and treatment tends to be monitored serologically (Marsden, 1994b; Berman, 1997). Failure to respond to SbV is an indication for using amphotericin B (1mg/kg for 20-40 doses or AmBisome), pentamidine (4mg/kg /48hours for 10-30 days) or paromomycin. Relapse and recurrence are documented for all regimens (Marsden, 1994b). Treatment can cause severe localized tissue inflammation which can be life-threatening in itself, especially in more advanced MCL, so treatment must be carried out in hospital.

1.8.4 Treatment of VL

WHO (1990) recommends SbV (im or iv, 20mg/kg/day -max 850mg/day) for VL in Africa, Brazil or Europe, treating for a minimum of 20 days or for 2 weeks after demonstration of apparent parasitological cure. The second-line drug of choice is amphotericin B, administered daily or 3x/week by slow iv infusion of 5-10mg (initial dose) rising by 5-10mg at each administration to 0.5-1mg/kg, continued until a maximum total dose of 1-3g is given. In India, where there have been many cases of SbV-resistance, amphotericin B has become the first-line drug of choice, with pentamidine (4mg/kg 3X/week; 15-25 injections) the second-line drug of choice (Berman, 1997). Aminosidine is also used. A new drug, miltefosine, a phosphocholine analogue which can be administered orally, is also showing promise in clinical trials for Indian VL (Jha *et al.*, 1999)

1.9 Control of leishmaniasis

The incidence of the leishmaniases has increased sharply since the early eighties: it is estimated that only 3 out of 10 of cases are actually reported (WHO, 1993), hence the current figure of 2 million new cases annually is likely to be a gross underestimate. Eighty-eight countries are endemic for leishmaniasis, 76 of which are developing countries. Up to 1993, 90% of all VL cases occurred in Brazil, Bangladesh, India and the Sudan; 90% of all MCL cases occurred in Brazil, Peru and Bolivia and 90% of all CL cases occurred in Brazil, Peru, Afghanistan, Iran, Saudi Arabia and Syria (WHO, 1993). The disease hampers social and economic growth of these countries and their peoples. Leishmaniasis is usually a disease of the poor for whom working practices, concomitant malnutrition and an inability to pay for treatment often lead to severe disease. Development programmes for resettlement (involving deforestation, the building of new roads and urban centres) have been associated with new outbreaks of the disease by creating conditions in which unexposed, and therefore unprotected, people are brought into contact with the parasite and its vectors (WHO, 1993).

The ultimate goal for controlling leishmaniasis is the development of a vaccine, which will prevent disease or infection (see section 1.9.5). At present, in the absence of such a vaccine, other measures continue to play a role in controlling and containing the spread of disease. Control strategies include: case detection and treatment; ecological management; vector-specific and reservoir host-specific measures. To be successful in interrupting the transmission cycle, control measures must be integrated

and involve community participation. Implementation of control measures in a given area requires knowledge of the transmission cycle operating, i.e. which parasite, vector species and behaviour and reservoir hosts are involved.

1.9.1 Case detection and treatment

For anthroponotic leishmaniasis i.e. where man is the reservoir of *Leishmania*, the active detection and treatment of human cases is essential for controlling the spread of disease. The treatment of infected individuals not only cures the patient of disease, but also reduces the supply of *Leishmania* available to the vector for subsequent transmission. Medical surveillance is ideally carried out continuously in such areas to identify new infections and enable the implementation of early treatment (WHO, 1990). The treatment of passively-detected cases also helps to control transmission of zoonotic leishmaniases when combined with other control measures.

1.9.2 Environmental management

Land clearance is used extensively in central and South American countries to control transmission of leishmaniases. Deforestation to at least 300 metres has been shown to be highly effective in isolating villages from vectors and reservoirs thus reducing transmission and disease (WHO, 1993). However, some sand fly species have proven highly capable of adapting to new environments and different host blood sources after interference with, or destruction of, their natural habitat (Killick-Kendrick, 1990).

1.9.3 Vector control

Anti-sand fly measures include personal protection, chemical control and environmental management methods. Personal protection methods include the application of repellents such as diethyltoluamide to the skin and clothing, the use of fine-mesh screens on windows and doors of houses and the use of permethrin- or deltamethrin-impregnated bednets (WHO, 1990; Marzochi & Marzochi, 1994). Vector control for domestic dogs, by way of dog collars impregnated with deltamethrin (Killick-Kendrick *et al*, 1997), is also used although the efficacy of this approach remains to be determined. These general anti-vector measures can be implemented, without knowledge of the vector's behaviour, to prevent sand fly-human contact. Spraying the inside and outside of houses with residual insecticides such as DDT, malathion and deltamethrin may reduce transmission of *Leishmania* by anthropophilic vector species (WHO, 1990; Marzochi & Marzochi, 1994). The

efficacy of residual insecticides in reducing *Leishmania* transmission has often been demonstrated as a side-effect of malaria control campaigns (WHO, 1990).

The implementation of targeted vector control usually requires a detailed knowledge of a vector's behaviour and ecology, gained from entomological surveys. Sand fly vectors are often difficult to target owing to the lack of basic information regarding, for example, the whereabouts of breeding and resting sites. Potential vector breeding and resting sites in domestic / peridomestic situations, including animal shelters close to human habitation, should also be sprayed and, where possible, destroyed. Vector control methods are not usually possible for sylvatic species.

1.9.4 Reservoir control

As with vector control, the implementation of reservoir control methods also requires knowledge of which species are involved along with knowledge of their ecology and behaviour. The application of reservoir control methods also depends on local conditions and customs. There are three groups of reservoirs: man, domestic / peridomestic animals and sylvatic animals, control strategies vary for each group and in different foci. When the reservoir is man, control is achieved through active case detection and treatment (section 1.9.1) plus the use of personal protection measures (section 1.9.3).

Dogs are the main reservoirs of VL (WHO, 1990) and are increasingly implicated as reservoirs in peridomestic CL foci (Falqueto *et al.*, 1991; Passos *et al.*, 1993; Rojas *et al.*, 1994; Davies *et al.*, 1995, Reithinger & Davies, 1999). Control strategies include the elimination of stray and feral dogs. Mass-screening of domestic dogs is carried out in many countries. In several European countries, pet dogs have a high sentimental value and treatment of infected dogs may be carried out, however, relapse is common (Vexenat, 1998). In other less affluent countries, treatment is not generally an option and dogs found to be infected are usually destroyed (WHO, 1990; Vexenat, 1998). The efficacy of current methods of dog-control in containing transmission is questionable since in Brazil, where large numbers of dogs from endemic areas are culled, the incidence of VL is rising (Vexenat, 1998). For a comprehensive review of the dog as a potential target for CL control, see Reithinger & Davies (1999). Research is also ongoing into vaccination of dogs (Mayrink *et al.*, 1996).

For many *Leishmania* species, the reservoir host(s) remains to be determined definitively: this is especially true of sylvatic *Leishmania* species causing NWCL. Methods to control a suspected reservoir may fail to reduce transmission if other reservoirs are present. In some instances, however, the reservoir is known and specific control methods may be highly effective in reducing transmission. For example, in some rural foci of CL in Jordan and Tunisia, the fat sand rat (*Psammomys obesus*) is the sole reservoir. Targeted control of this rodent is effective because its burrows are easily found and, in addition, the rat has a single food source, goosefoot. Control is achieved by ploughing burrows and destroying the goosefoot (WHO, 1993). In contrast, the use of reservoir control in reducing the transmission of sylvatic *L. V. braziliensis* is usually impossible because this parasite species infects a wide range of mammals and, in most foci, the definitive reservoir host is either unknown or not amenable to control methods.

1.9.5 Vaccination

The variable ecology and geographic distribution of the different *Leishmania* species precludes the use of the control methods described above when definitive vectors and reservoirs are unknown or not amenable to control. A vaccine would therefore be a practical and effective control measure for some leishmaniases. The acquired immunity observed following natural infection with *Leishmania* has provided the stimulus for vaccine development aimed at preventing infection and / or disease. Protective immunity induced by vaccination is dependent on the ability of the vaccine to evoke an immune response capable of controlling or eliminating an infection. In leishmaniasis, the generation of protective immunity depends on a cell-mediated immune response (section 1.6.6.1), more specifically on the generation of a T-cell response in which Th1 cytokines such as IL-12 prevail. Moreover, immunity is thought to be maintained after clinical cure by the continual stimulation of the immune system by persisting parasites (section 1.6.6.2) which, if correct, has serious implications for vaccine design. The outcome of any vaccination system depends on choice of antigens, route of administration and the use and type of adjuvant used: all these factors will influence the immune response mounted.

Since "leishmanization" (section 1.9.5.1), there have been two major approaches to the development of anti-*Leishmania* vaccines: the first involves the use of poorly-defined crude leishmanial antigen preparations and the second involves the use of

molecularly-defined antigens. "First generation" vaccines consist of crude antigen preparations; these have been used for vaccination purposes in both animals and humans and have shown highly variable efficacy. "Second generation" vaccines include attenuated, recombinant and DNA vaccines. Molecularly-defined antigens or DNA, which require the use of sophisticated technologies, present the ultimate prospect for a vaccine but the majority of research into these vaccine candidates is still at the preclinical stage.

1.9.5.1 Leishmanization

Leishmanization, a crude immunization programme in which live *L. L. major* promastigotes were inoculated into a site on the body not normally visible, was carried out in epidemic situations in Israel, Iran and the former USSR. Unfortunately, the use of this live "vaccine" led to severe, persistent lesions in around 2-5 % of "immunized" individuals who subsequently required treatment (WHO, 1990). Leishmanization has been abandoned owing to issues of ethics, persistent parasites and other problems associated with using a live pathogen for vaccination (Modabber, 1990).

1.9.5.2 Killed whole promastigote vaccines

Killed whole promastigote vaccines against cutaneous leishmaniasis, with or without adjuvants, are at various stages of Phase I (safety), II (reactivity) and III (efficacy) trials in humans (Modabber, 1995; Grimaldi, 1995; Armijos *et al.*, 1998; Marzochi *et al.*, 1998). Some "polyvalent" vaccines incorporate several different strains of *Leishmania* (Armijos *et al.*, 1998) whereas others are monovalent, i.e. based on a single species (Marzochi *et al.*, 1998). A vaccine composed of killed promastigotes of several *Leishmania* strains, "Leishvacin", has been developed and is undergoing trials in Brazil with encouraging results (Mayrink *et al.*, 1985). Killed promastigote vaccines (*L. L. major* + BCG) have also been shown to elicit immunity against visceral leishmaniasis in monkeys in India (Dube *et al.*, 1998).

1.9.5.3 Attenuated vaccines

Avirulent and low virulence *Leishmania* clones have been isolated (da Fonseca *et al.*, 1997), however, a lack of knowledge regarding the mechanisms rendering them avirulent plus the possibility of reversion to virulent phenotype has made the use of these strains unacceptable (Handman, 1997). Genetically manipulated strains in

which a defined, controlled mutation has taken place are more acceptable. An attenuated *L. L. major* vaccine strain lacking the essential dihydrofolate reductase / thymidylate synthetase (DHFR/TS) gene has been highly effective in mice (Titus *et al.*, 1995), and is now being developed for use in human trials (Handman, 1997). In addition to the potential for reversion, another problem impeding the use of attenuated vaccines is the difficulty of ensuring that strains grown on the large scale that would be required for vaccination programmes are fully-attenuated.

1.9.5.4 Recombinant vaccines

Most novel vaccine research involves the use of genetically-defined *Leishmania* antigens including the surface antigens gp63, PSA-2, gp46/M-2 and LPG. PSA-2 (promastigote surface antigen-2 from *L. L. major*) has been shown to induce a beneficial Th1 cytokine response in mice (Handman *et al.*, 1995). Defined antigens are also being incorporated into novel delivery systems such as immunostimulating complexes (ISCOMs; Papadopoulou *et al.*, 1998) and live vectors (attenuated *Salmonella typhimurium* and *Vaccinia* virus). *Salmonella* vaccine vectors incorporating gp63 from *L. L. major* have been shown to protect mice from infection with *L. L. major* (Xu *et al.*, 1995). Vaccinia vaccines have also been developed incorporating a membrane glycoprotein, gp46/M-2, from *L. L. amazonensis*. This vaccine has been shown to generate significant protection against challenge in mice (McMahon-Pratt *et al.*, 1993) however gp46/M-2 is not found in *Leishmania Viannia* species (McMahon-Pratt *et al.*, 1992).

In general, defined antigen or "subunit" vaccines elicit immunity which is highly protective but short-lived. It is thought that occasional "boosters", by way of natural infections, would maintain levels of immunity in endemic areas. Another potential problem with using subunit vaccines is that of non-responders, i.e. people who fail to mount an immune response to a particular antigen; this may be overcome by using polyvalent vaccines, those incorporating several antigens.

1.9.5.5 DNA vaccines

DNA vaccines consist of parasite antigen genes integrated into eukaryotic expression vectors, usually plasmids. After intramuscular or intradermal injection, DNA is taken up by cells and, following transcription and translation, antigen is produced and processed for presentation to the immune system. DNA vaccines for leishmaniasis

are particularly attractive because antigens may be produced in their native conformation throughout the course of "infection", mimicking what happens during natural infection (Handman, 1997). Candidate genes for anti-*Leishmania* DNA vaccines, including gp63 and PSA-2, have been tested in mice and shown to be effective in generating immunity to reinfection (Xu & Liew, 1995; Handman, 1997). Other advantages of DNA vaccines are that they are stable, easy and cheap to produce in large quantities and, furthermore, administration does not require the use of adjuvants (Handman, 1997). Potential problems of DNA vaccines include integration of vaccine DNA into the host genome (which may result in insertional mutagenesis) and the induction of autoimmune or immune complex disorders; there has, however, been no evidence of these problems in animal models so far (Wahren, 1996).

1.10 Molecular analysis of genetic variation

Genetic variation forms the basis for studying the relationships between groups or individuals, whether for epidemiological or taxonomic purposes. Two processes are responsible for the variation observed between individuals of the same species: mutation and genetic exchange. Genetic exchange, discussed in section 1.12, is constrained by speciation, hence mutation alone is responsible for the diversity observed between different species.

Mutations are spontaneous or induced alterations in an individual organism's genetic material and thus may be inherited. Mutations may be deleterious, advantageous or neutral to the survival of the individual: deleterious mutations are usually removed by natural selection; advantageous mutations are selected for and will usually become fixed; chance dictates whether neutral mutations, i.e. those which have no phenotypic effect, become established. The vast majority of mutations are neutral and occur in non-coding regions. Mutations may affect the number or morphology of chromosomes (e.g. translocations, inversions or deletions), or may result in small changes in DNA sequence (e.g. point mutations, deletions and insertions). Both types may affect the survival of the individual. At the gene level, mutations may affect gene function by altering expression of the gene (regulatory mutation) or by altering the amino acid sequence of the gene product (structural mutation). Single base mutations in a codon may result in the same amino acid being encoded (synonymous or silent mutation) or the generation of a different amino acid (non-synonymous mutation). Non-synonymous mutations give rise to abnormal gene products: a change

of amino acid ("mis-sense" mutation) may alter the form of the protein whereas a mutation which results in the formation of a termination codon ("nonsense" mutation) results in a truncated product.

Data indicate that kinetoplastid organisms are diploid at most loci: ploidy has been determined indirectly by comparisons of total nuclear DNA (Wells *et al.*, 1987; Iovannisci & Beverley, 1989) estimates of nuclear genome size (Leon *et al.*, 1978), RFLPs (Gibson *et al.*, 1985; Wells *et al.*, 1987) and, with the advent of PFGE, by karyotype analysis (e.g. Gibson *et al*, 1992; Dujardin *et al*, 1993a). Aneuploids and tetraploids have also been identified from the analysis of experimental crosses of *Trypanosoma brucei* (see section 1.12.1) and have been suggested to occur in natural populations of *Leishmania* (Bastien *et al.*, 1992; Cruz *et al.*, 1993). The *Leishmania* genome, as with other kinetoplastids, comprises both chromosomal or nuclear DNA and independently-replicating kinetoplast DNA (kDNA). *Leishmania* exhibit considerable inter- and intra-species genetic diversity which has complicated their taxonomic classification. This diversity and variation can be defined at the level of both the genome and individual genes, using numerous techniques.

1.10.1 Diversity in genome organization

1.10.1.1 Molecular karyotype analysis

Classical karyotype analysis is impossible for *Leishmania* since chromosomes do not condense during the cell cycle, however, the development of pulse-field gel electrophoresis (PFGE) has allowed chromosome-sized DNA to be resolved (Schwartz & Cantor, 1984). The haploid genome of *Leishmania* consists of 34-36 chromosomes, which are resolved into 20-25 chromosomal bands by PFGE (Wincker *et al.* 1996; Britto *et al.*, 1998). Bands ranged in size from 0.35-~3Mb, different sized homologues of the same chromosome were identified and some bands comprised more than one chromosome.

Molecular karyotype analysis couples PFGE with Southern blotting and the hybridization of gene- and/or chromosome-specific probes. Molecular karyotypes are defined by the assignment of genes to DNA "chromosome" bands after PFGE: strains and isolates sharing a molecular karyotype can be grouped into "karyodemes". Many *Leishmania* genes have been mapped to chromosomes including those for ribosomal DNA, the 70kb heat shock protein (*hsp70*:), α- and ß-tubulin, trypanothione

reductase, gp63, gp46/M2 and mini-exon (Leon *et al.*, 1978; Landfear *et al.*, 1983; Samaras & Spithill, 1985, 1987; Lee *et al.*, 1988; Giannini *et al.*, 1990; Hanekamp & Langer, 1991; Dujardin *et al.*, 1993a; Belli *et al.*, 1994; Espinoza *et al.*, 1995; Wincker *et al.*, 1996). Wincker *et al.*, (1996) used 41 known genes, 66 expressed sequence tags (ESTs) and 137 anonymous DNA sequences for molecular karyotype analysis which enabled them to compile physical linkage group maps constituting the genome of *L. L. infantum*. Linkage groups were shown to be conserved in other *Leishmania* species, despite being separated by large genetic differences.

Chromosomal plasticity is extremely common in *Leishmania*: molecular karyotyping has revealed considerable inter- and intra-species diversity, in both chromosome number and size (Spithill & Samaras, 1985; Scholler *et al.*, 1986; Bishop & Miles, 1987; Pages *et al.*, 1989; Bishop & Akinsehinwa, 1989; Hanekamp & Langer, 1991; Bastien *et al.*, 1992; Lighthall & Giannini, 1992; Cruz *et al*, 1993; Dujardin *et al.*, 1993a, 1994; Wincker *et al.*, 1996; Britto *et al.*, 1998). In terms of size, two types of chromosomes have been identified in different isolates of the same species: size-conserved (consensus) and size-variable (hypervariable) chromosomes (Samaras & Spithill, 1987). Lighthall & Giannini (1992) identified three levels of karyotype conservation in *Leishmania* species. At the first level the karyotype is highly conserved (e.g. *L. L. major*): strains collected from a wide geographical area are remarkably similar to a single consensus karyotype. At the second level more than one consensus karyotype can be identified within a small geographical range but strains show little variation from the consensus karyotypes present (e.g. *L. V. panamensis*). At the third level molecular karyotypes are highly variable, even from geographically-restricted isolates: few size-conserved chromosomes are identifiable, even from patients with the same clinical presentation (e.g. *L. L. amazonensis*). In some *Leishmania* species, polymorphisms occur to such an extent that each strain or clone has a unique karyotype (Bastien *et al.*, 1990; Lighthall & Giannini 1992; Dujardin *et al.*, 1993a, 1995a).

The chromosomal structure in *Leishmania* has been shown to be similar to that of other kinetoplastids: genomic DNA consists of conserved low copy number genes, multi-copy genes and many repetitive sequences (Blaineau *et al.*, 1991; Eresh *et al.*, 1993). Small changes in chromosomal size can be due to intra-chromosomal gene amplification or deletion (Cruz & Beverley, 1990). Variation in chromosomal size

may also result from homologous recombination between repetitive sequences (Iovannisci & Beverley, 1989; Dujardin *et al.*, 1994). Chromosomal translocation, as a means of generating variation, is thought to be of little significance in *Leishmania* owing to the high level of linkage group conservation exhibited (Beverley *et al.*, 1987; Wincker *et al.*, 1996). Gene amplification (often as a result of drug selection) has been noted for metabolic genes such as the bifunctional dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene (Beverley *et al.*, 1988). Amplification of the mini-exon array has been shown to account for a 50kb size variation between homologues of chromosome 2 in *L. L. major* (Iovannisci & Beverley, 1989). Rearrangements of gp63 and ribosomal DNA (rDNA) genes are associated with variation in the size of chromosomes bearing these genes in members of the subgenus *Viannia* (Dujardin *et al.*, 1994; Inga *et al.*, 1998).

1.10.1.2 Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis is based on the hybridization of molecular probes to specific DNA fragments which have been generated by digesting genomic DNA with restriction endonucleases (REs). Fragments are separated by electrophoresis and bound to membranes by Southern hybridization before probing. Fragment lengths are determined by the positions of RE recognition sites in the DNA sequence. RFLPs usually arise as a result of single base substitutions in a sequence: new RE sites may be created (resulting in fragment partition) or existing RE sites may be deleted (resulting in fragment 'fusion'). RFLPs may also arise from larger mutational events such as chromosomal rearrangements. Combinations of different enzymes and probes are used for RFLP analysis allowing strains and isolates to be typed to various taxonomic levels. The discrimination of RFLP analysis relies on the degree of similarity between sequences from different strains and on the level of conservation of the specific probe used. In addition, RFLP pattern variation may be used as a crude measure of the overall sequence divergence between strains.

RFLP analysis provides information on the genomic organization of genes and can identify polymorphisms between isolates, strains and species of *Leishmania*. *Leishmania* DNA contains many genes which are present as large multigene families. Copies of genes from multigene families may vary in number and location between species and may have diverse structure and sequence. For example, the genes

encoding the major surface glycoprotein, gp63, occur in tandem arrays and as dispersed single genes and are variably organized in different species. There is a tandem array of 5 genes plus a single dispersed gene in *L. L. major*, a tandem array of 7 genes plus at least 3 dispersed genes in *L. L. donovani*, a tandem array of 4 genes plus 14 dispersed genes in *L. L. chagasi*, 2 tandem arrays of 4 and 5 genes plus one dispersed gene in *L. L. mexicana* and 4 tandem arrays, one of which contains at least 22 gene copies in *L. V. guyanensis* (Button *et al.*, 1989; Webb *et al.*, 1991; Roberts *et al.*, 1993; Medina-Acosta *et al.* 1993; Steinkraus *et al.*, 1993). RFLP analysis, involving many of the same probes as used for molecular karyotyping, has been used in many studies to show variation in *Leishmania* genomic DNA (Beverley *et al.*, 1987; Reiner *et al.*, 1989; Ellis & Crampton, 1991; Kelly *et al.*, 1991; Macedo *et al.*, 1992; Belli *et al.*, 1994; Dujardin *et al.*, 1995b; Espinoza *et al.*, 1995; Gomes *et al.*, 1995; Mendoza-Leon *et al.*, 1995).

1.10.1.3 Kinetoplast DNA (kDNA) and schizodeme analysis

All kinetoplastids possess a single unusual mitochondrion and a "kinetoplast", an organelle that contains a unique DNA network consisting of thousands of concatenated circular DNA molecules. This kinetoplast DNA (kDNA) network consists of two classes of circular DNAs, termed maxicircles and minicircles. Maxicircles range in size (from 19 - 40kb) and number (approximately 10 - 50), depending on the species. Maxicircles are inherited uniparentally and are homogeneous in sequence within an individual (Gibson, 1989; Gibson & Garside, 1990; Turner *et al.*, 1995). Maxicircles carry genes encoding mitochondrial ribosomal RNAs and several protein complexes of the mitochondrial oxidative phosphorylation system (Shapiro & Englund, 1995). Minicircles also range in size (0.5 - 2.5 kb) and number (3000 - 30000) however, they are heterogeneous in sequence and inherited biparentally (Gibson & Garside, 1990). Minicircles encode the sequences for the guide RNAs involved in RNA editing (Pollard *et al.*, 1990). Minicircle sequence analysis reveals that each minicircle of *Leishmania* species carries a single copy of a 100-200bp conserved region which contains the origin of replication. Within this conserved region, there are three highly conserved sequences; one of these, the universal minicircle sequence (UMS), is identical in all kinetoplastids. Outside of these highly conserved regions, there is considerable diversity: *Leishmania* species-specific kDNA sequences have been identified and exploited for the development molecular diagnostic probes (Barker & Butcher, 1983;

Lopes & Wirth, 1986; Gramiccia *et al.*, 1992). In addition to RFLP analysis of genomic DNA (gRFLP), RFLP analysis of kDNA ("schizodeme analysis") has also been used for typing isolates of *Leishmania*, where a schizodeme is a group of isolates sharing the same kDNA-RFLP pattern (Lopes *et al.*, 1984; Degrave *et al.*, 1994a).

1.10.2 DNA sequence diversity

In addition to the diversity shown at the level of genomic organization, *Leishmania* display great diversity at the DNA sequence level, both in coding and non-coding regions. Variation in DNA sequence arises as the result of mutational events which may consist of additions or deletions of genetic material, nucleotide substitutions or sequence rearrangements. Sequence diversity may be demonstrated by phenotypic or genotypic methods. Phenotypic methods analyze the gene product itself and hence detect genetic changes indirectly. Phenotyping techniques, including isoenzyme analysis (IEA), isoelectric focusing (IEF) and monoclonal antibody (serodeme) typing, are described in sections 1.10.2.1 and 1.10.2.2. Genotypic methods can demonstrate changes in DNA sequence directly, in both coding and non-coding regions. Many methods of detecting genotypic variation involve the use of the polymerase chain reaction (Mullis & Faloona, 1987; Saiki *et al.*, 1988), including RFLP analysis of amplification products from PCR (PCR-RFLP), random amplified polymorphic DNA (RAPD) analysis, microsatellite analysis, DNA sequencing and mutation detection methods such as denaturing gradient gel electrophoresis (DGGE) and single-stranded conformational polymorphism (SSCP) analysis. Genotyping techniques are described in sections 1.10.2.3 through 1.10.2.7.

1.10.2.1 Isoenzyme analysis (IEA) and isoelectric focusing (IEF)

IEA and IEF are phenotypic methods which are based on the electrophoretic separation of enzymes coupled with their histochemical visualization. In IEA, enzymes are separated according to size and charge by electrophoresis though a suitable matrix which may be a starch, polyacrylamide or agarose gel (Miles, 1980a; Lanham *et al.*, 1981) or a cellulose acetate plate (Kreutzer & Christensen, 1980). In IEF, enzymes are separated by electrophoresis through a polyacrylamide gel containing a pH gradient; enzymes migrate to the position of their isoelectric point (pI) and thus are separated by charge only.

Isoenzymes are phenotypic characters since they result from the expression of an enzyme gene locus; they are, however, informative statistically since their interpretation has a genetic basis. Variations in electrophoretic mobility are evidence of nucleotide substitutions causing changes in the numbers of charged amino acids in the enzyme. For both IEA and IEF, banding patterns can usually be interpreted in terms of Mendelian genotypes for a given enzyme, based on previous experience of assigning genetic loci and alleles to banding patterns and knowledge of the enzyme structure. For reviews, see Harris & Hopkinson, 1976; Miles *et al.*, 1980b; May, 1992. The simplest interpretation of IEA patterns involve monomeric enzymes (e.g. phosphoglucomutase, PGM) which are composed of a single polypeptide subunit. Isolates homozygous for a monomeric gene locus produce a single band on IEA however, enzyme mobility may vary between species or isolates depending on the size and / or charge. An isolate heterozygous for a monomeric enzyme locus, as in a hybrid gaining one allele from each of two homozygous parents with enzymes of differing mobility, would show a double-banded pattern, one band resulting from each of the two parental alleles. Enzymes may also be dimeric (e.g. glucose phosphate isomerase - GPI), trimeric (e.g. nucleoside phosphorylase - NP) or tetrameric (e.g. lactate dehydrogenase - LDH), each having characteristic heterozygote banding patterns reflective of random associations between the polypeptide subunits. Enzymes may also be encoded by more than one locus (e.g. nucleoside hydrolase - NH); banding patterns are usually interpretable by treating each product in the same manner as if encoded by a single locus. "Missing" bands may be attributable to a particular polypeptide combination forming a non-functional enzyme. Interpretation of IEA / IEF data obviously requires a knowledge of basic genetics and experience.

IEA, also known as "zymodeme analysis", has been used extensively for the identification, classification, numerical taxonomy and epidemiological study of *Leishmania* species (Miles *et al.*, 1980b; 1981; Kreutzer & Christensen, 1980; Evans *et al.*, 1984; 1987; Cuba-Cuba *et al.*, 1985; Le Blancq *et al.*, 1986; Kreutzer *et al.*, 1987; Arana *et al.*, 1990; Rioux *et al.*, 1990; Grimaldi *et al.*, 1991; Kelly *et al.*, 1991; Revollo *et al.*, 1992; Felinto de Brito *et al.*, 1993; Cupolillo *et al.*, 1994, 1995; Dujardin *et al.*, 1995b; 1998; Chouicha *et al.*, 1997). *Leishmania* isolates are assigned to zymodemes based on accumulated data from 10 or more enzyme systems; identification is made by comparison with reference strains.

IEF has not gained widespread use despite its reported higher resolving power. IEF cannot be used with all enzymes; for some, migration to their pI results in the loss of activity making visualization impossible. In addition, the use of IEF in numerical taxonomy is precluded since insufficient enzyme systems can be studied. Nevertheless, IEF can be used to complement IEA data, providing further phenotypic evidence for inferred genotypic variance (Ebert, 1987; Piarroux *et al.*, 1994).

1.10.2.2 Monoclonal antibody typing

Monoclonal antibodies have been used for *Leishmania* species identification in reservoir hosts and sand flies, in diagnostic assays and for typing and epidemiological purposes (for review, see Grimaldi & Tesh, 1993). Many monoclonal antibodies have been raised against *Leishmania*: these have been used in a variety of immunoassay formats including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody (IFA) assays (Grimaldi *et al.*, 1987; Grimaldi & McMahon-Pratt, 1996). Monoclonal antibody typing ("serodeme analysis") has identified stage-, subgenus-, complex- and species-specific monoclonal antibodies in addition to others which have been shown to identify strains within a species (McMahon-Pratt & David, 1981; McMahon-Pratt *et al.*, 1982; Jaffe *et al.*, 1984; McMahon-Pratt *et al.*, 1985; Grimaldi *et al.*, 1987; Eperon & McMahon-Pratt, 1989; Hanman *et al.*, 1991). The use of monoclonal antibodies for serodeme analysis of New World *Leishmania* has been extensively studied (for review, see Grimaldi & McMahon-Pratt, 1996).

1.10.2.3 PCR-RFLP analysis

PCR-RFLP analysis combines amplification of a DNA sequence using PCR with the RFLP analysis of the PCR product. PCR-RFLP fragments are separated by electrophoresis and are visualized directly by staining, usually with ethidium bromide or silver. As with genomic RFLP analysis, PCR-RFLP analysis detects variability only in sequence which encodes restriction enzyme (RE) sites; fragment lengths are determined by the positions of RE recognition sites in the DNA sequence. Obviously, the number of fragments generated by digestion of a product with a particular RE depends on the number of relevant RE sites present. The frequency at which a RE cuts is dependent, in part, on the length of its recognition sequence: REs which recognize 4 nucleotides ("4-cutters") will generally cut more often than REs which recognize for example, 6 nucleotides ("6-cutters"). For PCR products, which are

small compared to genomic DNA, REs which recognize 4 nucleotides ("4-cutters") are used because their cutting sites occur more frequently and hence there is an increased chance of detecting an RFLP. The choice of REs used can be made from sequence restriction maps and, if maps are not available, knowledge of nucleotide bias (*Leishmania* have a GC-rich genome).

Riboprinting (Clark & Diamond, 1991) is a term coined specifically for PCR-RFLP analysis of the 18S (small subunit, SSU) ribosomal RNA (rRNA) gene. The term "ribodeme" describes populations of a species that share the same riboprint pattern (Clark & Pung, 1994). Riboprinting has been used to assess diversity in several protozoan genera, including *Trypanosoma cruzi* (Clark, 1992; Clark & Pung, 1994; Stothard *et al.*, 1998a).

PCR-RFLP analysis has been used to demonstrate variation in other *Leishmania* sequences including the internal transcribed spacer regions of the mini-exon and rRNA genes (Ramirez *et al.*, 1987; Fernandes *et al.*, 1994; Cupolillo *et al.*, 1995), the gp63 gene array (Victoir *et al.*, 1995; 1998) and a *Leishmania Viannia*-specific subtelomeric repeat sequence (Fu *et al.*, 1998).

1.10.2.4 Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis (Welsh & McClelland, 1990; Williams *et al.*, 1990), also known as arbitrarily primed PCR (AP-PCR), employs 10 - 30mer oligonucleotide primers of arbitrarily chosen sequence in low-stringency PCRs. The major advantage of RAPD analysis over other PCR-based assays is that it does not require prior knowledge of specific DNA sequence. The low stringency conditions employed allows DNA synthesis to be initiated from any site to which the primer is matched. A number of sites, flanked by complementary primer sequences and randomly distributed throughout the genome, are simultaneously amplified to produce DNA fragments of variable length which can be resolved by gel electrophoresis.

RAPD profiles (scored by the presence and absence of fragments) may reveal genus-specific and/or species-specific fragments which can be used as characters for identification purposes. In addition, isolate-specific fragments may be observed. The ease with which large amounts of RAPD data can be generated has led to the increased use of RAPD markers for population genetic and phylogenetic analyses.

The interpretation of RAPD data has, however, many limitations, with serious implications for subsequent analyses:

1. It is proposed that RAPD markers are inherited in a Mendelian manner (Williams *et al.*, 1990; Apostol *et al.*, 1996; Kazan *et al.*, 1993; Tibayrenc, *et al.*, 1993; 1995). For *Leishmania*, and other organisms in which mating experiments have, as yet, proven impossible, the Mendelian inheritance of observed RAPD variation is difficult to establish (Tibayrenc, 1995).

2. The origins of RAPD fragments are unknown hence fragments of identical size may not be homologous (i.e. may exhibit homoplasy and have arisen from separate areas of the genome). This means that loci, and therefore alleles / allelic polymorphisms, cannot be identified accurately or conclusively. Polymorphisms of a given fragment ("locus") may result in alleles having different- or same-sized bands - how do you tell which? For organisms in which crossing experiments are possible, it may be possible to infer RAPD loci (Kazan *et al.*, 1993) and hence score locus polymorphism.

3. RAPD markers are essentially binary characters that can be considered to exhibit "dominance" because only one of two possible states, present or absent, can be distinguished at a "locus" (Lynch & Milligan, 1994).. The presence of a band may be presumed indicative of a dominant allele (in either homozygous or heterozygous state), whereas the absence of a band may be assumed to indicate an unamplified, recessive, null allele.

One basic approach for population genetic analysis using RAPD data has been to carry out recombination tests which do not require the identification of individual loci and alleles (Tibayrenc, 1995). For a given primer, each distinct RAPD pattern is taken as a genotype (loci and alleles remain unknown), with genetic relationships inferred by scoring the presence and absence of bands between pairs of different genotypes. Many population genetic analyses carried out on RAPD data make assumptions the validity of which are highly questionable. Statistical methods have recently been developed for the population genetic and phylogenetic analyses of RAPD data, in which the limitations of RAPD data are acknowledged (Lynch & Milligan, 1994; Zhivotovsky, 1999). There remain conflicting views as to whether RAPD markers can be reliably used for defensible population genetic and phylogenetic analyses (Apostol *et al.* 1996; Nadler *et al.*, 1995; Noyes *et al.*, 1996; Stothard & Rollinson, 1996).

Nevertheless, RAPD data has shown parity with other molecular markers confirming that RAPD markers can provide valuable information (Tibayrenc *et al.*, 1993; Dujardin *et al.*, 1995b). Data from RAPD analysis has been used both for phylogenetic and population genetic analyses of *Leishmania:* results have shown good correlation with isoenzyme, RFLP and karyotype data. (Tibayrenc *et al.*, 1993; Dujardin *et al.*, 1995b; Gomes *et al.*, 1995; Pogue *et al.*, 1995; Schönian *et al.*, 1996). In the author's (and others) opinion, however, RAPD markers should be employed for population genetic and phylogenetic analyses only if specific DNA markers for a particular organism are lacking. In such cases, given that (i) alleles and genotypes cannot be identified conclusively, (ii) RAPD markers are considered dominant and (iii) identity by state assumptions are highly questionable, results should be interpreted with extreme caution and correlated with those obtained using other genetic markers.

1.10.2.5 Microsatellite analysis

A large part of non-coding DNA is organized in repeated sequences which often exhibit pronounced polymorphism. One class of repetitive DNA, satellite DNA, consists of tandemly-repeated sequence motifs which are present throughout the genomes of both prokaryotic and eukaryotic organisms. Satellite DNAs include so-called mini- and microsatellites which consist of arrays of repeats nominally 10-100 bp and 1-9bp in length respectively. Satellite DNA is tandemly-repeated between areas of conserved DNA sequence, allowing primers to be designed which enable the satellite region to be amplified by PCR. Alleles are generally distinguished by different copy numbers of the repeat, although diversity may also occur in the sequence of the flanking regions. Satellite DNA markers were initially developed (Jeffreys, 1985) and exploited for use in forensic medicine, parentage and pedigree analyses and in the study of human genetic disease (for review see Jeffreys *et al.*, 1993).

Microsatellites are tandemly-repeated sequences, 1-9 base pairs in length, which are widely dispersed in eukaryotic genomes (Hamada *et al.*, 1982; Tautz & Renz, 1984). Microsatellites consisting of dinucleotide repeats, e.g. $(CA/GT)_n$ (where n is the number of repeat units) are the most common type (Ashley & Dow, 1994). Most microsatellite loci are situated in non-coding areas of the genome and, since they have no known function, are assumed to be neutral markers (Ashley & Dow, 1994;

Schlotterer, 1998). Microsatellite loci are discrete and have co-dominant alleles which are inherited in a Mendelian manner (Ashley & Dow, 1994; Schlotterer, 1998). Alleles at microsatellite loci can be scored by size or by sequence analysis: the majority of studies are based on analysis of size variation after PCR amplification. Primers for PCR are designed from conserved sequences flanking the microsatellite locus. Most alleles at microsatellite loci vary in size according to the numbers of repeat units (Tautz, 1989; Ashley & Dow, 1994).

Population genetic and phylogenetic analyses require accurate sizing of microsatellite alleles. Accurate sizing is highly dependent on being able to resolve small (1-2bp) changes in size. Microsatellite alleles are most commonly visualized by radioactive or fluorophore labeling or silver staining following electrophoresis though long-range, high percentage denaturing PAGE gels since these combinations offer high levels of sensitivity and resolution. PCR products visualized by radiolabeling or silver-staining can be sized by running a sequencing ladder next to the PCR-amplified microsatellites. Such methods usually rely on visual scoring of bands by comparison with the ladder marker.

Automated fluorescence-based instruments, initially developed for sequence analysis, have recently been applied to microsatellite analysis by coupling to microsatellite-specific computer software (for a review, see David & Menotti-Raymond, 1998). PCR products are labeled with a fluorescent dye (fluorophore) either by using fluorescently-labeled PCR primers or by incorporating fluorescently-labeled dUTPs during PCR. Fluorophore-labeled PCR product is electrophoresed through a PAGE gel past a laser set at a fixed distance. The laser beam impinges on the fluorophore which becomes excited and emits fluorescence: the emission is detected, measured and recorded. The computer generates a gel image showing the bands that were detected; this data is analyzed using software such as Genescan® and Genotyper®.

Some automated instruments, i.e. those of Applied Biosystems (AB), can distinguish between multiple fluorophores. These systems enable PCR products from distinct microsatellite loci labeled with different fluorophores to be combined and electrophoresed in the same lane on a gel (multiplex analysis). To further increase throughput, loci of distinct sizes can be labeled with the same fluorophore and run in the same lane. In addition, a single gel can be used two or three times. A number of

fluorophores are available one of which is reserved for labeling a set of molecular weight markers. Markers are included in every lane during electrophoresis to serve as an internal lane standard, allowing constant band sizing within and between lanes. Automated fluorescence-based systems are becoming increasing popular as they eliminate the problems associated with radioactivity and manual scoring, have a high throughput and allow accurate sizing of microsatellite products.

The mechanisms creating microsatellite polymorphisms are only partially understood. It is generally thought that mutations in the number of repeats at microsatellite loci arise as a result of DNA slippage (mispairing of complementary bases) during DNA replication (Levinson & Gutman, 1987; Schlotterer & Tautz, 1992). Slippage, followed by DNA mismatch repair, results in the insertion or deletion of repeat units. Other mutational mechanisms, such as unequal sister-chromatid exchange (exchange of markers within same chromosome during mitosis and meiosis) and unequal crossing-over (exchange of markers between 2 different chromosomes during meiosis) may also occur. These mechanisms are, however, not thought to be as important as DNA slippage at microsatellite loci because of the relatively small size of microsatellite arrays (Ashley & Dow, 1994; Di Rienzo *et al.*, 1994).

Mutations involving changes in the number of dinucleotide repeats are generally thought to follow the stepwise mutation model (SMM) of Ohta & Kimura (1973) or the two-phase model (TPM) of Di Rienzo *et al.* (1994). The SMM, also known as the one-step model, assumes that alleles can be defined as characters: mutation at an allele results in an alteration of one step in a positive or negative direction. In terms of microsatellite alleles, this can be interpreted such that a mutation occurs by the gain or loss of a repeat. The TPM, a modification of the SMM, assumes that whilst most mutations involve changes of a single repeat, multiple repeat unit mutations can also occur. A few microsatellite loci have, however, been shown to fit the Kimura-Crow infinite allele model (IAM) (Kimura & Ohta, 1978) which predicts that each new mutation occurring in a population will be different from the existing alleles (Estoup *et al.*, 1995a).

Statistical analyses of microsatellite data are dependent on certain assumptions including: (i) mutation follows the SMM or TPM; (ii) no mutational pressures are operating within or across loci; (iii) mutation rates are high and constant across loci;

and (iv) all alleles can be called / scored (Schlotterer *et al.*, 1991). Whilst many loci studied appear to meet these criteria, there is also evidence that these assumptions do not always hold. For examples: some loci appear to follow the IAM model (Estoup *et al.*, 1995a); some alleles have specific mutation rates (Schlotterer *et al.*, 1998) with different mutation rates having been scored between alleles in a heterozygote (Amos *et al.*, 1996); there appear to be constraints on allele size in some loci (Garza *et al.*, 1995; Lehmann *et al.*, 1996a) and directional mutation of microsatellite loci has been demonstrated (Amos & Rubinsztein, 1996). It is obviously important therefore, that the above assumptions are shown to hold for a given locus prior to its use for population genetic analyses otherwise incorrect conclusions are likely to be drawn.

With the increase in microsatellite sequencing, evidence of mutations in the flanking regions has also emerged (Valdes *et al.*, 1993; Orti *et al.*, 1997; Grimaldi & Cronau-Roy, 1997; Viard *et al.*, 1998). Point mutations in the flanking regions are inapparent if using size-scoring only as such alleles usually appear identical in size (i.e. exhibit homoplasy) by electrophoretic techniques (Garza *et al.*, 1996; Estoup *et al.*, 1995b; Viard *et al.*, 1998). In addition, insertions or deletions in flanking regions may result in an incorrect repeat number being scored if these are calculated by subtracting the flanking region sizes from the size of the PCR product. Null alleles may also be scored (evident by the absence of a PCR product): these alleles presumably have a mutation(s) in one of the primer-binding sites such that no amplification occurs (Callen *et al.*, 1993; Pemberton *et al.*, 1995; Lehmann *et al.*, 1996a).

Many studies base population genetic and phylogenetic analyses solely on size-defined alleles. It is not possible to determine whether a particular allele arose from a longer or shorter allele (see *Figure 1.10.2.5a*). Nevertheless, alleles that are closely related in size are thought "more likely to share a more common recent ancestry than alleles widely different in size" (Di Rienzo *et al.*, 1994).

Phylogenetic analyses based solely on length differences between alleles are unlikely to reflect precise relationships between alleles since mutations outside the repeat region itself, which are likely to involve different mutational mechanisms, will be ignored. A more precise approach is to base analyses on mutations in the flanking regions, i.e. excluding the microsatellite repeat region from phylogenetic analysis (Ashley & Dow, 1994). Such analyses should, however, be interpreted with caution,

bearing in mind the assumptions and limitations of the analyses and, where possible comparing results with those obtained from the analysis of other molecular characters. For localized populations of closely-related individuals, however, estimates of microsatellite allele and genotype frequencies can be made and used for population genetic analyses. In addition, heterozygotes may be scored from the presence of different-sized alleles at microsatellite loci (see *Figure 1.10.2.5b*).

Figure 1.10.2.5a: **"Identity by state".**

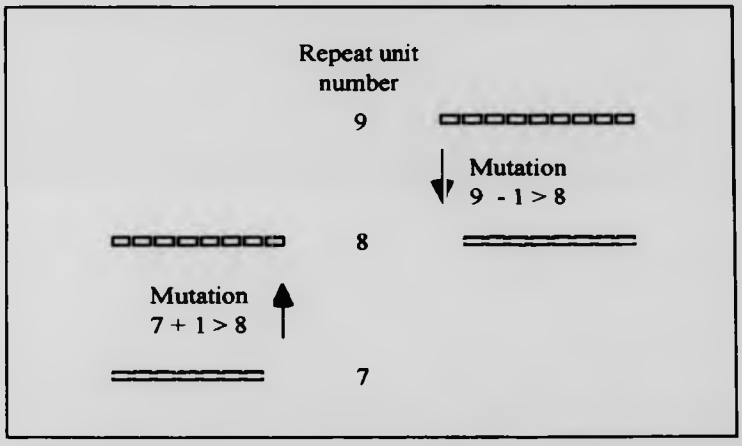


Figure 1.10.2.5a: "Identity by state". Two homoplasic microsatellite alleles, both with 8 repeat units, are shown. These alleles are derived from different ancestral alleles thus, despite identical lengths, they are less closely related to each other than, for example, the 8- and 9-repeat alleles. (Figure modified from Schlotterer, 1998).

Mini- and microsatellites are increasingly employed as molecular markers in analyses of genetic variation, population structure and phylogeny of parasites and parasite vectors. They have been employed for studies on *Trypanosoma brucei* (Barrett *et al.*, 1997), *T. cruzi* (Oliveira *et al.*, 1998), *Plasmodium* spp. (Van Belkum *et al.*, 1992; Su *et al.*, 1998), *Anopheles* spp. (Rongnoparut *et al.*, 1996; Lehmann *et al.*, 1996b, 1997) and *Rhodnius pallescens*, a vector of *T. cruzi*, (Harry *et al.*, 1998). Mini- and microsatellite sequences have been used for DNA fingerprinting of OW and NW *Leishmania* strains and species (Macedo *et al.*, 1992; Rossi *et al.*, 1994; Gomes *et al.*, 1995; Schonian *et al.*, 1996; Oliveira *et al.*, 1997). Macedo *et al.* (1992) constructed phylogenies for NW *Leishmania* species based on band-sharing observations from

DNA fingerprints. *Leishmania*-specific microsatellite loci (markers) have only very recently been described (Russell, *et al.*, 1999) and have not, until now (work described in this thesis), been used for population genetic and phylogenetic analyses.

Figure 1.10.2.5b **PCR amplification of a microsatellite locus.**

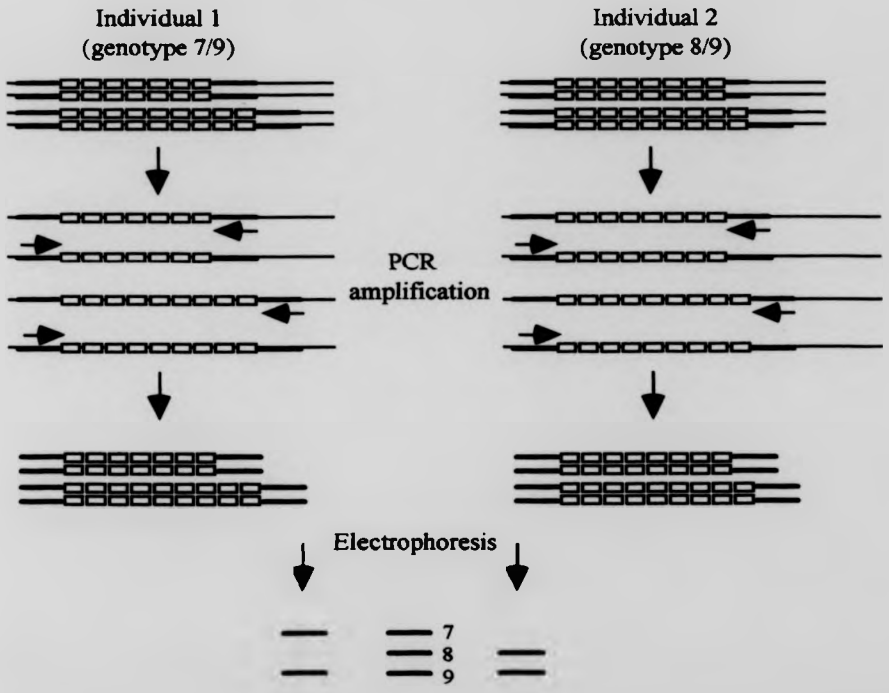


Figure 1.10.2.5b PCR amplification of a microsatellite locus. Heterozygous individuals A and B differ in repeat number at one allele. PCR products from A and B for this microsatellite locus, separated by gel electrophoresis, differ in size for this allele. (Adapted from Schlotterer, 1998).

1.10.2.6 Mutation detection methods

Many methods of detecting variation due to small mutational changes now exist including single-strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), RNase cleavage, chemical cleavage of mismatches (CCM) and heteroduplex analyses (for a comprehensive review, see Hawkins, 1997).

DGGE (Fischer & Lerman, 1983) and SSCP (Orita *et al.* 1989) are gel electrophoresis-based techniques capable of detecting polymorphisms and mutations in otherwise identical PCR products. DGGE is based on the fact that DNA heteroduplexes differing in a single base pair have slightly different melting characteristics. DNA molecules melt in discrete segments that are dependent on DNA sequence and base composition. When two similar heteroduplexes are electrophoresed through a denaturing gradient (chemical e.g. urea, or temperature), DNA melting will occur at slightly different positions within the gradient, migration is thus slowed down allowing resolution of the original similar heteroduplexes. SSCP analysis is based on the principle that the electrophoretic mobility of a molecule within a gel matrix varies with the size, charge and shape of the molecule. Native single stranded DNA has a folded structure dictated by its sequence: in SSCP analysis, a single nucleotide difference between two similar sequences is sufficient to alter the folded structure of one relative to the other. This conformational change is detected as a mobility difference upon gel electrophoresis. The technique differs from DGGE in that the PCR products are denatured prior to electrophoresis in a non-denaturing gel. Although DGGE and SSCP allow the determination of single base changes, DNA sequencing needs to be used to definitively characterize specific differences in gene sequence.

DGGE and SSCP have been used to identify sequence variation in rRNA gene sequences of *T. cruzi* (Stothard *et al.*, 1997; 1998b) and several other organisms including *Plasmodium falciparum* (Kain *et al.* 1996) and the flatworm, *Dugesia* (Carranza *et al.*, 1996).

1.10.2.7 DNA sequencing

DNA sequencing is the ultimate in molecular typing methods but is rarely required or used, except for comparisons of gene sequences or other sequences of interest for phylogenetic purposes or for designing probes aimed at identifying specific taxonomic groups. For example, DNA sequencing of *Leishmania* 18S rRNA genes has allowed species-specific DNA probes to be designed (Uliana *et al.*, 1991, 1994). With the advent of PCR-based cycle sequencing and automated fluorescent techniques however, high-throughput typing can be achieved with exquisite sensitivity allowing accurate phylogenetic and population genetic analyses to be carried out.

1.11 Genetic transformation

Genetic transformation is a powerful technique which has facilitated the study of gene function, expression and control mechanisms both in asexual organisms e.g. *Giardia* (Singer *et al.*, 1998; Sun *et al.*, 1998) and in organisms such as *Trypanosoma cruzi* and *Leishmania* in which sexual reproduction is thought possible but where genetic crossing experiments have not been successful. Genetic transformation of these organisms has allowed information to be gained on putative virulence factors and potential drug targets for vaccination and chemotherapeutic interventions respectively (for reviews, see Kelly, 1995a; 1997). Genetic transformation will be discussed in greater detail in Chapter 7.

1.12 Population structure and genetic exchange

For *Leishmania* and other trypanosomatids, a clonal theory of population structure has been proposed in which reproduction occurs predominantly by binary fission (Tibayrenc *et al.*, 1990). The clonal reproduction theory is based on the pronounced genotype linkage disequilibrium observed in natural populations of these organisms, as demonstrated by the presence of predominant genotypes over-represented in particular localities, over extensive geographical areas and long periods of time (Tibayrenc *et al.*, 1990; Tibayrenc & Ayala, 1991). According to this theory, sexual reproduction and genetic exchange make only minor contributions to the genetic diversity observed within populations and do not occur frequently enough to break the prevailing pattern of a basically clonal population structure. The applicability of the clonal theory to trypanosomatids has, however, been questioned as a result of the demonstration of genetic exchange in *Trypanosoma brucei* (Jenni *et al.*, 1986) and the finding of putative hybrid genotypes in three major trypanosomatid taxa: *Leishmania*, *T. brucei* and *T. cruzi* (Evans *et al.*, 1987; Darce *et al.*, 1991; Kelly *et al.*, 1991; Bonfante-Garrido *et al.*, 1992; Belli *et al.*, 1994; Carrasco *et al.*, 1996; Dujardin *et al.*, 1995b; Bogliolo *et al.*, 1996).

1.12.1 Genetic exchange in Trypanosomatids

The first proposal for the occurrence of genetic exchange in trypanosomatids came in 1980 (Gibson *et al.*, Tait): isoenzyme analysis (IEA) of *Trypanosoma brucei* obtained from wild-caught tsetse flies revealed classical Hardy-Weinberg equilibrium among alleles of polymorphic enzymes, leading to the suggestion that populations were randomly mating.

The first direct evidence that genetic exchange could occur in these organisms came from Jenni *et al.* (1986). In their experiments, two clones of *T. brucei* were mixed and passaged simultaneously through the tsetse fly vector. Analysis of the resulting progeny by IEA, RFLPs, molecular karyotyping and schizodeme analysis showed that, in addition to parental types, hybrid progeny had also been generated (Jenni *et al.*, 1986; Wells *et al.*, 1987; Sternberg *et al.*, 1988). Similar parental and hybrid patterns had been observed previously in isolates from natural populations (Gibson *et al.*, 1980; Tait, 1980). Initial suggestions were that exchange occurred at the procyclic stage in the tsetse midgut (Schweizer *et al.*, 1991) however Gibson & Whittington (1993), using transformed lines, have showed that the salivary glands are the likely site of exchange. Analysis of progeny from crossing experiments has shown that, since both parental and hybrid forms are generated, genetic exchange is not obligatory. Crosses with both homozygous and heterozygous parental strains have now been carried out, producing the equivalent of heterozygous F1 and homozygous backcross progeny respectively (Jenni *et al.*, 1986; Gibson, 1989; Turner *et al.*, 1990; Gibson & Whittington, 1993; Scwhweizer *et al.*, 1994).

The precise mechanism of genetic exchange remains unclear. Pandavoine *et al.* (1986) proposed a complicated model involving multiple fusions of trypanosomes with a subsequent loss of DNA to yield diploid organisms. Several groups have reported that some T. *brucei* hybrid clones were found to have a 3n DNA content (Wells *et al.*, 1987; Gibson & Bailey, 1994); in addition, trisomy of several chromosomes was demonstrated (Gibson *et al*, 1992). In conflict with earlier reports (Paindavoine *et al.*, 1986), all *T. brucei* hybrid clones generated and analyzed in Gibson's laboratory conformed to 2n or 3n status and clones with intermediate DNA contents were not found. Models based on the proposals of Gibson (1995) involve fusion of diploid and/or haploid nuclei and meiosis (Gibson & Stevens, 1999). Additional evidence for fusion and meiosis comes from the observed frequency of chromosomal recombination in hybrids (Gibson & Bailey, 1994) and the analysis of marker segregation (Turner *et al.*, 1990; Schweizer *et al.*, 1994). Indeed, most data now support the involvement of meiosis with exchange explainable in terms of classical Mendelian genetics (Sternberg *et al.*, 1989; Gibson, 1989; Turner *et al.*, 1990; Gibson & Whittington, 1993; Schweizer *et al.*, 1994). In the absence of evidence for a haploid life cycle stage, it is proposed that exchange involves the fusion of haploid nuclei as opposed to haploid cells (Gibson, 1995). In this case, the

mechanism of genetic exchange starts with the fusion of diploid organisms followed by meiosis and fusion of a single pair of haploid nuclei, with the rest being destroyed (Gibson, 1995).

Elucidation of the precise mechanism of exchange has also been complicated by the inheritance of kDNA: maxicircles are inherited uniparentally whereas minicircles are inherited from both parents (Gibson, 1989; Gibson & Garside, 1990; Turner *et al.*, 1995). Two models have been proposed to explain these observations (Birky, 1983; Shapiro & Englund, 1995). In the first model (Birky, 1983) one maxicircle type is lost during mitotic division by a random segregation process. In the second model (Shapiro & Englund, 1995) minicircles are exchanged between parental kDNA networks. For a comprehensive review of genetic exchange in trypanosomatids, see Gibson & Stevens (1998).

Indirect evidence of genetic exchange in natural populations can be demonstrated by population genetic analysis of data gained using molecular markers. These analyses can provide an indirect measure of gene flow within and between populations thus giving an insight into the reproductive strategy of, and subdivisions within, the population. All statistical approaches assume the null hypothesis of panmixia (i.e. random mating). Significant departures from this hypothesis, evidenced by the application of segregation or recombination tests, are indicative of non-sexual reproduction and/or population substructuring (for reviews, see Tibayrenc, 1995; Gibson & Stevens, 1998).

Evidence of genetic recombination has been demonstrated in other trypanosomatids including *Trypanosoma cruzi* and *Leishmania*. For *T. cruzi*, indirect evidence of diploidy and genetic recombination in natural populations comes from data generated by IEA, RAPD and RFLP analyses (Gibson & Miles, 1986; Tibayrenc *et al.*, 1986; Carrasco *et al.*, 1995; Bogliolo *et al.*, 1996). Experimental crosses have not been successful in *Leishmania* (Panton *et al.*, 1991), although naturally-occurring putative hybrids have been reported (Evans *et al.*, 1987; Darce *et al.*, 1991; Kelly *et al.*, 1991; Bonfante-Garrido *et al.*, 1992; Belli *et al.*, 1994; Dujardin *et al.*, 1995b; Banuls *et al.*, 1997). Spontaneous fusion of promastigotes of several *Leishmania* species has been reported in axenic culture and in the sand fly gut (Lanotte & Rioux, 1990).

1.12.2 Implications of genetic exchange

Although recombination experiments in the laboratory have shown that genetic exchange can occur in trypanosomatids, its epidemiological importance remains unclear. Truc and Tibayrenc (1993) point out that the demonstration of genetic exchange in the laboratory shows only that the potential for exchange is not lost. They propose that the demonstration of a correlation between two independent sets of genetic markers (isoenzymes and schizodemes), as shown for *T. brucei*, is classical evidence of clonality. Gibson (1990; 1995) points out however, that non-random sampling of a natural population with too many human and not enough wild vector / reservoir host isolates can give rise to the occurrence of seemingly predominant zymodemes generating substantial deviations from Hardy-Weinberg equilibrium, which Tibayrenc *et al.* (1993) have taken as evidence of clonality. In her analysis of sympatric *T. brucei* subspecies, individual zymodemes are seen to be generated by relatively few patterns for each enzyme, reassorting in every possible combination (Gibson *et al.*, 1990). Gibson thus concludes that genetic exchange is not a rare occurrence in undisturbed habitats.

Whilst the amount of genetic exchange occurring in or between a population is a controversial subject, its theoretical impact is not. The implications of genetic exchange are that drug resistance or virulence factor genes i.e. those encoding greater vector/host range, different clinical presentation in humans, etc. may be mobilized such that the gene may be able to spread more rapidly through the population. Obviously, gene flow would be quicker through a sexual population than through one that is obligately asexual. The selection of a particularly successful phenotype carrying such a gene, however acquired, may be responsible for increased incidence and prevalence of disease (Maynard-Smith *et al.*, 1993).

Evolution is driven at different rates by the divergent selective pressures operating on the parasite by vector and reservoir host combinations. Since the transmission of different *Leishmania* species involves such diverse patterns, the evolution of each species or population is likely to be distinct. For example, Dujardin (1994) proposed that chromosomal plasticity may be limited by the prevalent mode of reproduction within the species. Sexual reproduction, if common, would favour the conservation of chromosomal linkage groups in the population, resulting in conserved consensus karyotypes. However, if reproduction was prevailingly clonal, the genotype would be

replicated as a unit, and independently-propagating clonal lines could evolve highly divergent karyotypes. Bearing in mind the three levels of conservation observed by Lighthall & Giannini (1992), it may be the case that different species of *Leishmania* undergo varying amounts of genetic exchange within and between their populations.

1.13 Aim and objectives of the project

Tibayrenc *et al.* (1991) proposed, based on results from population genetic analyses, that the predominant mode of reproduction in *Leishmania* was asexual. Research into *Leishmania* variation initiated by Prof. Miles and Dr. Evans in the Pathogen Molecular Biology and Biochemistry Unit at LSHTM, provided evidence that genetic exchange could occur in these organisms (Evans *et al.*, 1987; Belli *et al.*, 1994).

The aim of the work described in this thesis is to expand on this initial research by investigating variation and relationships within and between *Viannia* species with a view to providing an insight into the mechanisms generating this variability.

Specific objectives of this work were to:

1. **Select populations of parasites from the same, and separate, *Viannia* species including putative hybrid stocks.**

   Leishmania reference strains and stocks were made available for the study from the WHO *Leishmania* cryobank at LSHTM and from other sources. In addition to reference strains, three populations were selected:

   I. *Viannia* stocks from Brazil, including stocks from the Brazilian States of Pernambuco, Pará and Amazonas (courtesy of Sinval Brandão-Filho, Jeff Shaw and Roberto Naiff) and from Três Braços / Corte de Pedra, Bahia State (courtesy of Cesar Cuba-Cuba and Philip Marsden). This population consisted mostly of *L. V. braziliensis* stocks and was selected to study variation between stocks of this species isolated from a wide geographical area and from different host and vector species.

   II. *L. V. braziliensis, L. V. panamensis* and putative hybrid stocks from Nicaragua (courtesy of Alex Belli). These stocks had previously been shown to include putative *L. V. braziliensis / L. V. panamensis* hybrid stocks (Belli *et*

al., 1994) and were selected to i) examine variation in and between *L. V. braziliensis* and *L. V. panamensis* stocks, ii) re-examine the evidence of genetic recombination between these species using a wider range of techniques and iii) for transformation to drug resistance for use in crossing experiments aimed at demonstrating genetic exchange in the laboratory.

III. Stocks isolated during an epidemiological survey of a cutaneous (CL) and mucocutaneous leishmaniasis (MCL) epidemic site in the Department of Huánuco, Peru (courtesy of Clive Davies, LSHTM and A. E. Llanos-Cuentas and N. Roncal, Peru). These stocks were from an area previously associated with *L. V. peruviana* but where MCL had recently been described, suggesting the presence of *L. V. braziliensis* parasites. This uncharacterized set of stocks provided a unique sample collected over a short time from a localized area and therefore would support the requirements of population genetics analysis.

2. Assess diversity in *Leishmania* reference strains and in the selected populations using phenotypic and genotypic methods.

- Compare the level of discrimination between WHO-designated *Leishmania* reference strains revealed by isoenzyme analysis (IEA) and other techniques including: isoelectric focusing (IEF); random amplified polymorphic DNA (RAPD) analysis; polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of ribosomal RNA small subunit (SSU) gene and internal transcribed spacer (ITS) region; denaturing gradient gel electrophoresis (DGGE).

- Apply techniques to stocks from defined parasite populations.

- Carry out phylogenetic and population genetics analyses of appropriate data obtained for defined parasite populations.

- Assess and implement any new methods or gene targets which become available.

3. Attempt to demonstrate genetic exchange in the laboratory.

- Construct transformation vectors from available trypanosomatid shuttle vectors, incorporating *L. V. braziliensis*-specific sequence for promoting integration.

- Transform putative parental *L. V. braziliensis* and *L. V. panamensis* clones from the Nicaraguan population to drug resistance using established transformation techniques.

- Perform crossing experiments between cloned transformed lines (in culture *in vitro*, in macrophage cell culture, experimental animals and sand flies) in an attempt to generate hybrid progeny, selectable by dual drug resistance.

- Analyze offspring generated from crossing experiments using previously established techniques.

CHAPTER 2 - MATERIALS & METHODS

2.1 *Leishmania* strains and stocks

2.1.1 Reference strains

Leishmania reference strains, selected as representative of species present in the study areas, were obtained from the cryobank at LSHTM, the Instituto de Medicina Tropical "Alexander von Humboldt", Lima, Peru, the Instituto Nacional de Pesquisas da Amazônia (INPA), Amazonas State, Brazil and the Instituto Evandro Chagas, Belém, Para State, Brazil. Reference strains are listed in *table 3.1* (Chapter 3).

2.1.2 *Leishmania* stocks

Leishmania subgenus *Viannia* stocks were selected from three South American countries: Brazil, Nicaragua and Peru. These stocks and their geographical origins will be discussed in greater detail in the relevant chapters (4-6).

2.1.2.1 Stocks from Brazil (see Chapter 4).

Stocks were selected from geographically separate areas and from Três Braços/Corte de Pedra, a localized region in Bahia State. Many of the Brazilian stocks have been analyzed previously (e.g. Cuba-Cuba *et al.*, 1985; 1991; Grimaldi *et al.*, 1991; Lainson *et al.*, 1981). Stocks from geographically separate areas of Brazil were selected mostly from sand fly vector and non-human reservoir sources. These stocks are listed in *Table 4.2* (Chapter 4). The Três Braços/Corte de Pedra stocks represent a population of *L. V. braziliensis* which has shown little variation using isoenzyme analysis (Cuba-Cuba *et al.*, 1985; 1991): stocks were selected from human, animal and sand fly vector sources, and are listed in *table 4.3* (Chapter 4).

2.1.2.2 Stocks from Nicaragua (see Chapter 5).

These human stocks, from geographically separate areas of Nicaragua, have also been partially analyzed (Noyes *et al.*, 1996; Belli *et al.*, 1994). The species *L. V. braziliensis*, *L. V. panamensis* and a putative *L. V. braziliensis/L. V. panamensis* hybrid form have been described (Belli *et al.*, 1994). Stocks were selected to include several representatives of each type, and are listed in *table 5.1* (Chapter 5).

2.1.2.3 Stocks from Peru (see Chapter 6).

These stocks, which represent a localized *Leishmania* population, were isolated from dogs with cutaneous lesions and from human cases of leishmaniasis in a new epidemic focus of cutaneous and mucocutaneous leishmaniasis in the Department of Huánuco, Peru. . Seven stocks from this area have previously been characterized (Dujardin *et al.*, 1995b), however the stocks used in this investigation have not been studied previously. Huánuco stocks are listed in *table 6.1* (Chapter 6).

2.2 Cultivation of *Leishmania*

2.2.1 Solid Media for cultivation of *Leishmania* promastigotes

Parasites held in the LSHTM cryobank were retrieved from liquid nitrogen storage, rapidly thawed and transferred immediately to bi-phasic 4N blood slopes (Evans *et al.*, 1989) overlaid with 250µL proline-balanced salt solution (PBSS). Recipes for culture media are given in *Appendix I*. Peruvian stocks, and Brazilian stocks not held in the LSHTM cryobank, were received on 4N slopes after a minimal number of passages and immediately transferred to fresh 4N blood slopes. Cultures were incubated at 23°C and observed twice weekly until rapidly dividing. Using standard methodology (Evans *et al.*, 1989), newly-received parasites were cryopreserved as soon as possible in order to minimize the number of passages thus retaining the original parasite type(s) present.

2.2.2 Liquid Media for cultivation of *Leishmania* promastigotes

Promastigotes growing well on solid medium were passaged in alpha-modified minimal essential medium (Sigma) supplemented with 10% heat-inactivated foetal bovine serum, 50µg/mL gentamycin, 30mM $NaHCO_3$, 40mM HEPES, 20mM D-glucose, 4mM L-glutamine, 10µM haemin, 30µM adenine, 10µM folic acid and 10µM D-biotin. (All supplements were purchased from Sigma).

2.3 Preparation of *Leishmania* enzyme lysates

Enzyme stabilates were prepared according to the method of Godfrey & Kilgour (1976). Promastigotes were harvested in exponential growth phase from liquid culture by centrifugation (3000*g* at 4°C for 10 minutes). The cells were washed once in ice-cold phosphate-buffered saline (PBS), pH 7.2 and centrifuged as previously. Excess PBS was removed from the pellet by inverting the tube onto tissue followed by

swabbing the inside of the tube using paper towel, carefully avoiding the pellet. The cell pellet was resuspended in a volume of enzyme stabilizer [2mM EDTA; 2mM ε-aminocaproic acid; 2mM dithiothreitol] approximately equal to that of the pellet. Suspensions were freeze-thawed three times in liquid nitrogen, transferred to microfuge tubes and centrifuged at top speed (13krpm) at 4°C for 30 mins. in an Eppendorf microfuge. The supernatant was "beaded" by dropping 15µL aliquots into liquid nitrogen. Frozen "beads" were stored under liquid nitrogen prior to use.

2.4 Preparation of genomic DNA

Genomic DNA was prepared according to the method of Kelly (1993). Promastigotes were harvested in exponential growth phase from liquid culture by centrifugation (3000*g* at 4°C for 10 minutes). The cells were washed once in ice-cold phosphate-buffered saline (PBS), pH 7.2, centrifuged as previously, then gently resuspended in cell lysis buffer [50 mM Tris, pH 8.0, containing 50mM NaCl, 50mM EDTA, 1% SDS] to a density of 10^9 parasites/mL. Proteinase K (Sigma) was added to a final concentration of 100µg/mL, and the mixture was incubated at 37°C overnight. The DNA was purified by extraction with equal volumes of phenol, phenol:chloroform (1:1), and chloroform respectively, followed by precipitation with 2 volumes of absolute ethanol. The precipitate was washed once with 70% ethanol, dried and redissolved in TE buffer (pH 7.2) or ddH_2O both containing 10µg/mL heat-treated RNAse A (Boehringer). The integrity, purity and concentration of DNA samples was assessed on agarose gels, and by measuring the absorbance at 260nm and 280nm in a spectrophotometer (1 OD unit @ A_{260} = 50mg/mL ds DNA).

2.5 Isoenzyme analysis (IEA)

2.5.1 Thin-layer starch-gel electrophoresis (TSGE)

Thin-layer starch-gel electrophoresis (TSGE) was carried out essentially as described by Godfrey & Kilgour and Harris & Hopkinson, (1976). Conditions for electrophoresis and enzyme development were based on those of Miles *et al.* (1980a, 1980b, 1981) and Evans *et al.* (1984) [see *Appendix III*]. Sixteen enzyme systems were investigated for discriminating stocks within *Leishmania Viannia* populations: these are listed in *table 2.5.1*. Eleven systems (bold type in *table 2.5.1*) were selected for IEA of all stocks based on reproducibility and the ability to make a reliable genetic interpretation of the banding patterns observed.

***Table 2.5.1*: Enzyme systems investigated for discrimination between *Leishmania* reference strains using IEA (TSGE and CAE*) and IEF§.**

Enzyme	Abbreviation	E.C. number†
mannose phosphate isomerase	**MPI§**	EC 5.3.1.8
nucleoside hydrolase [inosine substrate]	**NHi*§**	EC 3.2.2.1
nucleoside hydrolase [deoxyinosine substrate]	**NHd**	EC 3.2.2.**x**
esterase	**ES**	EC 3.1.1.1
proline dipeptidase	**PEPD*§**	EC 3.4.13.9
phosphoglucomutase	**PGM§**	EC 2.7.5.1
6-phosphogluconate dehydrogenase	**6PGD**	EC 1.1.1.44
glucose phosphate isomerase	**GPI*§**	EC 5.3.1.9
glucose-6-phosphate dehydrogenase	**G6PD**	EC 1.1.1.49
aspartate aminotransferase	**ASAT**	EC 2.6.1.1
alanine aminotransferase	**ALAT**	EC 2.6.1.2
malate dehydrogenase	MDH*	EC 1.1.1.37
isocitrate dehydrogenase [NADP+]	ICD	EC 1.1.1.42
superoxide dismutase	SOD*	EC 1.15.1.1
pyruvate kinase	PK	EC 2.7.1.40
malic enzyme	ME*	EC 1.1.1.40

Notes to table:

1. Enzyme abbreviation in **bold** type indicates those enzyme system which were used for IEA of all stocks.
2. * - enzyme systems investigated using CAE
3. § - enzyme systems investigated using IEF
4. † EC: Enzyme Commission
5. **x** - full EC number not assigned

2.5.2 Cellulose acetate electrophoresis (CAE)

CAE was carried out according to the methods described by Lanham *et al.* (1981) and according to the manufacturer's handbook (Helena Laboratories, Beaumont, Texas, USA). Conditions for electrophoresis were based on those of Miles *et al.* (1980a, 1980b, 1981) [see *Appendix III*]. Six enzymes (MDH, NHi, PEPD, GPI, SOD and ME) were analyzed using CAE*, see *table 2.5.1*. Staining reactions used for CAE were the same as those used for TSGE gels.

2.6 Isoelectric focusing (IEF)

Isoelectric focusing was performed on 0.5 x 120 x 240mm polyacrylamide gels using a refrigerated horizontal electrophoresis apparatus (FBE-300, Pharmacia). Enzymes used for IEA were tested for suitability in IEF analysis on a small set of stock samples using broad-range (pH 3.5 - 10) Ampholine (Pharmacia)-based polyacrylamide gels. Gels were cast on Gelbond™ (Pharmacia), prefocused at 2000V (15mA, 15W) for 20 mins; 1.5-5μL sample was applied to the gel either directly, or by using a casting applicator strip. Samples were focused at 2000V for 1 hour. Enzymes were detected using the same developers used for IEA. Five enzyme systems resolved well using IEF (see §, *table 2.5.1)*. These systems were reassessed using narrow range gels (range ascertained from pI locations of enzyme bands on broad-range gels prior to use for the IEF of samples). For IEF, GPI, MPI, and PGM were analyzed on pH 5-8 gels; NHi and PEPD were analyzed on pH 4-6 gels.

2.7 Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis was carried out on *Leishmania* reference strains and representative stocks of the putative parental and hybrid stocks from Nicaragua only (see chapters 3 and 5 for discussions). Subsequent RAPD analyses were found to be unreproducible (see section 3.4.4) and, with the development of microsatellite markers for *Leishmania Viannia* species (see section 2.10), this technique was not applied to other stocks.

2.7.1 Primers

Twenty-eight decameric (10-mer) oligonucleotides of arbitrary sequence were used to amplify genomic DNA from *Leishmania* reference strains and from representative stocks of the putative parental and hybrid stocks from Nicaragua. Oligonucleotide primers A1-A6, H1-H6 and L1-L6 were obtained from R&D products (Abingdon, UK), primers D1-D10 were obtained from D. Barker (University of Cambridge). RAPD primer sequences are listed in *Appendix IV*.

2.7.2 PCR amplification

Amplification reactions were performed to a protocol modified from that of Williams *et al.* (1990). PCR amplification reactions contained 10x NH_4 reaction buffer [160mM $(NH_4)_2SO_4$, 670mM Tris-HCl pH 8.8, 0.1% Tween-20 (Bioline)], 2.5mM $MgCl_2$, 0.2mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia LKB), 20pmol primer, 1U *Taq* DNA polymerase (Bioline) and 25ng genomic DNA in a total reaction

volume of 20µL. Double distilled water (used in the reactions and for preparation of reagents) was irradiated in a UV crosslinker to inactivate potentially-contaminating DNA. Reactions were overlaid with 30µL mineral oil (Sigma) and amplified in a Hybaid Thermal Reactor (Hybaid Ltd, Middx., UK) using: 1 cycle of 94°C for 5 min., 37°C for 1 min., 72°C for 1 min., followed by 40 cycles of 94°C for 1 min., 37°C for 1 min., 72°C for 1 min., followed by a final extension of 10 min. at 72°C.

2.7.3 Electrophoresis of RAPD PCR products

Amplification products were electrophoresed through 2% agarose in 1x TAE buffer. After staining with ethidium bromide, products were visualized by UV illumination and documented via a video copy processor (Mitsubishi).

2.7.4 Interpretation of RAPD results

RAPD profiles were scored visually to identify primers which discriminated: between (i) the *Viannia* and *Leishmania* subgenera, (ii) species within the *Viannia* subgenus and (iii) putative parental and hybrid strains from Nicaragua.

2.8 PCR-RFLP analyses: riboprinting and PCR-RFLP of the internal transcribed spacer (ITS)

Two PCR-RFLP techniques were employed to study variation in the *Leishmania Viannia* strains and stocks. Riboprinting, a specific term coined by Clark & Diamond (1991), is the PCR-RFLP analysis of the 18S [also known as the small subunit (SSU)] ribosomal RNA (rRNA) gene. Riboprinting was carried out on *Leishmania* reference strains and representative stocks of the putative parental and hybrid stocks from Nicaragua only (see chapters 3 and 5 for discussions). PCR-RFLP analysis of the internal transcribed spacer (ITS) region of the rRNA locus (also known as Intergenic Repeat Typing [IRT], Cupolillo *et al.*, 1995) was carried out on *Leishmania* reference strains and stocks from Nicaragua only. Both PCR-RFLP techniques were limited in terms of discriminatory power for *Viannia* species and, as for RAPD, with the development of microsatellite markers for *Leishmania Viannia* species (see section 2.10), these techniques were not applied to stocks from the Brazil or Huánuco populations. Since the methodologies employed are almost identical, and since the target sequence of each approach concerns the same gene locus, the techniques will be described together. The organization of the trypanosomatid rRNA locus, a consensus from studies of several genera, is shown in *figure 2.8.1*.

***Figure 2.8.1*: Organization of the trypanosomatid rRNA locus**

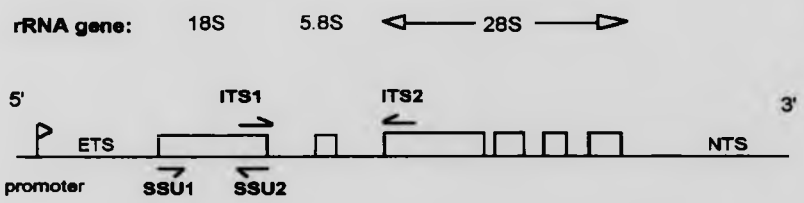


Figure 2.8.1: Organization of the trypanosomatid rRNA locus. Figure adapted from Cupolillo *et al.*, 1995, based on data from *Crithidia fasciculata*, *T. cruzi*, *T. brucei*, & *Leishmania* spp. ETS: external transcribed spacer; NTS: non-transcribed spacer. SSU1 & SSU2 are primers for ribotyping; ITS1 & ITS2 are primers for PCR-RFLP analysis of the ITS region (see *Appendix IV*). Not drawn to scale.

2.8.1 Primers

Primers for the amplification of the 18SrRNA gene were essentially those designed from multiple alignment of trypanosomatid 18S rDNA sequences by Uliana *et al.* (1991, 1994). Oligonucleotide primers SSU1 (S1) and SSU2 (S4) were obtained from R&D Products (UK). *Eco*RI and *Hind*III restriction endonuclease sites were added to the 5' ends of SSU1 and SSU2 respectively to facilitate cloning at a later date. Primers for amplification of the rRNA locus ITS were IR1 and IR2 as described by Cupolillo *et al.* (1995). Primer sequences are given in *Appendix IV*.

2.8.2 PCR reactions

For riboprinting analysis, PCR reactions contained 10x NH_4 reaction buffer [160mM $(NH_4)_2SO_4$, 670mM Tris-HCl pH 8.8, 0.1% Tween-20 (Bioline)], 1.5mM $MgCl_2$, 0.2mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia), 20pmol each primer, 1U *Taq* DNA polymerase (Bioline) and 25ng genomic DNA in a total volume of 100µL. Reactions were overlaid with 60µL mineral oil (Sigma) and amplified in a Hybaid Thermal Reactor (Hybaid Ltd, Middx., UK) using 1 cycle of 95°C for 5 min., 58°C for 60s, 72°C for 2 min., followed by 25 cycles of 95°C for 60s, 58°C for 30s, 72°C for 90s, followed by a single cycle of 72°C for 10 min. For PCR-RFLP analysis of the ITS region between the 18S and 28S rRNA genes, PCR parameters used were those of Stothard *et al.* (1996).

2.8.3 Electrophoretic visualization of the PCR product

Amplification products were checked for size and purity on 1.2% agarose gels in 1x TAE buffer [0.04M Tris-acetate. 0.001M EDTA], stained with ethidium bromide, visualized under UV and documented as described previously.

2.8.4 Preparation of the PCR product for RFLP analysis

PCR products were prepared by adding ddH_2O to a final volume of 300µL. 500µL of chloroform was added, the tube was inverted several times and then spun in an Eppendorf microfuge at top speed (13k rpm) for 5 mins. The diluted product (upper aqueous phase), free of mineral oil, was transferred to a sephadex G-50 spin column to remove unincorporated primers, dNTPs, etc. The column was centrifuged at room temperature for 3 minutes at 1500 rpm and the eluate transferred to a fresh tube. Prepared products were stored at +4°C before digestion.

2.8.5 RFLP analysis

Enzymes were selected for digestion of the PCR-products based upon data from a restriction map of the *L. V. braziliensis* 18SrRNA sequence (Genbank accession no.: M80292), high G/C base content (*Leishmania* have a GC-rich genome) and short length (4-cutters cut more often than enzymes of greater length). Under conditions recommended by the enzyme supplier, 20µL of each diluted amplification product (100-200µg DNA) was digested in 30µL (total volume) with each of the enzymes listed in *table 2.8.1*. Not all enzymes were used for both techniques: combinations are listed in *table 2.8.1*. Digestion mixes were incubated at 37°C for 2 hours (except *Taq*I which was incubated at 65°C for 2 hours).

2.8.6 Visualization of products from the digestion mix

For PCR-RFLP of the ITS only, products from the digestion were run out on 1.2% agarose gels, as previously described, to confirm complete digestion. Products were analyzed on 6% (T), 3.5%(C) polyacrylamide gels (see Appendix II) in 1x TBE buffer. PAGE gels were silver-stained according to the method of Vidigal *et al.* (1994) and photographed onto Polaroid print film (type 667) using a Polaroid MP-4 land camera.

2.8.7 Interpretation of PCR-RFLP PAGE gels

Fragments were scored visually, as for RAPD analysis, to identify enzymes which discriminated: between (i) the *Viannia* and *Leishmania* subgenera, (ii) species within the *Viannia* subgenus and (iii) putative parental and hybrid strains from Nicaragua.

Table 2.8.1: **Enzymes used for riboprinting and PCR-RFLP analyses.**

Enzyme	Recognition site	Technique	Supplier*
*Rsa*I	GT˅AC	Riboprinting	P
*Sac*II / *Nar*I	CCGC˅GG	Riboprinting	B
*Sau*3AI	˅GATC	Riboprinting	P
*Alu*I	AG˅CT	Riboprinting / PCR-RFLP (ITS)	P
*Hpa*II / *Msp*I	C˅CGG	Riboprinting / PCR-RFLP (ITS)	B
*Taq*I	T˅CGA	Riboprinting / PCR-RFLP (ITS)	P
*Hae*III	GG˅CC	Riboprinting / PCR-RFLP (ITS)	P
*Bst*UI	CG˅CG	PCR-RFLP (ITS)	N
*Cfo*I / *Hha*I	GC˅GC	PCR-RFLP (ITS)	N
*Csp*6I	G˅TAC	PCR-RFLP (ITS)	N
*Dde*I	C˅TNAG	PCR-RFLP (ITS)	N
*Dpn*I	GA˅TC	PCR-RFLP (ITS)	N
*Eco*RI	G˅AATTC	PCR-RFLP (ITS)	P
*Fsp*I	TGC˅GCA	PCR-RFLP (ITS)	N
ScrFI	CC˅NGG	PCR-RFLP (ITS)	N
*Sph*I	GCATG˅C	PCR-RFLP (ITS)	N

*Enzyme supplier: Promega [P], Boehringer [B], New England Biolabs [N]

2.9 Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed according to the method of Stothard *et al.* (1998), based on the protocols of Myers *et al.* (1987, 1990) and Michaelides *et al.* (1995). Perpendicular gradient gels (polyacrylamide gel containing 0 - 40% gradient of denaturant, 8M urea and 40% formamide) were used to determine the melting profile of one of the 18SrRNA amplification products in order to select the denaturation range of the product for subsequent analysis on parallel gradient gels. 30µL of *Alu*I-digested *L. V. braziliensis* PCR product was added to 60µL of loading buffer (1mL 10% sucrose, 1µL 1% bromophenol blue, 1% xylene cyanol). The sample was electrophoresed at 150V for 2.5 hours at 60°C, then silver stained. 10 - 25% parallel gradient gels were subsequently used to analyze 10µL of each of the complete range of amplification products. Parallel gels were electrophoresed at 65V for 6 hours at 60°C. Gels were silver stained and photographed as described previously.

2.10 Microsatellite analysis

2.10.1 Microsatellite analysis - background

Microsatellite DNA sequences specific for *Leishmania* subgenus *Viannia* species were identified by screening a genomic library of *L. V. peruviana* strain LC1116 with a $(CA)_{11}$ oligonucleotide by Russell *et al.* (1999). Positive clones were sequenced to identify microsatellite sequences of which three (AC01, AC16, AC52) were selected for further characterization and study (Genbank™ accession numbers AF139110, AF139111 and AF139112, respectively). Primers were designed from the flanking regions of these three microsatellites using DNAstar© software. Primer sequences are listed in *Appendix IV*.

2.10.2 PCR of microsatellite loci for PAGE analysis

Amplification reactions were carried out according to a protocol modified from that of Russell *et al.* (1999). PCR amplification reactions contained 10X NH_4 reaction buffer [160mM $(NH_4)_2SO_4$, 670mM Tris-HCl pH 8.8, 0.1% Tween-20 (Bioline)], 1mM (AC01, AC52) or 2mM (AC16) $MgCl_2$, 0.2mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia LKB), 5pmol each primer, 0.5% formamide (v/v), 1U *Taq* DNA polymerase (Bioline) and 25ng genomic DNA in a total reaction volume of 50µL. Reactions were overlaid with 30µL mineral oil (Sigma) and amplified in a Hybaid Thermal Reactor (Hybaid Ltd, Middx., UK) using 35 cycles of 95°C for 30s, 62°C (AC01 and AC52) or 60°C (AC16) for 30s, 72°C for 1 min, followed by a final extension period of 10 mins at 72°C. The lower annealing temperature was used for those stocks which gave poor yield at the higher temperature; in addition, for these stocks, the number of amplification cycles was increased to 38.

2.10.3 Visualization of microsatellite PCR products

PCR products were visualized using both agarose gel electrophoresis (AGE) and polyacrylamide gel electrophoresis (PAGE) as described previously. PCR product(s) were initially analyzed in 1.2% agarose gels after staining with ethidium bromide. This permitted sizing of the product and identified those stocks which failed to amplify. Stocks which failed to amplify under the standard PCR conditions were subjected to PCR again, using lower annealing temperatures and differing magnesium concentrations. DNA of stocks which consistently failed to amplify was tested for integrity by subjecting it to a PCR control using primers for the 18S ribosomal RNA gene (described in section 2.8).

Stocks producing a product were subsequently analyzed by non-denaturing PAGE and silver staining as for PCR-RFLP analysis (section 2.8). These techniques have improved resolution and sensitivity over agarose gel electrophoresis / ethidium bromide staining. Stocks were scored by, and divided into, distinct pattern types visually.

2.10.4 PCR of microsatellite loci for Genescan® and Genotyper® 2.0 analyses.

PCR reactions and conditions were as above (section 2.10.2) but with several modifications. Fluorophore-labeled reverse primers were used in the reaction for labeling the PCR product (each primer was included at 5pmol concentration). PCR amplifications were carried out in microtitre plates with sealed lids and used the heated-lid option. Using microtitre plates enabled multichannel pipettes to be used for multiplexing as the PCR reaction for each stock was carried out in the same position in a separate microtitre plate. The use of the heated lid option obviated the need for an oil overlay: the latter complicates multiplexing and interferes with Genescan® analysis. Reaction volumes were reduced to 10μL and PCR was carried out in an MJ Research PTC-200 Peltier thermocyler (Genetic Research Instrumentation Ltd., UK).

2.10.5 Genescan® and Genotyper® 2.0 analyses

To obtain an accurate size of the PCR product(s) for each stock at each microsatellite locus, stocks were subjected to Genescan® and Genotyper® 2.0 analyses. These methods, described in more detail in chapter 3, allow the detection and sizing of fluorescently-labeled PCR products from denaturing PAGE using the ABI 377 PRISM™ automated sequencing machine (Applied Biosystems, UK). PCR products are accurately sized by the inclusion of a fluorescently-labeled internal size marker.

Each reverse PCR primer was labeled with one of three fluorescent labels: AC01B-FAM™ (blue), AC16B-TET™ (green) or AC52B-HEX™ (yellow) (Perkin-Elmer, UK). As each different microsatellite locus PCR-product was labeled with a different fluorophore, all three PCR products for a given stock were run in the same lane of the gel, so called "multiplex analysis". PCR products were pooled in the ratio 1:50:10 (FAM:TET:HEX) for multiplex analysis. 2uL of the pooled product was added to 3.5uL of loading mix [containing ABI PRISM™ TAMRA™-500 (red), Perkin-Elmer, UK]: 2uL of this mix was loaded for analysis. Each gel was used twice: in between

sample runs, the gel was pre-run for 30-60 minutes to ensure that all size marker bands had completely run out before the next loading. Sizing of the PCR products was carried out using Genescan® and Genotyper® version 2.0 programs.

2.10.6 Sequencing reactions

Sequencing reactions were carried out using a dye terminator cycle sequencing kit (ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin-Elmer, UK), slightly modified from those given in the manufacturer's instructions. Sequencing reactions contained 4μL ABI sequencing reaction mix, 0.5pmol primer, 4μL ddH_2O and 25ng genomic DNA in a total reaction volume of 10μL. Reactions were overlaid with 10μL mineral oil (Sigma) and amplified in a Hybaid Thermal Reactor (Hybaid Ltd, Middx., UK) using 35 cycles of 95°C for 30s, 50°C for 15s and 72°C for 4 min.

2.10.7 Sample preparation for automated sequencing

Sequencing reaction products were prepared for automated sequencing by adding 90μL ddH_2O, followed by a pulse spin in a microfuge to separate the mineral oil and aqueous phase. The aqueous phase was transferred to a Sephadex G-50 spin column. The column was centrifuged at 1500 rpm for 3 minutes at room temperature and the eluate transferred to a fresh tube. The eluate, containing DNA from the sequencing reaction, was frozen in liquid nitrogen; DNA was precipitated from the eluate by freeze-drying overnight. DNA was stored dry at -20°C until ready for sequencing.

2.10.8 Automated sequencing

Dried DNA sequencing products were resuspended in 5μL loading buffer containing dextran blue; 2μL of this mix was loaded per lane. Automated sequencing was carried out on an ABI 377 PRISM™ automated sequencing machine (Applied Biosystems, UK). The sequences were edited and aligned using ABI PRISM™ Sequence Navigator version 1.01 (1984 -94, Applied Biosystems, UK).

2.11 Population genetic analyses

Population genetic analyses were carried out on the Huánuco stocks only. These stocks encompassed all those sampled, and hence represent an unbiased, indiscriminate sample. Furthermore, these stocks fulfill the criteria for population genetic tests (Tibayrenc & Ayala, 1991) in that they were collected from a small area

over a discrete time period. The tests used for population genetic analyses will be described and discussed further in Chapter 6.

2.11.1 Analyses of isoenzyme data

Isoenzyme data is phenotypic but it is possible to infer genotypes from these electromorphs based on knowledge of an enzyme's structure (May, 1992).

2.11.1.1 Hardy-Weinberg test

For each enzyme locus, genotype frequencies were calculated and tested for departure from Hardy-Weinberg (HW) equilibrium using the Genepop package (Raymond & Rousset, 1995) version 3.1d (March 1999) [http://www.cefe.cnrs-mop.fr/pub/pc/msdos/genepop]. Exact P-values associated with H_0 (i.e. null hypothesis of panmixia) were calculated using the probability test (exact HW test); no standard error value is computed for exact tests. In addition, the expected number of heterozygotes was calculated and used to determine the within-population fixation index (Fis, as described by Weir & Cockerham, 1984) for each locus.

2.11.1.2 Tests for detection of linkage disequilibrium

Three statistical tests for detecting linkage disequilibrium (LD) were performed: the Index of Association (I_A; Maynard Smith *et al.*, 1993); the D' index (Lewontin, 1964) and the r^2 (square of the correlation coefficient) index (Hill and Robertson, 1968).

2.11.2 Analyses of microsatellite data

Hardy-Weinberg and linkage disequilibrium analyses were carried out for the AC01 and AC16 loci only, since AC52 data was inconclusive for many of the Huánuco stocks. Tests were carried out as for enzyme data.

2.12 Phylogenetic analyses

2.12.1 Phylogenetic analysis of isoenzyme data

Isoenzyme phylogenies were constructed using quartet puzzling (Strimmer & von Haeseler, 1996), PUZZLE© version 4.0.2 (1999) [ftp://ftp.ebi.ac.uk/pub/software (European Bioinformatics Institute, UK)]. This programme reconstructs phylogenies of nucleotide, amino acid and two-state data using a maximum-likelihood (ML) approach. Analyses were performed on "Kilburn" (an Origin 2000 public-access

supercomputer based at Manchester Computer Centre). ML estimations were calculated for 10,000 quartet puzzling steps. The ML distance matrix was reconstructed using the neighbour-joining method (Saitou & Nei, 1987). The number of invariant loci was automatically estimated from the data. All alleles were equally weighted, therefore highly polymorphic enzyme loci were assumed to be more informative than less polymorphic loci. Phylogenetic trees were viewed and printed from the TreeView programme [http://taxonomy.zoology.gla.ac.uk/rod/treeview.html. (Roderic Page, unpublished)]. It was not possible to calculate bootstrap values for these data since ML approaches are highly reiterative processes and as such are computationally too expensive. However, "quartet supports", measures similar to bootstraps (Strimmer & von Haeseler, 1996) were calculated. These values are the percentage support of each branch, representing the number of quartets supporting each given branching order.

For comparison, isoenzyme phylogenies were also constructed using a similarity approach (based on a similarity coefficient matrix, SCM, produced according to Jaccard's method, 1908). Using this approach, equal weighting is given to all alleles, as with the ML approach, but the "over-weighting" of polymorphic loci is removed. Dendrograms were constructed by UPGMA (the unweighted pair-group method using arithmetic averages) using the SYN-TAX-pc package (Podani, 1993).

2.12.2 Phylogenetic analysis of microsatellite data

Molecular phylogenies for sequence data from the AC01 microsatellite locus were constructed using the neighbor-joining method for 1000 bootstrap replications, using the Kimura 3-parameter model in PAUP* 4.0 beta version (Swofford, 1997). This distance-based method uses a comprehensive algorithm which takes into account reverse mutations, transition-transversion ratios and transversion type. A maximum likelihood approach was not taken for this data set as it was computationally too expensive to perform due to the added complexity (4 nucleotide states) of the data.

2.13 Vector construction

2.13.1 Strategy

The aim of this work was to attempt to demonstrate genetic exchange in *Leishmania*. Representatives of the Nicaraguan "parental" *L. V. braziliensis* and *L. V. panamensis* stocks (XD28 and ZF01 respectively) and the *L. V. braziliensis* stock, M2903, from which the *hsp70* targeting fragment (see below) was derived, were selected for transformation to different drug resistance phenotypes. Hybrid offspring generated from crossing experiments would be selected by dual drug resistance phenotype.

2.13.2 Vectors

Kinetoplastids have unusual mechanisms for gene transcription. To ensure that these transformation vectors functioned in the *Leishmania* cell, these vectors carried, in addition to the resistance gene, processing elements involved in mRNA maturation (i.e. the 5'-spliced leader [SL] acceptor site and 3'-polyadenylation [pA] signal).

Vectors were designed so as to permit homologous integration of the vector DNA into the host cell's genome (as opposed to episomal vectors which replicate independently of the host cell's machinery). Integrative vectors were particularly required for the analysis of progeny generated from crossing experiments. The analysis of progeny is made easier with the use of integrative vectors because simple Mendelian inheritance is obeyed resulting in stable drug resistance phenotypes. With episomal vectors, the drug resistance phenotype of progeny may be unstable owing to the variable number of episome copies. To this end, *Leishmania*-specific gene targeting fragments were incorporated. Fragments targeting the *L. V. braziliensis hsp70* genes were isolated from the plasmid *pT3T719U-133P* (see below). The *hsp70* gene array is multicopy and constitutively-expressed to allow life-cycle-independent expression.

Two vectors, carrying genes conferring different drug resistance phenotypes, were required for transformation experiments: these were constructed from three existing plasmid vectors (*pT3T719U-133P*, *pTEX*, and *pTEX-ble*).

2.13.2.1 *pT3T719U-133P*

pT3T719U-133P is a *pUC*-based vector containing 133P, a 2472bp cDNA corresponding approximately to the C-terminal three-quarters of the coding sequence

and 3' untranslated region of the 70kDa heat shock protein gene (*hsp70*) from *L. V. braziliensis* strain M2903 (Amorim *et al.*, 1996). The coding region of 133-P contains a unique *Bgl*II site. The structure of *pT3T719U-133P*, is shown in *figure 2.13.2.*

Figure 2.13.2: pT3T719U-133P

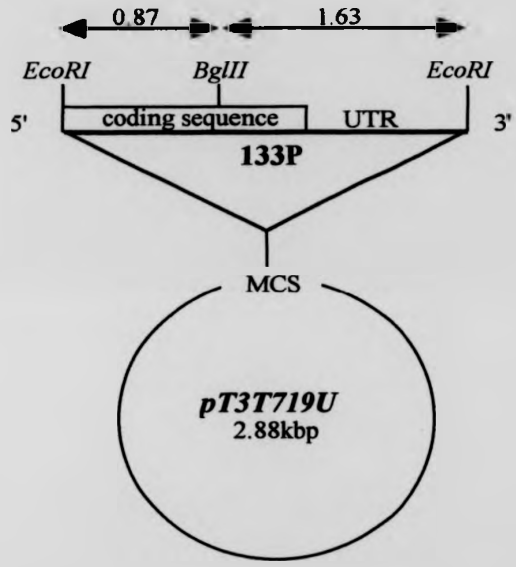


Figure 2.13.2: structure of *pT3T719U-133P*. MCS – multiple cloning site; UTR – untranslated region. *pT3T719U-133P* was a gift from M. Carrington (Cambridge)

2.13.2.2 *pTEX*

pTEX is a *pBluescript*-based vector containing the gene encoding neomycin phospho-transferase II and has been described previously (Kelly *et al.*, 1992). The neomycin resistance gene (*neo*^{r}) and multiple cloning site (MCS) are both flanked by the 5'-upstream and the 3'-downstream regions of the *T. cruzi* genes encoding glyceraldehyde phosphate dehydrogenase (*gapdh*). *pTEX* has been shown to confer resistance to neomycin in *T. cruzi* and *Leishmania* spp. (Kelly *et al.*, 1992). The vector also contains the ampicillin resistance gene (*amp*^{r}) derived from *pBluescript*. *pTEX* is maintained episomally in transformed parasites.

2.13.2.3 *pTEX-ble*

pTEX-ble was constructed from *pTEX* (Nozaki & Cross, 1994) by replacing the *neo*^{r} of pTEX with the phleomycin resistance gene (*ble*^{r}) from *Streptoalloteichus hindustanus*. *Ble*^{r} confers resistance to phleomycin derivatives (Drocourt *et al.*, 1990).

2.13.2.4 Construction of the vector conferring neomycin resistance

pT3T719U-133P was digested with *Eco*RI. The digest was run out on a 0.8% agarose gel in TBE buffer containing ethidium bromide. The 2.5kb *Eco*RI-cut 133P fragment was excised from the gel and purified using the Geneclean® kit (Bio101 Inc, CA, USA) according to the manufacturer's instructions. *pTEX* was linearized with *Eco*RI and then treated with shrimp alkaline phosphatase (Boehringer) to prevent subsequent religation. The linearized *pTEX* and 133P insert were ligated overnight at room temperature using T4 DNA ligase (Boehringer).

2.13.2.5 Construction of the vector conferring phleomycin resistance

pTEX-ble was digested with *Pvu*II and *Bam*HI to isolate the gene for phleomycin resistance (*ble*^{r}). The digest was run out on a 0.8% agarose gel in TBE buffer containing ethidium bromide. The 0.5kb fragment containing *ble*^{r} was excised from the gel and purified as described previously. For ligation, the fragment was blunt-ended by DNA polymerase Klenow fragment using standard protocols (Sambrook *et al.*, 1989). *pT3T719U-133P* was linearized with *Bgl*II and blunt-ended using DNA polymerase Klenow fragment (Boehringer). This was then treated with shrimp alkaline phosphatase to prevent subsequent religation. The *pT3T719U-33P* vector and *ble*^{r} fragment were ligated overnight as previously described.

2.13.3 Transformation of competent *E. coli*

Ligated *pTEX-133P* DNA was transformed into competent *E. coli* (strain DH5α) cells. After heat-shocking (3mins at 42°C), cells were plated out on LB agar plates containing ampicillin (250U/mL) and incubated at 37°C overnight.

2.13.4 Selection of *E. coli* transformants

Colonies were picked and inoculated into LB broth containing ampicillin (250U/mL) and were incubated at 37°C overnight. Plasmid DNA was harvested from each culture using a standard alkaline lysis method (Birnboim & Doly, 1979) and was analyzed by

electrophoresis in 0.8% agarose gel. The expected size for the *Neo*^{r} (*pTEX-133P*) vector was 7.9kbp and for the *Ble*^{r} (133P-*ble*) vector was 5.4kbp. Plasmid DNA of the required size was subjected to digestion with *Bgl*II, *Nco*I and *Sal*I to confirm the presence and orientation of the inserted DNA.

2.13.5 Bulk culture of plasmid vector DNA

Cultures containing the required vector DNA were subcultured into 200mL of LB broth containing 250U/mL ampicillin and grown up overnight at 37°C. Plasmid DNA was harvested for transformation as previously.

2.14 Transformation of *Leishmania*

2.14.1 Electroporation

Electroporation of *L. V. braziliensis* stocks M2903 and XD28, and *L. V. panamensis* stock ZF01 was carried out according to the method of Kelly *et al.* (1995b), based on conditions used for electroporation of *Trypanosoma cruzi*. The *L. V. braziliensis* stock M903 was included as an integration targeting control as this stock was the source of the *hsp*70 targeting sequence. Briefly, cells were washed in PBS, resuspended to 10^9/mL in 2 x 1mL electroporation buffer [272mM sucrose, 7mM sodium phosphate, pH 7.2], and 1mL aliquots transferred to labeled wells of a 24-well microtitre plate. After a 5 minute incubation on ice, 25µg *pTEX-133P* vector DNA was added to the required wells (DNA+); cells were pulsed (400V/99ms) 9x using the Hoeffer Progenitor electroporator (model PG1). The plate was incubated at 23°C overnight.

2.14.2 Selection of *Leishmania* transformants

Approximately 24 hours after electroporation, electroporated cultures were checked microscopically for viability. Subcultures were made from each electroporated well into αMEM liquid culture medium containing 0, 25, 50, 75 and 100µg/mL of the neomycin analogue, G418. Cells were incubated at 23°C, and checked daily for growth.

2.14.3 Southern analysis of G418-resistant *Leishmania*

Genomic DNA was extracted from uncloned transformed cells grown in liquid medium as described in section 2.3.1. Approximately 5µg genomic DNA was digested

using *Sal*I, *Bam*HI and *Pst*I separately, and *Sal*I/*Nco*I combined. Products were run out at 50V for 6 hours on a 1.2% agarose gel containing ethidium bromide. After photographing the gel, DNA was transferred to nylon membrane (Hybond N, Amersham) using standard Southern blotting protocols (Sambrook *et al.*, 1989), and subsequently bound to the membrane by UV irradiation. Probes for the neomycin resistance gene (from pGEM-*neo* and pTEX) were radiolabelled with [α-^{32}P]dCTP using the Rediprime® random-primer DNA labeling kit (Amersham International plc) according to the manufacturer's instructions. The membrane was hybridized overnight at 42°C in a solution containing 6X SSC [0.3M NaCl, 0.03M sodium citrate, pH 7.0], 5X Denhardt's, 0.5% SDS, 40% formamide, 100µg/mL sonicated salmon sperm DNA. Blots were washed after hybridization in 2x SSC and 0.2x SSC at 55°C before autoradiography at -70°C.

CHAPTER 3 - COMPARISON OF REFERENCE STRAINS

3.0 Introduction

This work aimed to clarify relationships of species within the *Leishmania* subgenus *Viannia*. To this end, new and established techniques were assessed for their ability to discriminate within and between the major *Leishmania* species found in the New World. Techniques found able to discriminate between the species of the subgenus *Viannia* were subsequently used to study the diversity of parasites from three areas (chapters 4-6).

3.1 *Leishmania* reference strains

Reference strains, selected as representative of *Leishmania* species present in the areas to be studied, were obtained from the *Leishmania* cryobank at LSHTM and from R. Naiff† (Instituto Nacional de Pesquisas Amazonas, Manaus, Amazonas, Brazil). The strains are listed in *table 3.1*.

***Table 3.1*: *Leishmania* reference strains**

ISOLATE CODE NO. (Abbreviation)	HOST SPECIES	ORIGIN	*LEISHMANIA* SPECIES	LESION TYPE
MHOM/BR/84/LTB300* (Lb)	Human	Bahia / Brazil	*L. V. braziliensis*	MCL
MHOM/PA/71/LS94* (Lpa)	Human	Panama	*L. V. panamensis*	CL
MHOM/BR/75/M4147* (Lg)	Human	Para / Brazil	*L. V. guyanensis*	CL
MHOM/PE/84/LC39 (Lpe)	Human	Ancash/Peruvian Andes	*L. V. peruviana*	CL
IWHI/BR/94/M15065 (Ls)	Sandfly	Parana / Brazil	*L. V. shawi*	-
MHOM/BR/81/M6426* (Ll)	Human	Para / Brazil	*L. V. lainsoni*	CL
ISQU/BR/86/IM2832†§ (IM)	Sandfly	Amazonas / Brazil	*L. V.* sp. n. gp III	-
MHOM/BZ/82/BEL21* (Lm)	Human	Belize	*L. L. mexicana*	CL
MHOM/BR/73/M2269* (La)	Human	Para / Brazil	*L. L. amazonensis*	CL/DCL
MHOM/BR/74/PP75* (Lc)	Human	Brazil	*L. L. chagasi*	VL

* indicates WHO-designated reference strains; § Grimaldi *et al.*, 1991

3.2 Isoenzyme analysis

Eleven enzyme systems (twelve loci) were employed which were found to discriminate between all of the reference strains. *Table 3.2.1* lists the enzyme systems found to discriminate between reference strains of species from the *Viannia* subgenus. No additional data was gained using CAE since the resolution of bands from *Viannia* stocks was greater using TSGE.

Table 3.2.1: **Enzyme systems discriminating between *Viannia* reference strains.**

	Lb					
Lpe	1, 3	***Lpe***				
Lg	1, 2, 5, 6, 7, 9, 10, 11	1, 2, 3, 5, 6, 7, 9, 10, 11	***Lg***			
Lpa	1, 2, 5, 6, 7, 10, 11	1, 2, 3, 5, 6, 7, 10, 11	5, 9	***Lpa***		
Ls	1, 2, 5, 6, 7, 9, 10, 11	1, 2, 3, 5, 6, 7, 9, 10, 11	5, 6	5, 6, 9	***Ls***	
Ll	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	***Ll***
IM	1, 4, 5, 6, 7, 8, 9, 10, 11	1, 3, 4, 5, 6, 7, 8, 9, 10, 11	1, 2, 4, 5, 6, 7, 8, 9, 10, 11	1, 2, 4, 5, 6, 7, 8, 9, 10, 11	1, 2, 4, 6, 7, 8, 9, 10, 11	1, 2, 3, 4, 5, 7, 8, 9, 10

Species: *Lb - L. V. braziliensis; Lpe - L. V. peruviana; Lg - L. V. guyanensis; Lpa - L. V. panamensis; Ls - L. V. shawi; Ll - L. V. lainsoni;* IM - IM2832 (*L. V.* sp. n. gpIII).

Enzyme: 1 - MPI; 2 - NHi; 3 - NHd; 4 - ES; 5 - 6PGD; 6 - PGM; 7 - PEPD; 8 - GPI; 9 - G6PD; 10 - ASAT; 11 - ALAT

Banding patterns observed in the reference strains are shown for the enzymes NHi, PEPD, 6PGD and ES in *figure 3.2.1*. For each enzyme locus, bands were scored from 1 - *n*, where 1 was the fastest migrating band and *n* was the slowest. Banding patterns are shown schematically for all enzymes studied in *figure 3.2.2*.

***Figure 3.2.1*:** **Isoenzyme banding patterns of *Leishmania* reference strains.**

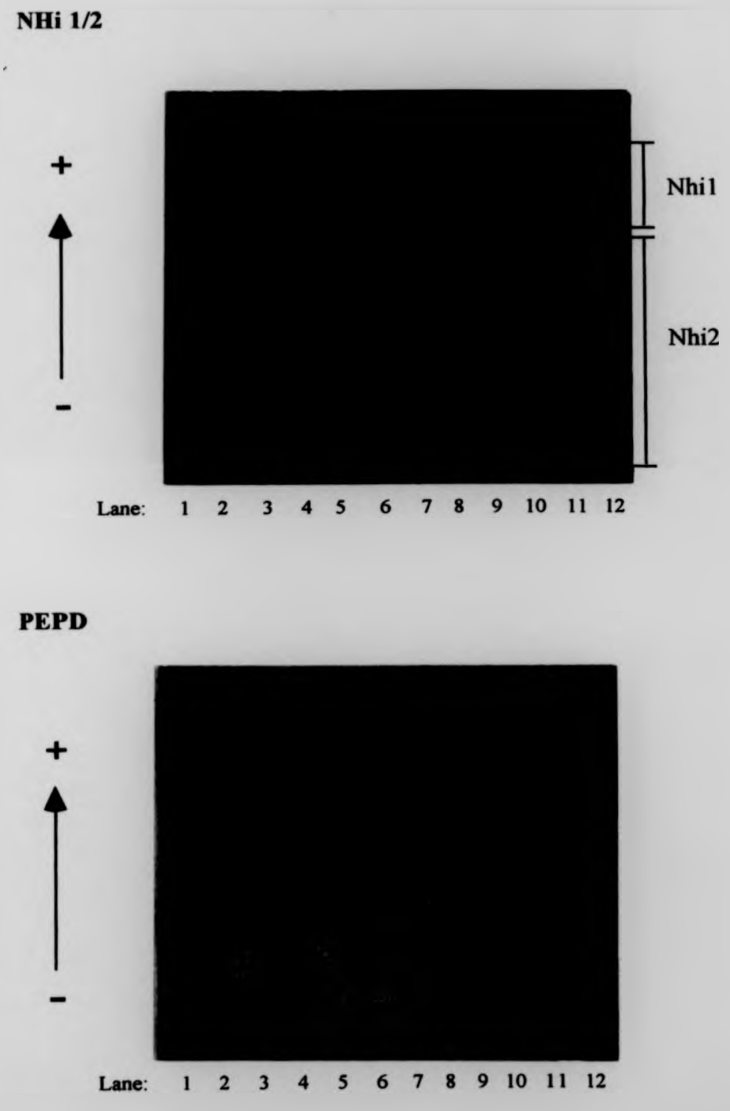
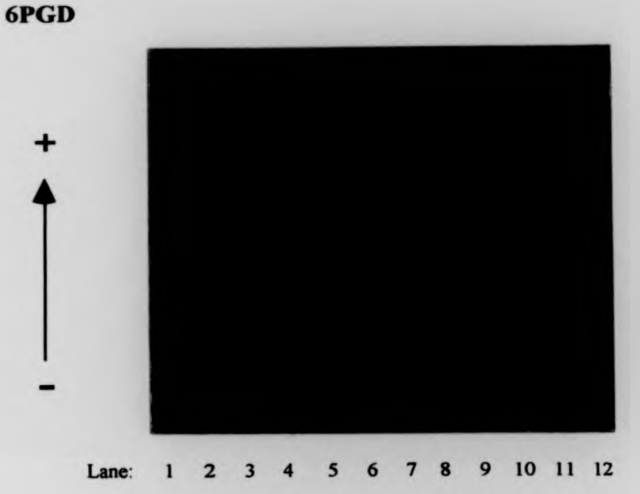


Figure 3.2.1 : Isoenzyme banding patterns of the reference strains. Lanes 1 & 2 - *L. V. braziliensis*; lanes 3 & 4 - *L. V. peruviana*; lane 5 - *L. V. guyanensis*; lane 6 - *L. V. panamensis*; lane 7 - *L. V. shawi*; lane 8 - *L. V. lainsoni*; lane 9 - *L. V.* sp. (see text); lane 10 - *L. L. amazonensis*; lane 11 - *L. L. mexicana*; lane 12 - *L. L. chagasi*. Arrow indicates direction of electrophoresis.

***Figure 3.2.1*:** **Isoenzyme banding patterns of *Leishmania* reference strains (continued).**



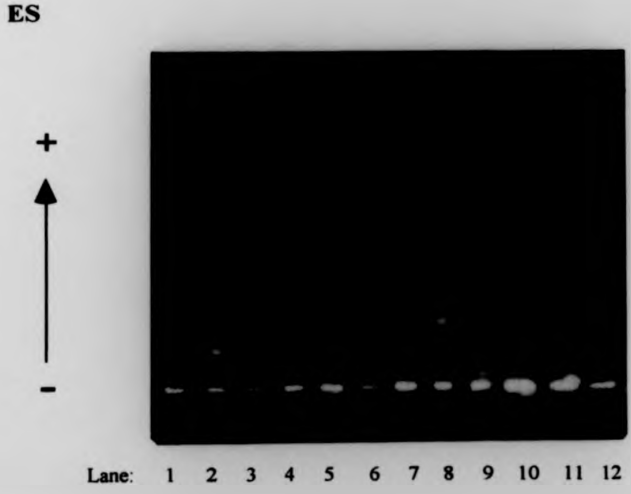


Figure 3.2.1 : Isoenzyme banding patterns of the reference strains. Lanes 1 & 2 - *L. V. braziliensis*; lanes 3 & 4 - *L. V. peruviana*; lane 5 - *L. V. guyanensis*; lane 6 - *L. V. panamensis*; lane 7 - *L. V. shawi*; lane 8 - *L. V. lainsoni*; lane 9 - *L. V.* sp. (see text); lane 10 - *L. L. amazonensis*; lane 11 - *L. L. mexicana*; lane 12 - *L. L. chagasi*. Arrow indicates direction of electrophoresis.

***Figure 3.2.2*:** **Diagrammatic representation of isoenzyme discrimination between *Leishmania* reference strains**

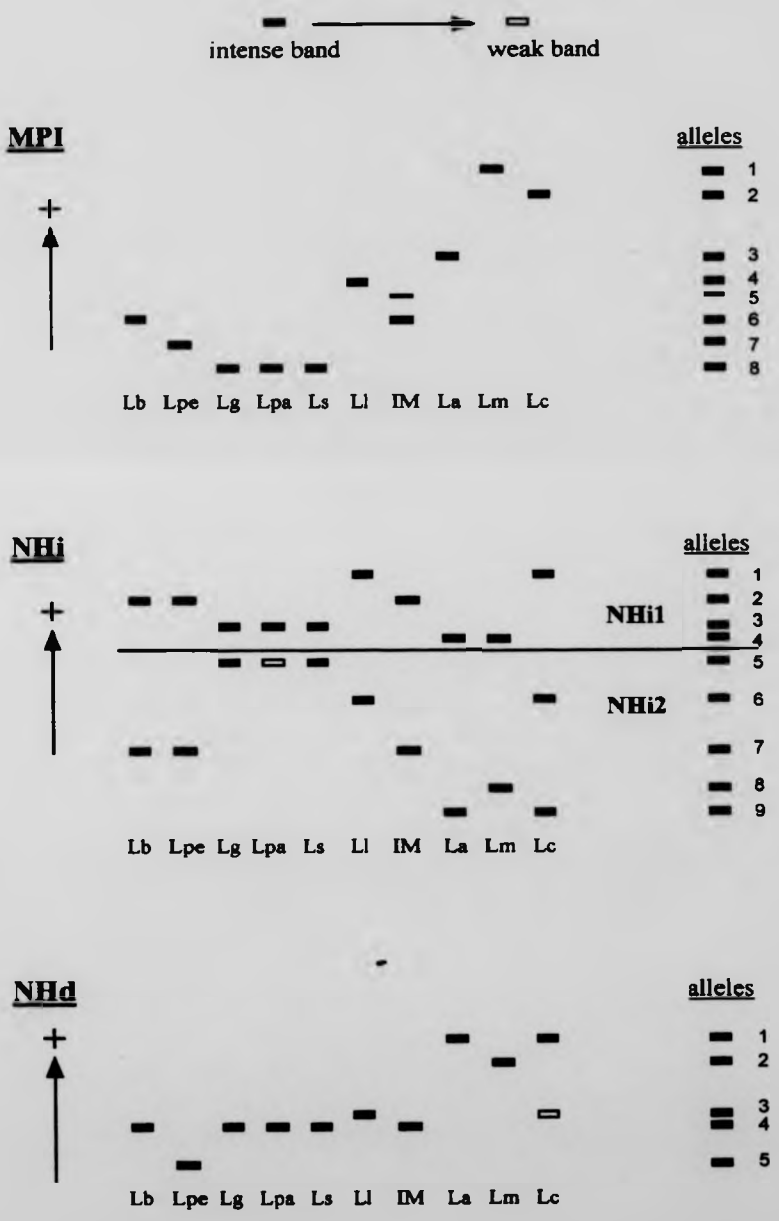


Figure 3.2.2: **Diagrammatic representation of isoenzyme discrimination between *Leishmania* reference strains (continued)**

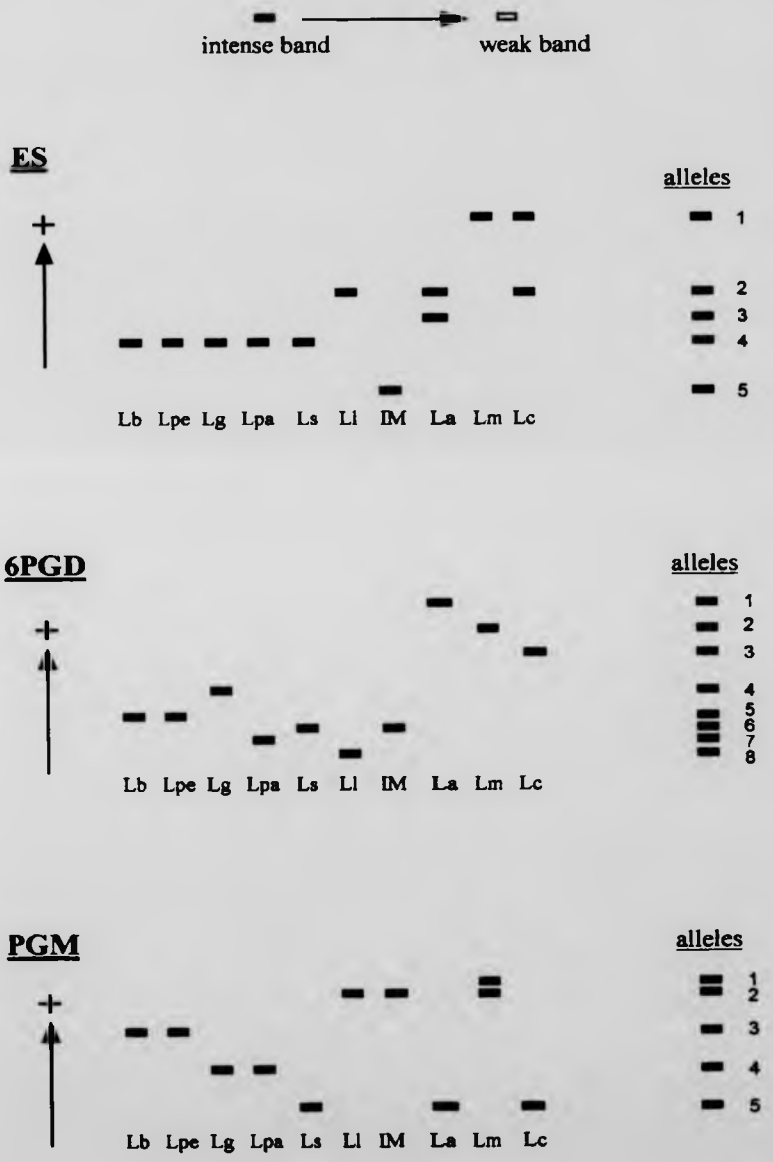


Figure 3.2.2: **Diagrammatic representation of isoenzyme discrimination between *Leishmania* reference strains (continued)**

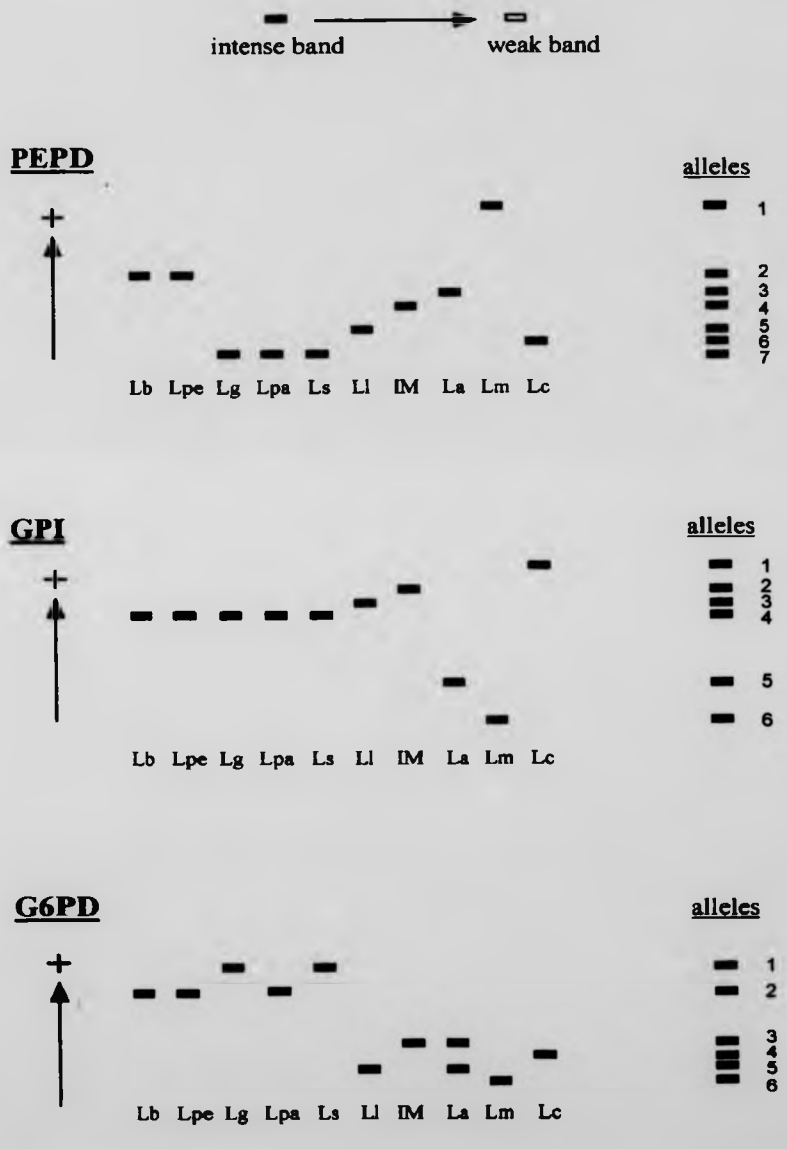
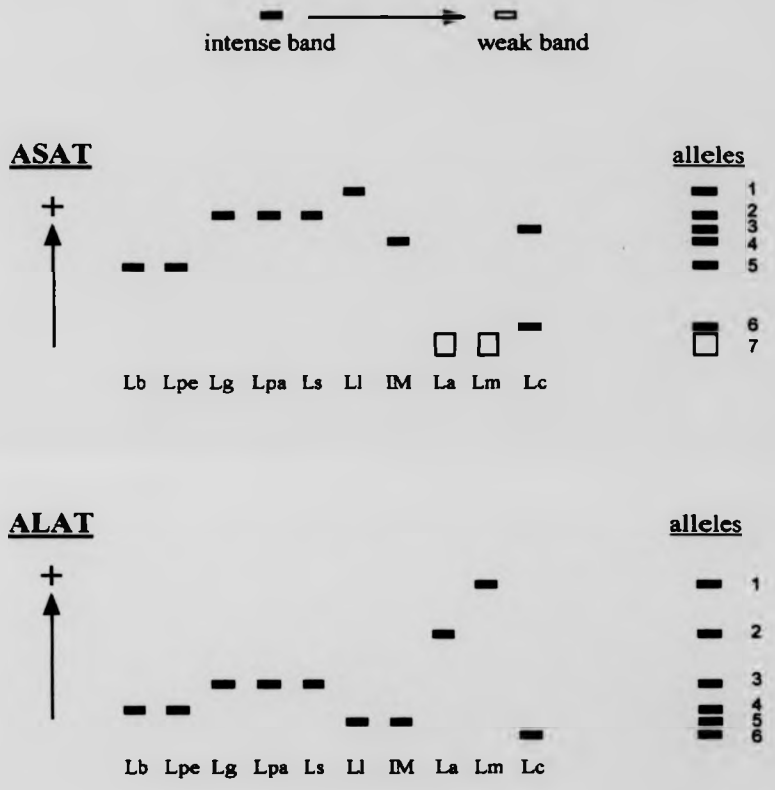


Figure 3.2.2. **Diagrammatic representation of isoenzyme discrimination between *Leishmania* reference strains (continued)**



A genetic interpretation, in terms of alleles present, was possible for all twelve loci studied. Some stocks presented heterozygous genotypes, i.e. presented more than one band. Profiles for non-monomeric enzymes included additional heterozygous bands which were not scored (e.g. the central band of a triple-banded pattern produced by a heterozygote at a dimeric enzyme locus). *Table 3.2.2* summarizes the allele(s) present at each enzyme locus for each reference strain and gives the zymoderne designation for each. Each strain belonged, not surprisingly, to a separate zymoderne.

Table 3.2.2: **Reference strain allele and zymodeme designations.**

REFERENCE STRAIN CODE NO.	ENZYME / SPECIES	MPI	NHi 1	NHi 2	NHd	ES	PEPD	PGM	6PGD	GPI	G6PD	ASAT	ALAT	Z*
LTB300	*L. V. braziliensis*	6 / 6	2 / 2	7 / 7	4 / 4	4 / 4	2 / 2	3 / 3	5 / 5	4 / 4	2 / 2	5 / 5	4 / 4	LON200
LC39	*L. V. peruviana*	7 / 7	2 / 2	7 / 7	5 / 5	4 / 4	2 / 2	3 / 3	5 / 5	4 / 4	2 / 2	5 / 5	4 / 4	LON201
LS94	*L. V. panamensis*	8 / 8	3 / 3	5 / 5	4 / 4	4 / 4	7 / 7	4 / 4	7 / 7	4 / 4	2 / 2	2 / 2	3 / 3	LON202
M4147	*L. V. guyanensis*	8 / 8	3 / 3	5 / 5	4 / 4	4 / 4	7 / 7	4 / 4	4 / 4	4 / 4	1 / 1	2 / 2	3 / 3	LON123
M15065	*L. V. shawi*	8 / 8	3 / 3	5 / 5	4 / 4	4 / 4	7 / 7	5 / 5	6 / 6	4 / 4	1 / 1	2 / 2	3 / 3	LON203
M6426	*L. V. lainsoni*	4 / 4	1 / 1	6 / 6	3 / 3	2 / 2	5 / 5	2 / 2	8 / 8	3 / 3	5 / 5	1 / 1	5 / 5	LON204
IM2832	*L. V.* sp.†	5 / 6	2 / 2	7 / 7	4 / 4	5 / 5	4 / 4	2 / 2	6 / 6	2 / 2	3 / 3	4 / 4	5 / 5	LON205
BEL21	*L. L. mexicana*	1 / 1	4 / 4	8 / 8	2 / 2	1 / 1	1 / 1	1 / 2	2 / 2	6 / 6	6 / 6	7 / 7	1 / 1	LON206
M2269	*L. L. amazonensis*	3 / 3	4 / 4	9 / 9	1 / 1	2 / 3	3 / 3	5 / 5	1 / 1	5 / 5	3 / 5	7 / 7	2 / 2	LON207
PP75	*L. L. chagasi*	2 / 2	1 / 1	9 / 9	1 / 3	1 / 3	6 / 6	5 / 5	3 / 3	1 / 1	4 / 4	3 / 6	6 / 6	LON208

Z = zymodeme; * LON numbers above 200 have been reassigned by the author as previous records have been lost (zymodemes are assigned based on the IEA conditions used, i.e. for *Leishmania Viannia* species). For enzyme names, see chapter 2. See *figure 3.2.2* for allele scoring scheme. Data given assumes diploidy, see introduction.

3.3 Isoelectric focusing (IEF)

IEF generated enzyme banding patterns using 5 out of the 11 enzyme systems (PGM, MPI, GPI, NH and PEPD) tested. Presumably, those enzymes which failed to produce visible products did so because they were rendered inactive at their pI. *Figure 3.3.1* shows the IEF result for NHi for reference strains and a selection of stocks from the Nicaraguan and Huánuco populations which were known to show different IEA banding patterns. For the 5 enzymes which could be visualized, IEF generated patterns similar to those seen by IEA for the reference strains (e.g. PGM, lanes 1-9, *figure 3.3.1*). Some IEF reference strain bands were, however, not at the equivalent position to that observed using IEA, for example *L. V. lainsoni*, lane 5, with NHi. It is not surprising that some pattern differences were observed since IEF separates by charge only, in contrast to IEA which separates both by size and charge.

***Figure 3.3.1*: IEF banding patterns of *Leishmania* reference strains NHi.**

Figure 3.3.1: IEF banding patterns of *Leishmania* reference strains using NHi. Lane 1 - *L. V. braziliensis*; lane 2 - *L. V. peruviana*; lane 3 - *L. V. guyanensis*; lane 4 - *L. V. panamensis*; lane 5 - *L. V. lainsoni*; lane 6 - *L. V.* sp. (see text); lane 7 - *L. L. chagasi.*; lane 8 - *L. L. mexicana*; lane 9 - *L. L. amazonensis*. Lanes 10-12 - Nicaraguan stocks: 10: XD28 (*L. V. braziliensis*); 11: XD09 (putative *L. V. braziliensis* / *L. V. panamensis* hybrid); 12: ZF01 (*L. V. panamensis*). Lanes A-I - Huanuco stocks: A: HR80; B: HR108; C: HR110; D: HR399; E: HR410; F: HR413; G: HR419; H: HR434; I: HR529. Arrow indicates direction of electrophoresis.

IEF generated less informative banding patterns than IEA for NH, PEPD and MPI and equivalent information for GPI and PGM. Since only five enzymes could be studied using IEF yielding no further information, IEF was discontinued.

3.4 RAPD analysis

Of the 28 primers screened for discrimination between the selected reference strains (see *Appendix IV*), 19 yielded amplification products which were polymorphic and scorable. Nine primers generated no or too few bands or smears. Scorable bands ranged in size from approximately 500 - 2500 bp. Band intensity within and between profiles for a given primer was variable. *Figures 3.4.1-3* show the RAPD profiles generated using 3 of the primers used (H4, L3, and H2 respectively).

3.4.1 Discrimination between *Leishmania* subgenera

RAPD analysis clearly discriminated between reference strains of the *Leishmania* and *Viannia* subgenera. Eleven primers (A4, A5, D1, D5, D6, D9, D10, H1, H2, H4, L3) generated profiles which included bands specific to (at least) the four main species of the *Viannia* subgenus i.e. *L. V. braziliensis, L. V. peruviana, L. V. guyanensis* and *L. V. panamensis*. *Figure 3.4.1* and *figure 3.4.2* show the profiles generated by the reference strains with primers H4 and L3 respectively: *Viannia* subgenus-specific bands are indicated by arrows marked "V". In both figures, the *L. L. mexicana* complex species (*L. L. mexicana* and *L. L. amazonensis*, lanes 6 and 7) also generate distinct profiles from that of *L. L. chagasi* (*L. L. donovani* complex, lane 8).

3.4.2 Discrimination within the subgenus *Viannia*

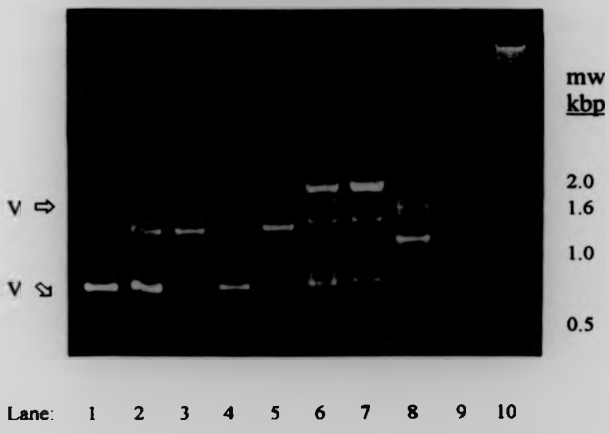
RAPD analysis was able to discriminate between species complexes of the *Viannia* subgenus. Fifteen primers (A1, A3, A4, L1, L3 - 5 H1, H2, D3, D4, D6, D7, D9 and D10) generated profiles which discriminated between the two most important groups of the subgenus i.e. the *L. V. braziliensis* and *L. V. guyanensis* species complexes. *Figures 3.4.2* and *3.4.3* show profiles generated by the reference strains with primers A3 and H2 respectively. Bands specific to the *L. V. braziliensis* complex or the *L. V. guyanensis* complex are indicated by arrows marked with "B" or "G" respectively.

RAPD analysis was also able to discriminate between member species of the *L. V. braziliensis* and *L. V. guyanensis* complexes. Three primers (A1, H1, H2) could differentiate between the *L. V. braziliensis* and *L. V. peruviana* reference strains e.g.

band indicated by arrow marked "Pe" for primer H2 (*figure 3.4.3*). Ten primers (A2, A5, D10, H1, H2, H4, L1, L3, L4, L5) could differentiate between the *L. V. guyanensis* and *L. V. panamensis* reference strains e.g. bands indicated by arrow marked with "Pa" for primer L3 (*figure 3.4.2*).

Whilst several primers generated similar profiles for the four major *Viannia* species reference strains (lanes 1-4, *figure 3.4.1*, primer H4), *L. V. lainsoni*, a more disparate species of the subgenus, commonly generated a profile distinct from those of the other *Viannia* species, e.g. lane 5 in *figures 3.4.1* and *3.4.2*.

Figure 3.4.1: **RAPD profiles generated from reference strains after PCR amplification with primer H4**



Figures 3.4.1-3 : RAPD profiles generated from reference strains after PCR amplification with primers H4, L3 and H2 respectively, visualized after electrophoresis through 1.2% agarose gel and ethidium bromide staining. Lane 1 - *L. V. braziliensis*; lane 2 - *L. V. panamensis*; lane 3 - *L. V. guyanensis*; lane 4 - *L. V. peruviana*; lane 5 - *L. V. lainsoni*; lane 6 - *L. L. mexicana*; lane 7 - *L. L. amazonensis*; lane 8 - *L. L. chagasi;* lane 9 - negative control; lane 10 - 1kb molecular weight (mw) marker (Gibco BRL, UK). Arrows (marked with V, B, G, Pe, Pa) indicate bands specific for the *Viannia* subgenus, *braziliensis* complex, *guyanensis* complex, *L. V. peruviana* and *L. V. panamensis* respectively.

Figure 3.4.2: **RAPD profiles generated from reference strains after PCR amplification with primer L3**

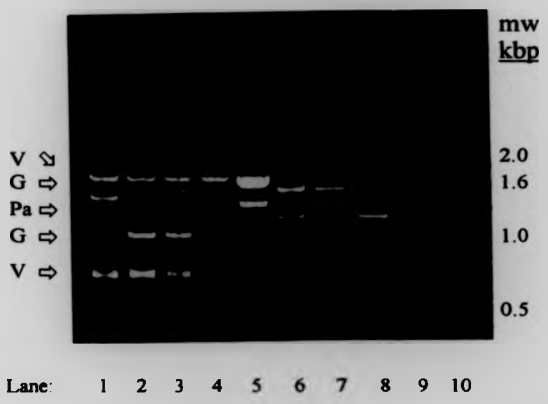


Figure 3.4.3: **RAPD profiles generated from reference strains after PCR amplification with primer H2**

Figures 3.4.1-3 : RAPD profiles generated from reference strains after PCR amplification with primers H4, L3 and H2 respectively, visualized after electrophoresis through 1.2% agarose gel and ethidium bromide staining. Lane 1 - *L. V. braziliensis*; lane 2 - *L. V. panamensis*; lane 3 - *L. V. guyanensis*; lane 4 - *L. V. peruviana*; lane 5 - *L. V. lainsoni*; lane 6 - *L. L. mexicana*; lane 7 - *L. L. amazonensis*; lane 8 - *L. L. chagasi*; lane 9 - negative control; lane 10 - 1kb molecular weight (mw) marker (Gibco BRL, UK). Arrows (marked with V, B, G, Pe, Pa) indicate bands specific for the *Viannia* subgenus, *braziliensis* complex, *guyanensis* complex, *L. V. peruviana* and *L. V. panamensis* respectively.

3.4.3 Subsequent RAPD analyses

Despite initial success with this technique, subsequent analyses were found to be unreproducible. Visualization of later PCRs using the same DNA from the same reference strains revealed missing bands at both the higher and lower size range. The DNA was shown to be intact by electrophoresis and by PCR amplification of other, specific loci. The reagents used for RAPD analyses were rigorously controlled throughout and the same thermocycler was used but the irreproducibility remained unresolved. With the availability of other molecular techniques, and with the advent of microsatellite analysis, RAPD analysis was not investigated further.

3.4.4 Discussion of RAPD

Not unsurprisingly, most published works in which the RAPD technique has been applied comment favourably on its reproducibility. However, it is my experience that the technique is extremely sensitive to experimental parameters to the extent that reproducibility is hindered. Other groups have also discontinued the use of RAPD analysis after encountering difficulties (Cupolillo *et al.*, 1995). Private communications with co-workers has revealed that this lack of reproducibility is by no means isolated. Even authors who report the technique's applicability acknowledge that reproducibility, in terms of the number and intensity of bands, is not absolute (Noyes *et al.*, 1996).

The generation of reproducible RAPD fragment profiles, as with all techniques based on PCR amplification, is highly dependent on the PCR reagents (including source and concentrations of polymerase, DNA, nucleotides and magnesium chloride) and conditions used (cycling parameters, in particular the annealing temperature, and electrophoretic techniques). Since, to the best of my ability, these factors were kept constant, the lack of reproducibility encountered cannot be attributed to variation therein. In addition, RAPD analysis also employs short arbitrary-sequence primers and low-stringency annealing temperatures. As a consequence, amplification can result from contaminating DNA sequences, resulting in variation of the number of bands produced. Amplification of contaminating DNA was not a problem here, as evidenced by the lack of bands in negative control lanes. Short arbitrary sequence primers and low-stringency annealing temperatures also permit amplification from only partially-matched priming sites, giving rise to less intensely-staining bands. It has been reported that the number of bands generated by RAPD is highly sensitive to fluctuations in the annealing temperature, with as little as 1°C change affecting the

reproducibility of the profile (Williams *et al.*, 1993; Noyes *et al.*, 1996). It has been recently been suggested that the *Taq* source may affect the outcome - the negative control lanes were generally negative so it is unlikely that the *Taq* source used here (Bioline) was contaminated, but others (Carrasco *et al.*, 1996 and Yeo *et al.*, unpublished data) have reported that superior results are achieved using *Taq* obtained from Stratagene.

It is widely recognized that RAPD fragments of the same size are not necessarily homologous in sequence. Their use for inferring relationships is, therefore, questionable. Despite the limitations discussed here and in chapter 1 (section 1.10.2.4), phylogenetic and population genetic analyses of *Leishmania* stocks based on data from RAPD analysis have shown parity with isoenzyme, RFLP and karyotype data (Tibayrenc *et al.*, 1993; Dujardin *et al.*, 1995b; Gomes *et al.*, 1995; Pogue *et al.*, 1995; Schönian *et al.*, 1996).

In summary, RAPD analysis is reported by workers in some laboratories to be a valid method for generating data for inferring relationships between *Leishmania* stocks (Tibayrenc *et al.*, 1993; Dujardin *et al.*, 1995b; Noyes *et al.*, 1996; Banuls *et al.*, 1997). It is my opinion, however, that the information which may be obtained from the analysis of RAPD data is more consistently obtained using data from other techniques (see sections 3.6 and 3.8 below). Reproducibility between laboratories, and even co-workers in the same laboratory, is fundamental to the adoption of a technique. I concede that RAPD analysis is a means of generating large amounts of molecular data but believe that the technique is most powerful for, and applicable to, the study of organisms for which no other specific molecular markers are available.

3.5 Riboprinting

PCR amplification of the 18S (SSU) rRNA gene generated a product of approximately 2.2kb, the expected size calculated from sequence data. *Figure 3.5.1* shows digests of this product using the enzymes *Taq*I and *Hpa*II, visualized after electrophoresis through 1.2% agarose gel and ethidium bromide staining. Analysis of the reference strains using 4 of the enzymes (*Alu*I, *Taq*I, *Sau*3AI and *Rsa*II) discriminated clearly between *L. L. chagasi* and *L. Viannia* spp. (e.g. *L. L. chagasi* band of >0.5kb, arrow marked "C", in lane 8 *Taq*I digests *figure 3.5.1*). However, all 8 enzymes failed to discriminate between species within the *Viannia* subgenus, (e.g. *Hpa*II digests, lanes 2-7 in *figure 3.5.1*).

Figure 3.5.1: **Riboprint profiles generated from *Leishmania* reference strains after digestions with *Taq*I and *Hpa*II.**

***Taq*I**

***Hpa*II**

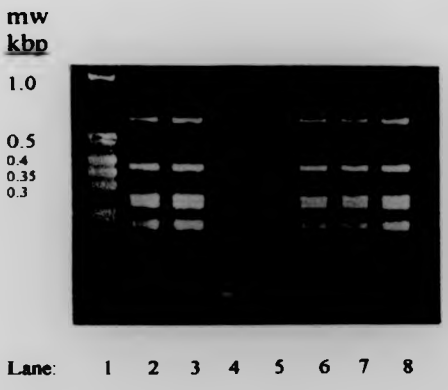


Figure 3.51: Riboprint profiles generated from reference strains after digestion of the 18S (SSU) rRNA gene PCR amplification product with *Taq*I and *Hpa*II, visualized after electrophoresis through 1.2% agarose gel and ethidium bromide staining. Lane 1 - 1kb molecular weight (mw) marker (Gibco BRL, UK); lane 2 - *L. V. braziliensis*; lane 3 - *L. V. peruviana*; lane 4 - *L. V. guyanensis*; lane 5 - *L. V. panamensis*; lane 6 - *L. V. lainsoni*; lane 7 - *L. V. braziliensis*; lane 8 - *L. L. chagasi*. Arrow (marked with C) indicates bands specific for *L. L. chagasi*.

To maximize resolution and sensitivity of detection, digested products were also analyzed on polyacrylamide gels (PAGE) stained with silver. *Figure 3.5.2* shows *Sau*3AI and *Rsa*II digests analyzed by PAGE and silver staining. Although detection using PAGE followed by silver staining is more sensitive than by agarose electrophoresis and ethidium bromide staining, no further information was gained by analyzing riboprinting products using this approach. Riboprinting was unable to differentiate between species of the subgenus *Viannia* and its further use in this study was therefore precluded.

***Figure 3.5.2*: Riboprint profiles generated from *Leishmania* reference strains after digestions with *Sau*3AI and *Rsa*I.**

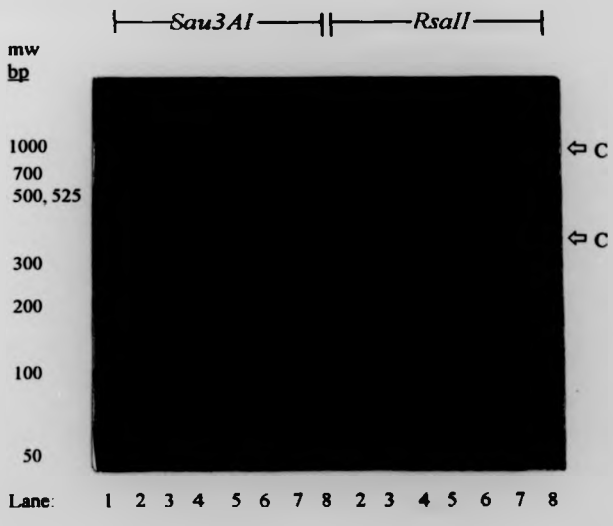


Figure 3.5.2: Riboprint profiles generated from reference strains after digestion of the 18S (SSU) rRNA gene PCR amplification product with *Sau*3AI and *Rsa*I, visualized after electrophoresis through 6% polyacrylamide gel and silver staining. Lane 1 - 1kb molecular weight (mw) marker (Gibco BRL, UK); lane 2 - *L. V. braziliensis*; lane 3 - *L. V. panamensis*; lane 4 - *L. V. guyanensis*; lane 5 - *L. V. peruviana*; lane 6 - *L. V. lainsoni*; lane 7 - *L. V. braziliensis*; lane 8 - *L. L. chagasi*.

It is perhaps not surprising that riboprinting failed to discriminate within the *Viannia* subgenus since the major species of this subgenus are known to be very closely

related. Riboprinting was able, however, to differentiate between reference strains of the more distantly-related *Leishmania* and *Viannia* subgenera. For further discussion of riboprinting, see section 3.6.3.

3.6 PCR-RFLP analysis of rRNA gene ITS region

3.6.1 PCR amplification of the rRNA gene ITS region

ITS PCR amplification products of *Viannia* species were approximately 1kb, which is the expected size, and in agreement with previous reports (Cupolillo *et al.*, 1995). *Figure 3.6.1* shows the rRNA ITS PCR products obtained from *Leishmania* reference strains, visualized after agarose gel electrophoresis and ethidium bromide staining. PCR products from species of the subgenus *Leishmania* (i.e. *L. L. mexicana*, *L. L. amazonensis* and *L. L. chagasi*) were larger (approx. 1.1-1.2kb) and, as such, could be clearly distinguished from the products of *Viannia* species on product size alone, without RFLP analysis.

***Figure 3.6.1*: Amplification products of the rRNA ITS region from *Leishmania* reference strains.**

Figure 3.61: Amplification products of the ribosomal RNA (rRNA) internal transcribed spacer (ITS) region from *Leishmania* reference strains, visualized after electrophoresis through 1.2% agarose gel and ethidium bromide staining. Lane 1 - 1kb molecular weight (mw) marker (Gibco BRL, UK); lane 2 - *L. V. braziliensis*; lane 3 - *L. V. peruviana*; lane 4 - *L. V. guyanensis*; lane 5 - *L. V. panamensis*; lane 6 - *L. V. lainsoni*; lane 7 - *L. L. chagasi*; lane 8 - *L. L. amazonensis*; lane 9 - *L. L. mexicana*; lane 10 - *L. V. braziliensis*. Arrows (marked with V, C, M) indicate bands specific for the *Viannia* subgenus, *L. L. chagasi* or *L. L. mexicana* complex respectively.

3.6.2 PCR-RFLP analysis

PCR-RFLP products were visualized by electrophoresis through 6% polyacrylamide gels and silver staining to maximize both the fragment resolution and sensitivity of detection. RFLP fragments were scored visually to identify enzymes which discriminated between the *Viannia* and *Leishmania* subgenera and between species within the *Viannia* subgenus.

RFLP analysis revealed polymorphism among the *Leishmania* reference strains using all enzymes tested. *Figure 3.6.2* shows the RFLPs produced using two of these enzymes: *Fsp*I and *Scr*FI. All enzymes used differentiated between reference strains of the *Leishmania* and *Viannia* subgenera: fragments indicated by arrows marked with "M" and/or "C" in *figure 3.6.2* are unique to the *L. L. mexicana* complex and *L. L. chagasi* respectively. RFLPs of the *L. L. mexicana* complex species were similar but could always be differentiated; *L. L. mexicana* and *L. L. amazonensis* could clearly be separated for example, by the presence of a fragment of approx. 140bp in *L. L. amazonensis* using *Fsp*I (arrow marked "A", lane 8 of *Fsp*I digest, *figure 3.6.2*).

The overall RFLPs of the *Viannia* reference strains were very similar using all enzymes (see lanes 2-6, *figure 3.6.2*). Subgenus-specific bands were commonly observed, e.g. fragments of approx. 150bp (*Fsp*I) and 50bp (*Scr*FI) indicated by arrows marked with "V" (lanes 2-6, *figure 3.6.2*). Five enzymes (*Fsp*I, *Bst*UI, *Scr*FI, *Taq*I, *Sph*I) also differentiated between the species complexes of the subgenus. For example, species of the *guyanensis* complex (*L. V. guyanensis* and *L. V. panamensis*, lanes 4 and 5) could be differentiated from other *Viannia* species (lanes 2, 3 and 6) by the absence of another fragment of approx. 170bp using *Fsp*I (*figure 3.6.2*). *L. V. lainsoni* could be separated from other members of the subgenus using 5 of the enzymes used (*Sph*I, *Scr*FI, *Csp*6I, *Dpn*I, *Taq*I), clearly indicating its more distantly related position within the group.

In addition to discrimination at the species complex level, several enzymes generated RFLPs which, using a combination of fragment presence and absence, allowed individual species to be identified. For example, *L. V. guyanensis* could be separated from *L. V. panamensis* by the presence of a *Fsp*I fragment of approximately 250bp (arrow marked "G", lane 4, *figure 3.6.2*). Members of the *guyanensis* complex could be separated using 3 enzymes (*Fsp*I, *Taq*I, *Sph*I).

Figure 3.6.2: **RFLP analysis of rRNA ITS amplification products from *Leishmania* reference strains.**

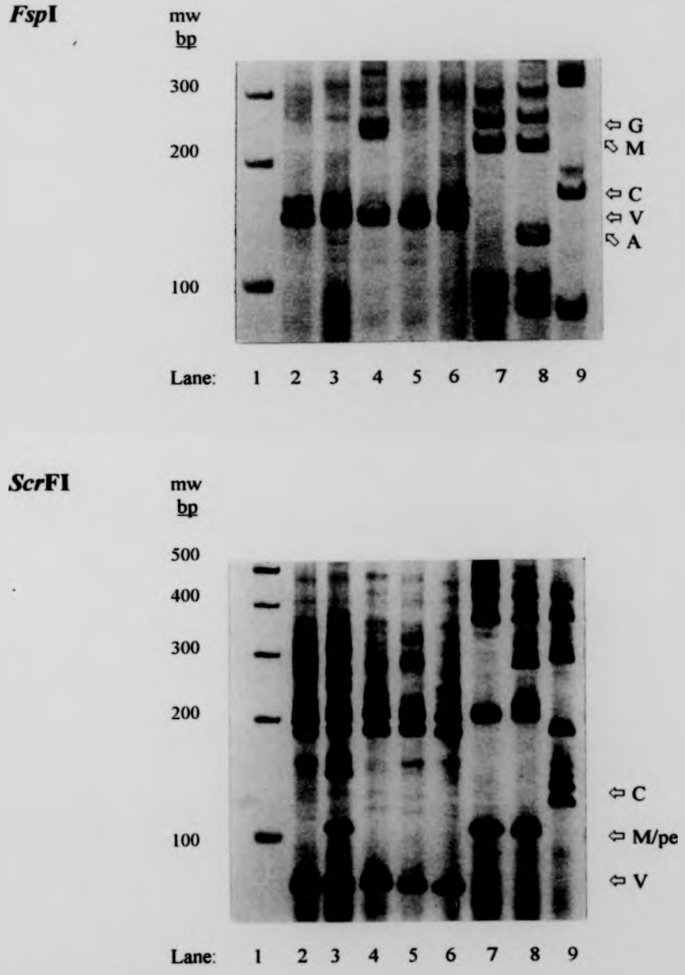


Figure 3.6.2: RFLP analysis of ribosomal RNA (rRNA) internal transcribed spacer (ITS) region amplification products from *Leishmania* reference strains, visualized after electrophoresis through 6% polyacrylamide gel and silver staining. Lane 1 - 100bp molecular weight (mw) marker (Gibco BRL); lane 2 - *L. V. braziliensis*; lane 3 - *L. V. peruviana*; lane 4 - *L. V. guyanensis*; lane 5 - *L. V. panamensis*; lane 6 - *L. V. lainsoni*; lane 7 - *L. L. mexicana*; lane 8 - *L. L. amazonensis*; lane 9 - *L. L. chagasi*. Arrows (marked with V, C, M) indicate fragments specific for the *Viannia* subgenus, *L. L. chagasi* or *L. L. mexicana* complex respectively. The arrows marked with "G" and "A" indicate fragments unique to *L. V. guyanensis* and *L. L. amazonensis* respectively. The arrow marked with M/pe indicates a fragment shared by the *L. V. peruviana* reference strain and members of the *L. L. mexicana* complex.

Within the *braziliensis* complex, polymorphism was limited to 2 enzymes, *Scr*FI and *Bst*UI. Using both of these enzymes, the RFLP patterns of the *L. V. peruviana* reference strain presented fragments not seen in any other *Viannia* reference strain. These fragments were not unique to *L. V. peruviana*, but appeared to be shared with members of the *Leishmania* subgenus. For example, the *L. V. peruviana* fragment of approx. 110bp indicated by the arrow marked "M/pe" generated using *Scr*FI (*figure 3.6.2*) is shared with members of the *L. L. mexicana* complex.

3.6.3 Discussion of PCR-RFLP analysis of the rRNA ITS region

PCR-RFLP analysis of the rRNA ITS region generated data that discriminated the *Leishmania* reference strains to the species and species-complex level, in agreement with previously published work for *Leishmania* (Cupolillo *et al.*, 1995). A great advantage of this technique is that it can be carried out on clinical material without recourse to isolation of the parasite as, in contrast to RAPD analysis, the PCR primers employed are specific for *Leishmania*. In the work presented here however, PCR amplification was carried out on DNA extracted from cultured *Leishmania*.

PCR amplification of the rRNA ITS region revealed single bands using agarose gel electrophoresis and ethidium bromide staining (*figure 3.6.1*), however, RFLP analysis revealed complex fragment patterns which were found to be not entirely reproducible using products from different PCR reactions (data not shown). Polyacrylamide gel electrophoresis and silver staining of the PCR amplification products revealed that additional bands were generated during PCR which had not been visible using agarose gel electrophoresis and ethidium bromide staining (data not shown). It was not possible to determine whether these additional bands were artefactual or due to ITS sequence variants, however these bands did not disappear after extensive work to re-optimize the PCR conditions. The problem of reproducibility was circumvented by extracting and "cleaning" the major PCR product prior to RFLP analysis. RFLPs generated by digestion of "cleaned" PCR amplification products were reproducible. Minor, less intensely-staining bands observed after RFLP analysis of "cleaned" products were also seen in some strains and possibly indicate sequence heterogeneity of ITS sequences within these individuals (section 3.5; Stothard *et al.*, 1996).

The requirement for PCR products to be "cleaned" in order to generate reproducible RFLP patterns entailed a great deal of work bearing in mind the number of stocks to

be studied. Since the discriminatory power of this technique within the *Viannia* subgenus was greatest at the species complex level, PCR-RFLP analysis of the rRNA ITS region was only carried out on stocks from the Nicaraguan population. These stocks consisted of parasites belonging to both *braziliensis* and *guyanensis* complexes i.e. *L. V. braziliensis*, *L. V. panamensis* and putative hybrids thereof. Stocks from the Huánuco and Brazilian parasite populations, which mostly comprised parasites from the *braziliensis* complex, were not analyzed by this technique.

For genes such as the rRNA genes, variation in gene coding sequence is likely to be lacking in closely related taxa due to the conservation imposed by functional constraints on the gene product. Analysis of gene sequence variation between such closely related taxa by the restriction enzyme digestion of such a conserved gene is, therefore, somewhat limited in that enzyme recognition sequences cover only a fraction of the small number of potentially variable sites in the gene. The lack of discrimination between *Viannia* species by riboprinting is evidence of the conserved nature of the 18S rRNA gene. The conserved nature of this gene among *Viannia* species was also reported by Uliana *et al.* (1994). In their study, synthetic oligonucleotides corresponding to areas of known sequence diversity in the *Leishmania* SSUrRNA gene were hybridized to PCR products from different *Leishmania* species. Discrimination of all the major *Leishmania* species complexes was evident except those within the *Viannia* subgenus.

In contrast to riboprinting however, PCR-RFLP analysis of the rRNA ITS was found to discriminate among species of the subgenus *Viannia*. This discrimination is evidence that the spacer region is less conserved than the rRNA genes themselves, which are constrained due to their essential function. *L. V. braziliensis* is estimated to have in the region of 160 copies of the rRNA repeating unit making up this multigene family (Villalba & Ramirez, 1982). Sequence heterogeneity of the rRNA repeat unit is thought to arise from concerted evolution, involving the mechanisms of unequal crossing over and biased gene conversion, resulting in the rapid accumulation of mutations in such families. Macedo *et al.* (1992) point out that sequence heterogeneity could also be due to mixed parasite populations in an isolate. Mixed populations can be excluded here since all of the reference strain parasites used were derived from clones.

3.7 Denaturing gradient gel electrophoresis (DGGE)

The rRNA repeat unit is highly conserved among closely-related taxa. The 18S rRNA gene in particular has been widely used for constructing phylogenies with the assumption that its sequence, within an organism, is highly conserved. It has recently been reported however, that sequence heterogeneity can be found in rRNA genes of parasites using DGGE (Gasser *et al.*, 1996). Sequence heterogeneity amongst gene copies of the 18S rRNA multigene family in *Plasmodium falciparum* and the free-living flatworm *Dugesia mediterranea* has also been reported (Kain *et al.*, 1996; Carranza *et al.*, 1996).

DGGE is a relatively new technique which has been shown to be capable of distinguishing sequence variants among a DNA product containing sequence variants of identical size (Lessa *et al.*, 1992; Gasser *et al.*, 1996; Stothard *et al.*, 1998b). The melting profile of a DNA fragment (i.e. the point at which strand-separation occurs) is dependent upon the sequence of the molecule (Michaelides *et al.*, 1995). DNA strands of divergent sequence, when denatured through a suitable gradient, will melt successively according to their sequence: single strands of different sequence exhibit distinct mobilities which can be visualized. It has been reported that DGGE permits the detection, under optimal conditions, of single base changes (Myers *et al.*, 1987; 1990).

DGGE was used here to assess homogeneity within the 18S rRNA gene of *Leishmania* subgenus *Viannia* species. To establish the melting profile of the 18S (SSU) rRNA PCR product, *Alu*I-digested *L. V. braziliensis* (LTB300) product was electrophoresed through a perpendicular gradient gel in which the direction of electrophoresis is at right angles to the chemical gradient (urea - gradient range 0 - 7M). *Figure 3.7.1* shows the melting profile of this product using perpendicular gradient DGGE.

At lower concentrations of urea (left-hand side of the gel), the *Alu*I-digested 18S rRNA gene PCR product fragments run as native, double-stranded (ds) DNA. Progressive strand separation (melting) occurred in the range 0.7 - 1.75M (10 - 25%) urea. The smaller digestion products (C and D) remained as single bands after denaturation, indicating the homogeneity of the sequences in both strands of these fragments. The largest fragment (A), which had 2 melting domains, resolved into 2 single strands after denaturation, indicating the presence of two distinct sequences in

this fragment. The second largest fragment (B) denatured resolving 4 single stranded curves, indicating that four distinct sequences were present in the strands of this fragment. The 18S rRNA gene copies of *L. V. braziliensis* (LTB300) were thus shown to consist of at least 4 heterogeneous sequences.

***Figure 3.7.1*: Melting profile of the *Alu*I-digested *L. V. braziliensis* 18S (SSU) rRNA PCR amplification product generated using perpendicular gradient DGGE.**

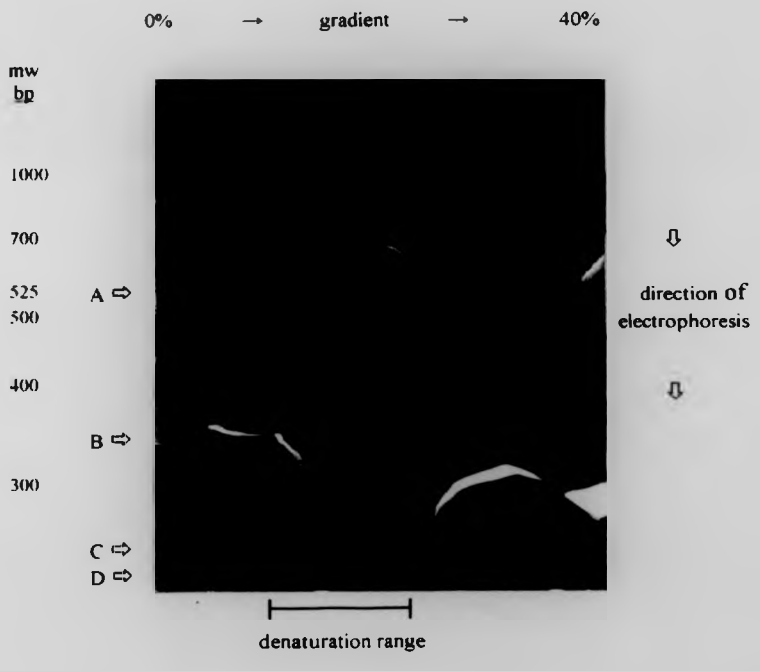


Figure 3.7.1 Melting profile of *Alu*I-digested *L. V. braziliensis* 18S (SSU) rRNA PCR amplification product generated using perpendicular gradient DGGE. Progressive denaturation occurred across the gel such that at 40% urea gradient (right of gel) all products are single-stranded. Arrows (A - D) indicate native (double-stranded) *Alu*I digestion products.

To see if sequence heterogeneity in 18S rRNA gene copies was conserved across the *Viannia* subgenus, parallel DGGE analysis was carried out on the *Alu*I digestions of 18S rRNA PCR products from six *Leishmania* stocks. In parallel DGGE, the denaturation gradient runs from top (low concentration) to bottom (higher concentration) in the same direction as electrophoresis. Parallel DGGE gels examine a narrow "window" of the original perpendicular gel and allow the comparative visualization of single-stranded product(s) from different strains or stocks. A parallel gradient gel, with the gradient spanning 10-25% 7M urea (the denaturation range identified in perpendicular DGGE), was used. *Figure 3.7.2* shows parallel DGGE analysis of the *Alu*I-digested 18S rRNA PCR products from 6 *Leishmania* strains.

***Figure 3.7.2*:** **DGGE analysis of *Alu*I-digested 18S (SSU) rRNA gene amplification products from *Viannia* reference strains.**

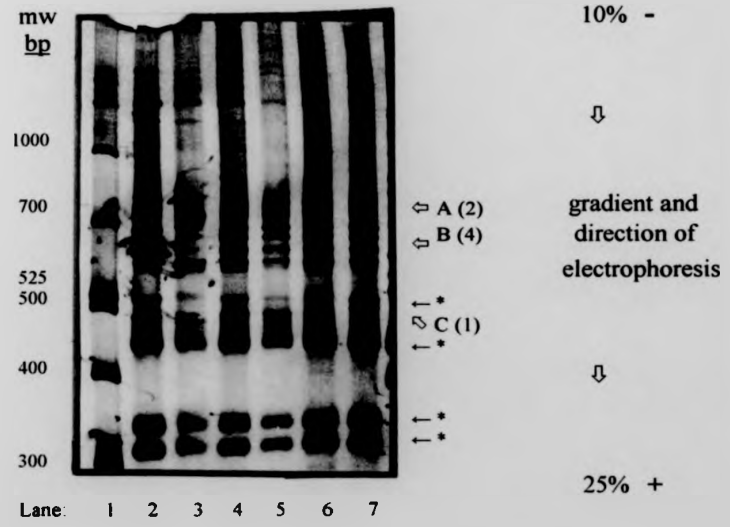


Figure 3.7.2: DGGE (parallel gradient) analysis of *Alu*I-digested 18S (SSU) rRNA PCR amplification products from *Leishmania* reference strains. Lane 1 - 1kbp moleclar weight marker; lane 2 - *L. V. braziliensis* (LTB300); lane 3 - *L. V. peruviana* (LC39); lane 4 - *L. V. panamensis* (LS94); lane 5 - *L. V. guyanensis* (M4147); lane 6 - *L. V. shawi* (M15065); lane 7 - *L. L. chagasi* (PP75). Arrows (A - C) correspond to single-stranded species arising from denaturation of native (double-stranded) *Alu*I digestion products A-C in *figure 3.7.1*, numbers correspond to the number of single-stranded species determined after denaturation. Faint / diffuse bands e.g. ← * arise from the denaturation curves of the digestion products.

The sequence variants resolved from fragments A (2) and B (4) are present in all species tested. Denatured fragment C (homogenous) is also visible; fragment D (also homogenous) is not evident because its position falls outside of the parallel gel "window". Other, more diffuse, bands can also be observed in this "window". Bands interspersed with the sequence variant bands resolved from fragments A and B are other portions of the fragment melting curves (stain reddish-brown not black, not reproducible in print); higher bands are likely to be denatured undigested DNA and are not relevant here. Hence, the number of sequence variants resolved for each fragment in parallel DGGE was identical for all *Viannia* species examined.

3.7.1 Discussion of DGGE

rRNA genes are amongst the slowest evolving (Olsen *et al.*, 1986) and hence many phylogenetic analyses of parasitic protozoa have been inferred from rRNA gene sequence data (Sogin *et al.*, 1986; Fernandes *et al.*, 1993; Maslov *et al.*, 1996; Vickerman, 1994). The majority of analyses have compared single or few sequences from a small number of species/strains to construct phylogenies (e.g. Fernandes *et al.*, 1993). Homogeneity in gene copies of a multi-gene family, such as the rRNA gene family, is maintained by concerted evolutionary mechanisms (Zimmer *et al.*, 1980). Here, DGGE analysis of PCR products from the 18S rRNA gene showed the presence of at least 4 sequence variants in gene copies from cloned strains of *Viannia* species. The existence of these 4 rRNA sequence types in all of the *Leishmania* species tested suggests that the variation in these sequences evolved prior to speciation within the genus; it would be interesting to see if Old World species also exhibit this variation.

Sequence variants have also been reported among 18S rDNA copies in other parasite taxa (Carranza *et al.*, 1996; Stothard *et al.*, 1996). Sequence diversity in such genes is most likely to occur at silent sites (in which case the function of the gene product remains unaffected). As has been suggested recently (Carranza *et al.*, 1996; Gasser *et al.*, 1996; Stothard *et al.*, 1996) diversity in 18S rRNA gene copies from a single organism should lead to a more cautious use of the gene for inferring phylogeny.

3.8 Microsatellite analysis

Microsatellite analysis as a technique for discriminating between species of the *Leishmania* subgenus *Viannia* has only very recently been described (Russell *et al.*, 1999). Here, results are reported for reference strains of the *Viannia* subgenus for three microsatellite loci: AC01, AC16 and AC52. Microsatellite analysis of all stocks was carried out and will be discussed further in chapters 4-7.

3.8.1 Specificity of the microsatellite loci for the subgenus *Viannia*

In agreement with Russell *et al.* (1999) PCR amplification products were not generated for any of the three microsatellite loci from reference species of the *Leishmania* subgenus (*L. L. mexicana* -BEL21, *L. L. amazonensis* - M2269 and *L. L. chagasi* - PP75). DNA from these stocks did however amplify the 18SrRNA gene indicating that the DNA itself was intact.

3.8.2 Amplification of microsatellite loci

All *Viannia* reference strains amplified the AC01 locus, generating a product of the expected size range (approx. 220 - 250bp). *Figure 3.8.2.1* shows the AC01 microsatellite products from reference strains visualized using agarose gel electrophoresis and ethidium bromide staining.

Figure 3.8.2.1 **Amplification products of the AC01 microsatellite locus from *Leishmania* reference strains.**

Figure 3.8.2.1: Amplification products of the microsatellite AC01 from *Leishmania* reference strains, visualized after electrophoresis through 1.2% agarose gel and ethidium bromide staining. Lane 1 - *L. V. braziliensis*; lane 2 - *L. V. peruviana*; lane 3 - *L. V. guyanensis*; lane 4 - *L. V. panamensis*; lane 5 - *L. L. chagasi*; lane 6 - negative control; lane 7 - 100bp molecular weight (mw) marker (Gibco BRL, UK); Arrow indicates the AC01 product.

Products from members of the *guyanensis* complex appeared slightly larger than those of the *braziliensis* complex (see *figure 3.8.2.1*). All *Viannia* reference strains, with the exception of IM2832, amplified the AC16 and AC52 loci, with product sizes ranging from approx. 240 - 270bp and 280 -310bp respectively (data not shown). The band sizes of all products were reproducible. The amplification products from the reference strains were also visualized using non-denaturing PAGE and silver staining as this approach allows higher resolution and greater sensitivity. However, in the case of the reference strains no further information was gained in terms of additional bands and the data is not shown

3.8.3 Genescan® and Genotyper® 2.0 analyses

PCR product(s) for each reference strain from each microsatellite locus were accurately sized using Genescan® and Genotyper® software. These programmes, allow the detection and sizing of fluorescently-labeled PCR products from denaturing PAGE using the ABI 377 PRISM™ automated sequencing machine (Applied Biosystems, UK). This approach has not been used previously for *Leishmania*.

Genescan® is a software programme which detects fluorochrome emission from fluorescently-labeled DNA after laser beam-excitation. *Figure 3.8.3.1* shows a typical gel image generated by Genescan® for these 3 microsatellite loci. Odd numbered lanes are loaded and run-in first followed by even-numbered lanes, giving rise to the staggered appearance of the bands. Size standard markers (ABI PRISM™ TAMRA™-500) are red and appear symmetrically distributed across the lanes on the gel. Lanes are indicated by blue triangles at the top of the gel. Manual manipulation of lane "tracker lines" was required to ensure that the software scored all bands in any given lane. This is necessary for every new run to overcome variations in the gel. The microsatellite products are represented as coloured bands: blue for the AC01 locus, green for the AC16 locus and yellow for the AC52 locus. These bands are not symmetrically distributed, reflecting the different allele sizes present.

Data generated by Genescan® was imported into Genotyper® for the analysis of loci within individual lanes. The output of each lane is visualized, by microsatellite locus, as a chromatogram in which peaks represent the labeled DNA. *Figure 3.8.3.2* shows the chromatogram of *Viannia* reference strains for the AC01 locus.

Figure 3.8.3.1: **Genescan® gel image showing microsatellite loci and size standard markers.**

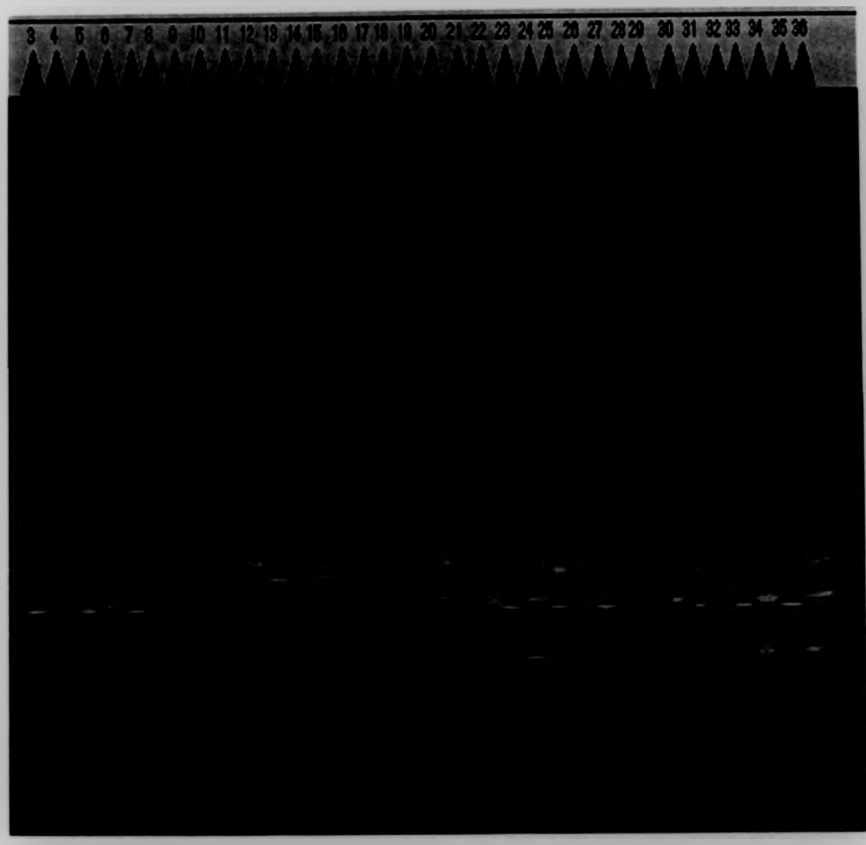


Figure 3.8.3.1 Genescan® gel image showing microsatellite loci (AC01 - blue, AC16 - green, AC52 - yellow) and size standard markers (ABI PRISM™ TAMRA™-500 - red). Lanes are shown by blue triangles at the top of the gel. Odd numbered lanes are loaded and run in first followed by even-numbered lanes, giving rise to the staggered appearance of the bands. The data generated by Genescan® is imported into Genotyper® for analyses of loci within individual lanes.

Figure 3.8.3.2 **Genotyper® output for the AC01 locus for *Viannia* reference strains.**

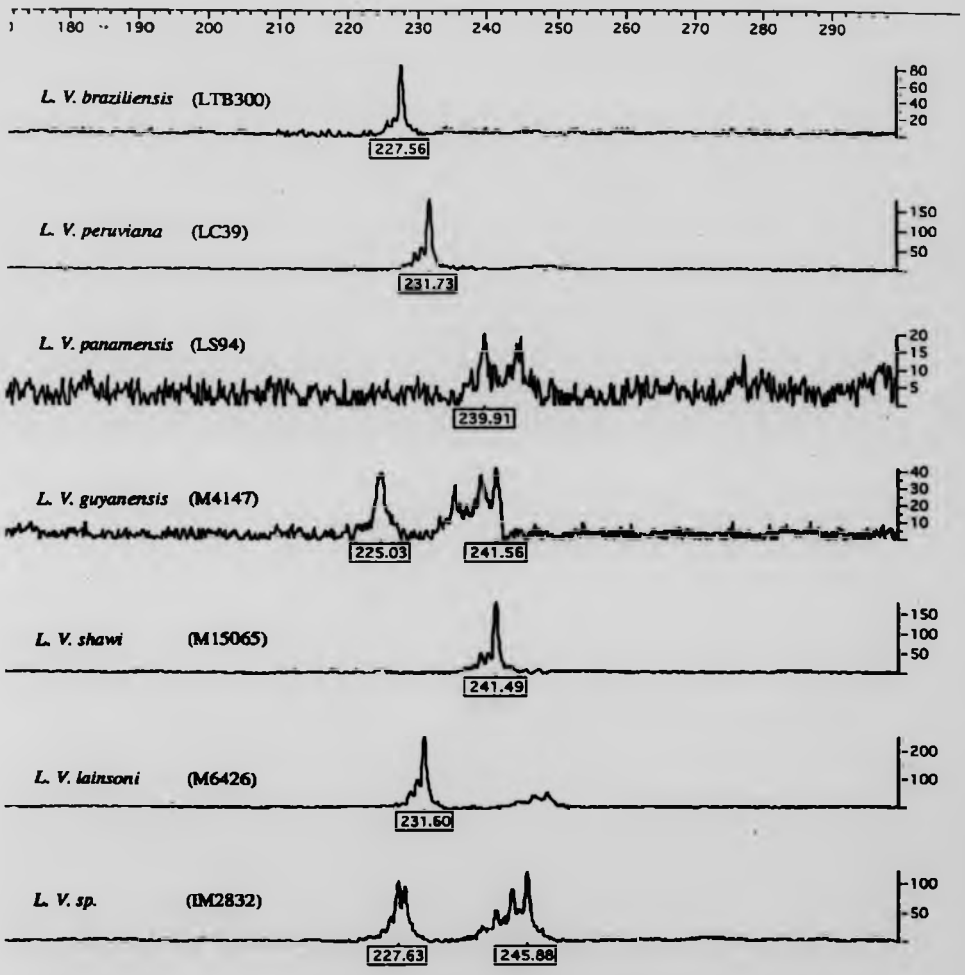


Figure 3.8.3.2: Genotyper® output for the AC01 locus for *Viannia* reference strains. Numbers across the top of the *figure* represent size, in bases. The exact sizes of individual peaks were scored automatically by the software. Exact peak sizes were assigned to "bins" which allows grouping of alleles, manually (see text). The scale to the right of each trace indicates the peak intensity. Intensities of less than 30 units were not scored (the *L. V. panamensis* peak was scored from another Genescan run). Where more than one peak was present, other criteria were taken into account. Criteria included: 1) peak height: all bands of equal intensity were scored; 2) pattern of the peaks - stutter bands (see text) were ignored; 3) information from other electrophoretic/staining methods was taken into account when deciding whether a peak was real (i.e. an allele) or artefactual. See text for further discussion.

Allele scoring was subject to visual interpretation which required a degree of knowledge regarding the locus concerned. The Genotyper® results for the AC01 locus were generally very clear-cut and could be corroborated using data from PAGE and sequencing analyses. Sequencing data was not available from the AC16 and AC52 loci: since PAGE analysis of these loci revealed complex banding patterns, allele scoring results for these two loci are preliminary and require confirmation. Several basic rules were applied for scoring peaks as alleles. The scale to the right of each trace (see *figure 3.8.3.2*) indicates the peak intensity: peaks with intensities of less than 30 units were not scored unless unambiguous. All peaks of equal intensity were scored: stocks showing two peaks were considered to be heterozygous at that locus. Single peaks were assumed to indicate homozygosity although it is possible that the second allele was "null" and therefore failed to amplify. Indeed, homozygous null alleles were identified among stocks of the three populations under study (see chapters 4-6). In cases where peaks of unequal intensity were observed, information from other electrophoretic/staining methods was taken into account when deciding whether a peak was real (i.e. an allele) or artefactual (see below). For example, many stocks from the Nicaraguan population sample generated 2 peaks for the AC01 locus: PAGE analysis suggested that these stocks were heterozygous at this locus, however the Genotyper® peaks were of very unequal intensities (see chapter 5). Both peaks corresponded to allele sizes found in other stocks and hence, despite their unequal intensities, both peaks were scored giving rise to a heterozygous genotype. It has been suggested that in such cases, the allele generating the peak of low intensity has a polymorphism in the PCR priming site such that binding of the associated PCR primer is reduced (Wang *et al.*, 1999).

Additional peaks were also observed in some stocks. "Stutter peaks", additional bands which are thought to result from DNA slippage during *in vitro* PCR amplification (Schlotterer, 1998), were commonly observed, especially for the AC52 locus which made assigning alleles difficult. The pattern of stutter bands is generally locus-specific: the pattern is usually observed in the traces of all samples at that locus (Schlotterer, 1998) and can also be visualized in PAGE analysis. Stutter bands for the loci studied here were smaller than the actual allelic band and were evident as a ladder, with peaks of increasing intensity up to the allelic peak (e.g. LC39 peak, *figure 3.8.3.2*). Stutter bands were not scored. In addition to stutter peaks, other peaks were also sometimes observed above the size range expected for the locus (data not shown);

these peaks were generally ignored. It is possible that these bands result from the terminal transferase activity of *Taq* polymerase, used for PCR amplification, as described by Schlötterer (1998).

Peak sizes were calculated automatically by the Genotyper© software. Peaks were manually assigned to "allele bins": each bin contains groups of alleles likely to contain microsatellite repeats of the same size. This was done by using a well-resolved peak to determine whether the size of the allele was odd or even (in numbers of base pairs) and to establish a correction factor to apply to the other alleles. At the AC01 locus, for example, the LTB300 peak size was scored as 227.47: the allele size was rounded to the nearest whole number, i.e. 227. The correction factor to apply to all other peak sizes was thus -0.47 (227 - 227.47). All other AC01 peak sizes were then assigned to bins by subtracting 0.47 before rounding to the nearest number. For example, the *L. V.* sp. reference strain IM2832 generated 2 peaks of sizes 227.63 and 245.88: applying the correction factor (giving 227.16 and 245.41 respectively) and rounding to the nearest number gives allele sizes of 227 and 245 respectively. Since the microsatellite repeat unit consisted of dinucleotides, allele sizes generally varied by units of 2 base pairs (excluding insertions and deletions in the flanking region, see section 3.8.4.).

It has been reported that identical alleles usually migrate within 0.5bp of each other on a gel, with larger variations observed between identical alleles from different gels (David & Menotti-Raymond, 1998). In order to maintain consistent allele bin designation between gels, the *L. V. braziliensis* reference strain, LTB300, was included as a control on all gels. Samples producing no or poorly resolved peaks, or peaks of low intensity, were re-run and / or repeated. The reference strains of *L. V. braziliensis* and *L. V. panamensis* (LTB300 and LS94 respectively) were homozygous at all three loci. The *L. V. peruviana* reference strain (LC39) was homozygous at AC01; its status at AC16 and AC52 was not determined. The reference strain of *L. V. guyanensis* (M4147) was heterozygous at AC01, homozygous at AC16, with its status at AC52 undetermined. The reference strains of *L. V. shawi* (M15065) and *L. V. lainsoni* (M6426) were homozygous for AC01 and heterozygous for both AC16 and AC52. *L. V.* sp. (IM2832) was heterozygous at AC01 but had "null" alleles, i.e. failed to amplify, the AC16 and AC52 loci. The sizes of the PCR amplification products (alleles) of the *Viannia* reference strains are summarized, by microsatellite locus, in *table 3.8.3.1*. The peaks obtained reflect sizes of the alleles at each locus; single

peaks were assumed to indicate homozygous alleles. Each strain was assigned a distinct microsatellite multilocus genotype on the basis of combined locus genotypes observed.

Table 3.8.3.1 **Summary of the product (allele) sizes of the *Viannia* reference strains for each microsatellite locus.**

			Microsatellite locus					
			AC01		AC16		AC52	
Viannia species	Strain Code No.	Multilocus genotype	allele 1[a]	allele 2[b]	allele 1	allele 2	allele 1	allele 2
braziliensis	LTB300	LON1	227	227	244	244	283	283
peruviana	LC39	LON2	231	231	ND	ND	ND	ND
guyanensis	M4147	LON3	225	241	242	242	ND	ND
shawi	M15065	LON4	241	241	246	248	297	299
panamensis	LS94	LON5	239	239	244	244	291	291
lainsoni	M6426	LON6	231	231	240	270	281	283
sp. n.	IM2832	LON7	227	245	X	X	X	X

Table 3.8.3.1: [a] allele bin size, in base pairs, of the smaller allele. Assignment of allele bins is explained in the text. [b] allele bin size, in base pairs, of the larger allele. Where alleles 1 and 2 are given as identical, it has been assumed that the sample is homozygous at that locus. It is also possible that a second allele was "null", i.e. failed to amplify. Scoring of peaks is described in the text. ND indicates than Genescan® analysis failed to determine a peak which could be called with any confidence. X indicates that no PCR amplification was achieved, probably due to mutation in the primer binding site such that alleles at this locus are "null".

3.8.4 Sequencing of the AC01 microsatellite locus

Sequencing was carried out for the AC01 locus only. Sequencing of products from the AC16 locus was attempted but failed to generate readable sequence data, probably due to overlapping of multiple DNA sequences (data not shown). These mixed sequences arose from the presence of multiple PCR amplification products, discussed in *section 3.8.2*. Isolation and purification of individual bands for this locus proved to be technically impossible in the short time available due to the lack of band resolution in agarose gel. Sequencing of the AC16 locus, and similarly the AC52 locus, was therefore not carried out.

All *Viannia* reference strains generated readable AC01 sequence except for *L. V.* sp. (IM2832). Each reference strain was sequenced at least twice; the sequence was reproducible. *Figure 3.8.4.1* shows a typical electropherogram from sequencing analysis of the AC01 microsatellite locus, shown here for the *L. V. braziliensis* reference strain LTB300. The microsatellite repeat sequence $(TG)_n$ can be seen between bases 50 - 70; for LTB300, n = 8.

Sequence at the extreme 5' end was often ambiguous hence for phylogenetic analyses, sequences were aligned commencing with the motif of bases "ATG" which was present in all samples sequenced. *Figure 3.8.4.2* shows the alignment of sequence from the AC01 microsatellite locus for reference strains of the *Viannia* subgenus. The sequences of the *L. V. braziliensis* and *L. V. peruviana* reference strains were identical before the microsatellite repeat motif and varied at only 5 heterologous peaks downstream of this. Complex-specific variation was observed: all *guyanensis* complex species had "C" not "T" at positions 28 and 135, the number of dinucleotide repeats was 14 or greater. Within this complex, each reference strain could be differentiated: *L. V. shawi* and *L. V. guyanensis* had "CG" insertions in the repeat motif. *L. V. shawi* also had a 4 base pair insertion immediately before the repeat motif.

Notes to *figure 3.8.4.2*. (see page 117) Alignment of sequence data from *Viannia* reference strains at the AC01 locus. *L. V. b - L. V. braziliensis; L. V. pe - L. V. peruviana; L. V. g - L. V. guyanensis; L. V. s - L. V. shawi; L. V. pa - L. V. panamensis; L. V. l - L. V. lainsoni*. Numbers above sequence data indicate the base position used for alignment purposes. "." represents an identical base to that seen in the LTB300 (*L. V. braziliensis*) sequence; "-" represents a gap. Gaps between positions 88 - 91 have been introduced to account for additional repeats in stocks from other populations. The sequences were edited and aligned using ABI PRISM™ Sequence Navigator version 1.01 (1984 -94, Applied Biosystems, UK). Ambiguous bases were scored visually: nucleotide codes were assigned to peaks according to the standard coding system [see Appendix IV] which is the format required by phylogenetic packages. Sequencing of the AC01 locus for the *L. V.* sp. reference strain IM2832 repeatedly failed to generate readable sequence data. Sequence alignments were made using 165 bases of the total sequence generated since this region gave the most readable sequence data and contained the dinucleotide repeat region; approximately 60-90 bases at the 3' end of the product were not scored. Bases in blue type form the microsatellite repeat region; bases in red type illustrate the TCCGC end motif; bases in green type are invariant downstream in reference strains.

Figure 3.8.4.1 **A typical sequencing electropherogram showing data from the AC01 microsatellite locus for *L. V. braziliensis*, LTB300.**

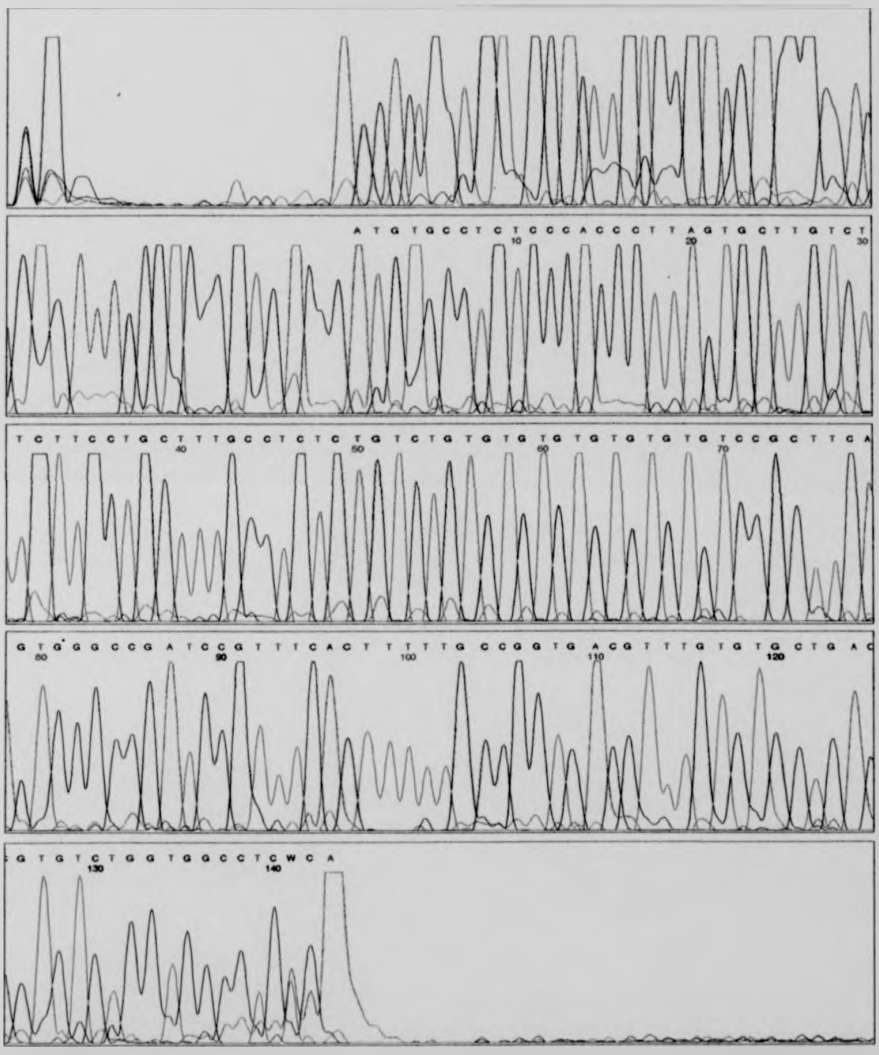


Figure 3.8.4.1 electropherogram illustrating the nucleotide sequence of the AC01 microsatellite locus, shown here for the *L. V. braziliensis* reference strain LTB300. Bases called are given above the individual peaks along with base numbers. The microsatellite repeat sequence $(TG)_n$ can be seen between bases 50 - 70. For this sequence, n = 8. For phylogenetic analyses, sequences were aligned commencing with the motif of bases "ATG" (base numbers 1-3) since for numerous stocks the sequence at the 5' end was ambiguous.

Figure 3.8.4.2: **Alignment of sequence data from *Viannia* reference strains at the AC01 locus.**

Species §	Strain /stock* code no.	10 20 30 40 50 60 70 80
L. V. b	**LTB300**	ATGTGCCTCT CCCACCCTTA GTGCTTGTCT TCTTCCTGCT TTGCCTCTCT GTC----TGT GTGTGTGTGT GTG-------
L. V. pe	**LC39**	....----... ....TGTG---
L. V. pa	**LS94**	....C..C.. ....----... ....TGTGTGT
L. V. g	**M4147**	....C.. ....C ...----... ....TGYGYGT
L. V. s	**M15065**	....C.. ....TGTC... ....TGTGCGT
L. V. l	**M6426**	....A....C.. .....C ......T.GC C..----... ....TGTG---

Species §	Strain /stock* code no.	90 100 110 120 130 140 150 160 165
L. V. b	**LTB300**	---------- -TCCGCTTCA GTGGGCCGAT CCGTTTCACT TTTTGCCGGT GACGTTTGTG TGCTGACGTG TCTGGTGGCC TCWCA
L. V. pe	**LC39**	---------- -..... ....K.... ...W..... ..K..... W.A..
L. V. pa	**LS94**	GTGTG----- -.....YW.. ......M... ....Y..... ..K..SS..W ....CG.... ...A..Y... ..K..K.... ..A..
L. V. g	**M4147**	GTGYGYS--- -.S...CA.. .K...SA..Y S...CW.... ..GY.SS..A S...CG.... ..SA..Y... .SG..K.... W.A..
L. V. s	**M15065**	GCG------- -..... ....K..... ....CK.... ...W..... ....A..
L. V. l	**M6426**	---------- -..... .....T. ..A..... ...C..... ....W..... ....A..

Table 3.8.4.1 compares information gained from sequence data for the *Viannia* reference strains. The length of the sequence varied depending on the size of the microsatellite repeat and the presence of any insertions in the flanking regions. Generally, sequence was more variable in the 3' flanking region (downstream) of the microsatellite repeat. This is likely due to the creation of the microsatellite itself, i.e. due to DNA slippage during replication and inaccurate subsequent DNA mismatch repair (Levinson & Gutman, 1987; Schlötterer & Tautz, 1992). Point mutation, insertions or deletions and other mutational mechanisms such as unequal sister-chromatid exchange and unequal crossing-over mechanisms may also operate (Levinson & Gutman, 1987; Schlötterer & Tautz, 1992; Ashley & Dow, 1994; Di Rienzo *et al.*, 1994), adding to sequence variation at the locus.

Table 3.8.4.1 **Summary of information from AC01 sequence data**

Species	Strain Code No.	sequence length from Genotyper®	sequence length from alignment†	* n =	% identity to LTB300 (5' end)	% identity to LTB300 (3' end)
L. V. braziliensis	LTB300	227	143	8*	100	100
L. V. peruviana	LC39	231	147	10*	100	93.2
L. V. shawi	M15065	241	157	13	98.1	93.2
L. V. panamensis	LS94	239	155	14*	96.2	79.7
L. V. guyanensis	M4147	225/241[a]	157	15	96.2	64.9
L. V. lainsoni	M6426	231	147	10*	86.8	93.2

* perfect TG repeat: *L. V. guyanensis* and *L. V. shawi* had "CG" insertions within the microsatellite repeat. † 84 bases at the 5' end were not used for alignment since this region was not clearly readable in all strains / stocks. The "ATG" motif was common to all samples sequenced and was chosen as an arbitrary place to start alignments. [a] Genotyper® indicated that *L. V. guyanensis* strain M4147 was heterozygous at the AC01 locus: the sequence length is concordant with the 241 allele having been sequenced however the ambiguity within and downstream of the microsatellite repeat motif may indicate that, in fact, both alleles were sequenced and that the sequence read contained the overlapping sequences from both.

Genotyper® data indicated that *L. V. guyanensis* strain M4147 was heterozygous at the AC01 locus, with allele sizes of 225 and 241 bp. The sequence read length obtained is compatible with the 241 allele having been sequenced, however, the variation observed in this strain both within and downstream of the microsatellite repeat motif

indicates that both alleles were, in fact, sequenced and that the electropherogram contained the overlapping sequences from both. Scoring of overlapping sequences would explain why *L. V. guyanensis* was so divergent from the other reference strains (64.9% identity) in the 3' flanking region: the 5' flanking region is highly conserved hence a difference in total sequence length caused by different numbers of dinucleotide repeats would cause ambiguity only after the repeat. It is possible also that the sequences of the two alleles of the reference strain *L. V.* sp. IM2832 were so divergent that, when co-sequenced, the overlapping sequence outputs rendered the output unreadable.

3.8.5 Microsatellite analysis discussion

Microsatellite analysis was found to be a highly informative technique for discriminating between the *Viannia* reference strains. This approach was capable of differentiating all reference species tested and, importantly, was able to discriminate between reference strains of *L. V. braziliensis* and *L. V. peruviana*.

Initial analysis of the microsatellite loci PCR products was carried out using non-denaturing PAGE analysis and silver staining. Results for the reference strains varied from single bands for the AC01 and AC16 loci to several bands for AC52: the banding patterns at all loci were reproducible. The resolution of individual bands using standard PAGE gels and silver staining was not generally sufficient to accurately differentiate or size products from different strains. In addition, an allelic interpretation of patterns consisting of multiple bands (i.e. AC52) was generally not possible using this approach. Bands additional to the microsatellite allele(s) were probably conformational variants, such as dimers, or stutter bands as described in section 3.8.3.

The availability of Genescan® / Genotyper® and the automated sequencing machine led to the described approach being taken. Genescan® and Genotyper® have not previously been used to study variation at these microsatellite loci. Genescan® analysis utilizes long-range denaturing PAGE gels: the long range of the gel allowed maximal resolution of bands, which were accurately sized by comparison with internal size standards. Conformational variant bands were eliminated under the denaturing conditions of Genescan® PAGE analysis. Stutter bands could generally be identified after Genotyper® analysis and were ignored. In some stocks, mostly at the AC52

locus, it was not possible to infer alleles (and hence genotypes) from Genotyper® since more than two peaks were present. In such cases, alleles/genotypes were scored as "not determined - ND". The analysis of these stocks was repeated but failed to resolve less than two peaks. Possible explanations for these additional peaks is that they were the result of non-specific amplification from another part of the genome, or that the stock in question was not diploid at that locus.

Unsurprisingly, sequencing analysis revealed the most diversity among reference strains. Each strain had a unique AC01 locus sequence. Sequencing strengthened the interpretation of AC01 results from Genescan® / Genotyper® in that it was generally possible to explain the size variation of the product simply in terms of variation in the numbers of dinucleotide repeats. The sequence of a few stocks did, however, contain insertions which would complicate the typing / population and phylogenetic analyses of stocks based on locus product size alone. The number of dinucleotide repeats resolved at the AC01 locus by sequence analysis is in agreement with that of Russell *et al.*, 1999, however, there are a few nucleotide discrepancies in the absolute AC01 sequence (Roslin Russell, personal comm.) of *L. V. guyanensis* (M4147) and *L. V. panamensis* (LS94). Since I provided the DNA from both of these strains for their analyses and we both sequenced the reference strains repeatedly using automated DNA sequencing machines, it is not clear why these sequences differed.

In addition to sequencing AC01, Russell *et al.* (1999) also reported sequencing results from the AC16 and AC52 loci. The microsatellite repeat in the AC16 locus was found to be composed of simple (TG)n repeats, where n ranged from 6 - 48 for the reference species used here. The microsatellite repeat in the AC52 locus was more complex and many species contained (TA) insertions. The AC52 repeat length varied among reference strains from 18 - 72 nucleotides. It is not clear how the authors managed to generate readable sequence data from the AC52 locus when they reported that multiple alleles were present (Russell *et al.*, 1999).

Table 3.8.5.1 compares the data obtained from sequence analysis of the AC16 and AC52 loci (Russell *et al.*, 1999; Roslin Russell, pers.comm.) with data obtained from Genescan® / Genotyper® analyses. Comparisons were made by calculating the expected product size assuming that allele sizes differ by changes in the microsatellite repeat number alone (see notes to table).

***Table 3.8.5.1*: Comparison of data obtained for the AC16 and AC52 loci.**

AC16 locus

Viannia species and code no.	Dinucleotide repeat*	Relative difference (in bases)±	Expected product size †	Genotyper® data§	Comment (see notes)
L. V. braziliensis LTB300	$(TG)_7$	2	244	244	1
L. V. peruviana LC39	$(TG)_{48}$	84	326	F	F
L. V. panamensis LS94	$(TG)_7$	2	244	244	1
L. V. guyanensis M4147	$(TG)_6$[a]	0	(242)[b]	242[b]	b
L. V. shawi M15065	$(TG)_8$	4	246	246 / 248	2
L. V. lainsoni M6426	$(TG)_{20}$	28	270	240 / 270	2

AC52 Locus

Viannia species and code no.	Dinucleotide repeat*	Relative difference (in bases)±	Expected product size †	Genotyper® data§	Comment (see notes)
L. V. braziliensis LTB300	$(CA)_9$[a]	0	(283)[b]	283[b]	b
L. V. peruviana LC39	$(CA)_{18}(TA)_2(CA)_2$	26	333	F	F
L. V. panamensis LS94	$(CA)_{10}TA(CA)_2$	8	291	291	1
L. V. guyanensis M4147	$(CA)_9TA(CA)_2$	6	289	F	F
L. V. shawi M15065	$(TG)_{17}$	16	299	297 / 299	2
L. V. lainsoni M6426	$(CA)_5TA(CA)_3(TA)_2$	4	287	281 / 283	3

Notes to *table 3.8.5.1*:

* Dinucleotide repeat size, in bases, from Russell *et al.* (1999) and Roslin Russell (pers. comm.): data from sequence analysis. ± Relative difference (in bases) expected in locus product size assuming no insertions or deletions in flanking regions. Calculated by subtracting the number of dinucleotide repeats observed in the smallest product[a] from the total number of dinucleotide repeats (including TA repeats in AC52 locus) and multiplying by 2 to convert to number of bases. Expected product size calculated by adding the relative difference to the size of the smallest AC16 allele observed using Genotyper®. § Data from this thesis.

Comments:

1: Exact correlation observed between Genotyper® results (this thesis) and the expected size calculated from sequencing data (Russell *et al.*, 1999, R. Russell, personal communication).

2: Unambiguous Genotyper® results: 2 peaks of equal intensity scored, see *figure 3.8.5.1* for AC16 alleles (AC52 data not shown). One of these alleles correlated with the expected size calculated from sequence data of Russell *et al.* (1999).

3: Unambiguous Genotyper® results: 2 peaks of equal intensity scored (data not shown). Neither peak correlated with the expected size calculated from sequence data of Russell *et al.* (1999).

F: Genotyper® analysis failed repeatedly, no comparison possible.

b: Genotyper® size of smallest allele, used to calculate expected size of other alleles.

Several discrepancies were found for both loci, in both cases involving the reference strains of *L. V. shawi* and *L. V. lainsoni*. Genotyper® results for both of these strains show that 2 AC16 peaks are clearly present, see *figure 3.8.5.1*.

Figure 3.8.5.1 **Genotyper® output for the AC16 locus for 4 *Viannia* reference strains.**

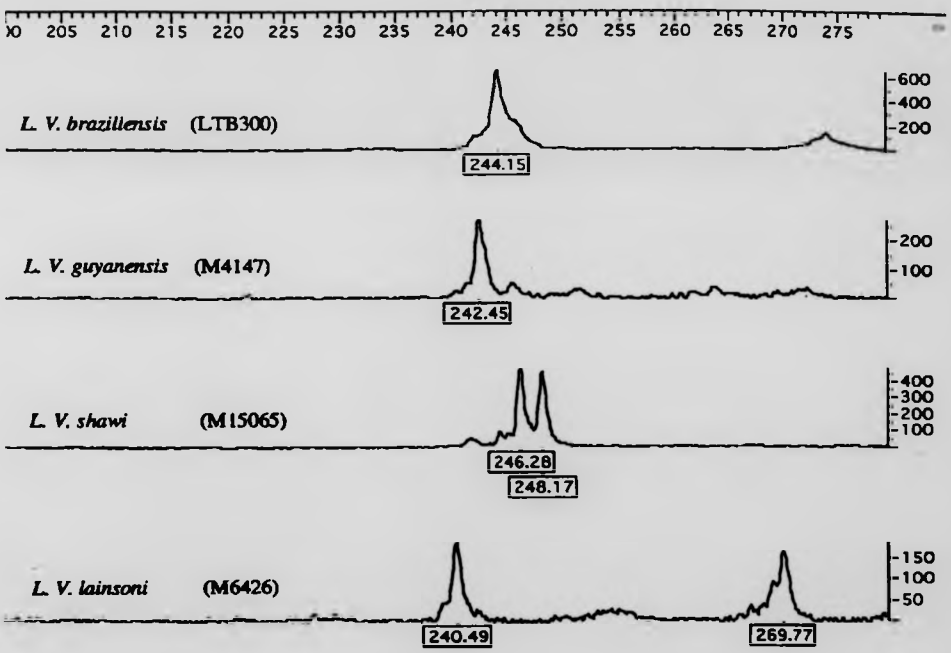


Figure 3.8.5.1: Genotyper® output for the AC16 locus for 4 of the*Viannia* reference strains. Numbers across the top of the *figure* represent size, in bases. The exact sizes of individual peaks were scored automatically by the software. Exact peak sizes were assigned to "bins" which allows grouping of alleles, manually (see text). The scale to the right of each trace indicates the peak intensity. For *L. V. braziliensis*, *L. V. guyanensis* and *L. V. panamensis* (data not shown), an exact correlation was observed between Genotyper® results (this thesis) and the expected size calculated from sequencing data (Russell *et al*., 1999, Roslin Russell, personal communication). For *L. V. shawi* and *L. V. lainsoni* however, Genotyper® results showed 2 peaks of equal intensity. Only one of these alleles correlated with the expected size calculated from sequence data of Russell *et al.* (1999).

Two peaks of equal intensity were unambiguously scored by Genotyper® (making it unlikely that one of the peaks is a stutter band), hence it is not clear why only one allele was observed by Russell *et al.* (1999) in these strains using sequence analysis. For both *L. V. shawi* and *L. V. lainsoni* at the AC16 locus, and for *L. V. shawi* only at the AC52 locus, one of the alleles identified by Genotyper® corresponded to the expected product size calculated from sequence data. Genotyper® data for *L. V. lainsoni* at the AC52 locus however also revealed 2 peaks, but in this case, neither peak (281 and 283) correlated with the expected size (287) from sequence data.

Microsatellite analysis of *Leishmania Viannia* has only very recently been described (Russell *et al.*, 1999) and until now has not yet been subjected to evaluation by other laboratories. Described in chapters 4 - 6, microsatellite analysis has been applied here to the examination of populations of *Leishmania* consisting of i) stocks mostly of a single species, *L. V. braziliensis* (chapter 4, Brazil / Três Braços), ii) putative hybrids between parasites belonging to different *Viannia* species complexes (chapter 5, Nicaragua) and iii) putative hybrids between parasites belonging to the same *Viannia* species complex and which have been collected from a discrete geographical area (chapter 6, Huánuco, Peru). Microsatellite analysis will be discussed in greater detail in chapters 4-7.

3.9 Comparative assessment of techniques used for discriminating between *Leishmania* reference strains.

Of the techniques assessed for discrimination between *Viannia* reference strains, IEA, PCR-RFLP and microsatellite analysis revealed the most variability: in addition, the results from each of these methods could be interpreted genetically.

IEA is the "gold standard" method of classifying *Leishmania* to the species level. The major drawback of the technique is that parasite isolation and culture are required to provide sufficient material for analysis. Parasite isolation is prone to contamination and has a variable success rate. Some *Leishmania* species, notably *L. V. peruviana*, have been found particularly difficult to expand in *in vitro* culture. In addition, isolation and long-term culture, *in vivo* or *in vitro*, may result in strain selection (the loss of variant parasite types present during the original infection). Cultivation is expensive and time consuming. IEA itself is also expensive and time consuming to perform, although fairly simple, generating results which are easy to read and readily

interpretable in terms of genotype. IEF was assessed as an alternative to IEA because it is possible using IEF to run many more samples per gel and hence increase throughput. This technique however, proved to be less informative than IEA owing to the reduced number of examinable loci.

RAPD analysis was assessed because of its apparent popularity, as based on the literature at the time. Initially promising in terms of discrimination between reference strains, problems of reproducibility were encountered which led to its use being discontinued. The disadvantages of the RAPD technique were described at length in section 3.4. In addition, the use of non-parasite-specific PCR primers means that isolation and culture of the parasite is, like IEA, a necessity to eliminate contaminating DNA.

Most other molecular-based approaches are based on PCR using parasite-specific primers which eliminates the requirement for the parasite isolation and culture: strain selection is thus minimized and infections involving mixed species or strains may be detected.

The RFLP technique can be applied to PCR products from any locus. The variability shown depends on the conservation level of the locus being examined. Multigene families, such as rRNAs, are usually organized as arrays of tandem repeated units and, as such, provide a number of loci suitable for PCR-RFLP analysis. The rRNA genes of all organisms are functionally and evolutionarily homologous and are highly conserved both in structure and nucleotide sequence. The intergenic regions and flanking non-transcribed regions of the arrays are less conserved. Riboprinting was unable to discriminate between *Viannia* species and so was not used further. PCR-RFLP analysis of the rRNA ITS region, however, did differentiate between the various reference strains: the discriminatory power was found to be greatest at the species complex level. Hence PCR-RFLP analysis of the rRNA ITS region was used only to examine stocks of the Nicaraguan population which encompassed more than one species complex.

Microsatellite loci are ideal molecular markers because they are discrete and have co-dominant alleles which are inherited in a Mendelian manner (Ashley & Dow, 1994; Schlötterer, 1998). They have no known function and it is therefore assumed that there is no functional constraint limiting diversity and that they are selectively neutral

(Ashley & Dow, 1994; Schlötterer, 1998). Microsatellite analysis is an important new technique for differentiating species of the subgenus *Viannia*. This technique was shown, in agreement with Russell *et al.* (1999), to reveal a large amount of variation among *Viannia* strains and stocks. Combining the data from Genescan® / Genotyper® and sequencing allowed the unique identification of each reference strain.

The application of Genescan® and Genotyper® to the analysis of these loci has not been described previously. Genescan® and Genotyper® analyses were relatively easy to perform and proved superior to the PAGE / silver staining method. A great advantage of the Genescan® approach was multiplex analysis which allowed the simultaneous analysis of multiple loci. Here, PCR products were pooled manually: even greater throughput could have been obtained by multiplexing the PCR reactions themselves. Another advantage is that peak sizes were scored automatically by the software in comparison with internal size standards. This meant that size scoring was accurate, consistent within and between gels and that any possible bias due to manual interpretation were eliminated.

The sequencing of any locus provides the ultimate data for typing, population genetic and phylogenetic analyses. For small numbers of samples, sequencing may be a viable approach, however, it has limited applicability to the analysis of large numbers of stocks or for long sequences. Here, sequencing of the AC01 locus was carried out to examine the variation between stocks and to see if variation was limited only to variable numbers of the dinucleotide repeat. Although size variation was generally found to correlate with variable numbers of repeats, mutations in the flanking regions were also demonstrated by sequencing. Phylogenetic and population genetic analyses are likely to be inaccurate (insertions / deletions) or underestimated (point mutations) if such mutations are present: phylogenetic analyses here were based on sequence data.

Another advantage of microsatellite analysis using Genescan® / Genotyper® is that heterozygotes at a single locus are readily detectable as double peaks. Russell *et al.* (1999) reported finding a single heterozygote among their stocks. Two of the populations examined in this thesis contained putative hybrids identified using IEA, RAPD and RFLP analyses. The analysis of heterozygous parasites are reported and discussed in detail in chapters 5 and 6.

CHAPTER 4 – BRAZIL: *L. V. BRAZILIENSIS* STOCKS FROM DIFFERENT GEOGRAPHICAL AREAS INCLUDING TRÊS BRAÇOS / CORTE DE PEDRA

4.1 Leishmaniasis in Brazil - general introduction

Brazil, the largest country in South America, stretches some 4350 kilometres from the Andes eastwards to the Atlantic Ocean, bordering on every country of the continent bar Chile and Ecuador. Brazil, as would be expected in such a vast country, has a diverse vegetation ranging from the tropical rain forest ('selva') of the Amazon region, through savannah or tropical grassland areas to low, scrub and thorn forests ('caatinga'). Brazil has rich agricultural (coffee, sugar, cacao, soybeans, timber) and mineral resources (iron ore and gold) however, despite the recent modernization of its industry, it has an economy that is hampered by a huge foreign debt. The rapid population growth rate, particularly in urban areas, has exacerbated unemployment, lack of housing etc. such that around 45% of the population (65 million people) live in poverty (J. Arias, personal comm.).

Cutaneous leishmaniasis is endemic in Brazil, with foci in virtually every state. The worst affected regions are those of the north, northeast and central/west, see *table 4.1* (from Arias, 1996). The incidence of cutaneous leishmaniasis in Brazil has increased from 10.45 cases per 100, 000 inhabitants in 1985 to 22.81 in 1995. Over all regions, there has been a 261% increase in the number of cases reported during this time period. Whilst improved detection and reporting have been implemented, there is no doubt that CL is on the increase. Until relatively recently, CL transmission most commonly occurred in rural forested areas which enabled control measures to be centered around the treatment of infected individuals coupled with health education. CL epidemics and new endemic foci have traditionally been associated with rural workers colonizing sylvatic habitats (i.e. settlement in deforested areas etc., e.g. Barrett & Senra, 1989). More recently however, changes have been noted in transmission patterns. There are increasing reports of transmission occurring in peri-domestic and domestic habitats in the absence of direct contact with the forest (Yoshida *et al.*, 1990; Felinto de Brito *et al.*, 1993; Brandão-Filho *et al.*, 1999) and in peri-urban foci (Oliveira-Neto *et al.*, 1988; Passos *et al.*, 1993). These changes are

Table 4.1: **Cutaneous leishmaniasis in Brazil: new cases reported and cases / 100, 000 inhabitants for 1985 and 1995, by region and by all regions combined.**

Region	States in region	1985		1995		% increase in cases reported (1985-95)
		cases. reported (new)	cases /100, 000 inhabitants	cases. reported (new)	cases /100, 000 inhabitants	
North	**Pará**, Rondõnia, **Amazonas**, Acre, Roraima, Amapá, Tocatins	5, 935	82.48	13, 017	112.13	219.3
Northeast	Maranhão, **Bahia**, Ceará, **Pernambuco**, Piauí, Paraíba, Sergipe, Alagoas, Rio Grande do Norte	4, 402	11.56	13, 883	30.87	315.4
Southeast	**Minas Gerais**, **São Paulo**, Espírito Santo, Rio de Janeiro,	844	1.50	2, 605	3.93	308.7
South	**Paraná**, Rio Grande do Sul, Santa Catarina	420	2.06	796	3.44	189.5
Central & West	Mato Grosso, Goiás, Mato Grosso do Sul, Distrito Federal	2, 038	23.41	5, 343	52.01	262.2
Brazil	**All Regions / States**	**13, 639**	**10.45**	**35, 644**	**22.81**	**261.3**

Table 4.1: new cases of cutaneous leishmaniasis and cases / 100, 000 inhabitants reported in Brazil for 1985 and 1995. Figures are given by region and for all regions combined. Data from Arias, 1996. Stocks examined in this thesis were sampled from States written in **bold** type: all Regions bar Central / West were represented.

alarming as it likely involves the adaptation of parasite and/or vector species to non-sylvatic habitats and / or alternative reservoirs.

Leishmania species causing CL in Brazil include *L. V. braziliensis, L. V. guyanensis, L. V. lainsoni, L. V. naiffi, L. V. shawi* and *L. L. amazonensis*. VL (caused by infection with *L. L. chagasi)* is also widespread and common in Brazil and is spreading, especially through areas of greater poverty (Desjeux, 1992b).

4.2 Leishmaniasis in Três Braços and Corte de Pedra, Brazil

Três Braços and Corte de Pedra are situated 600-800m above sea level (a.s.l.) in the south west of the state of Bahia, (northeast region) Brazil, see *Figure 4.2*. The region is endemic for cutaneous and mucocutaneous leishmaniasis: Três Braços and Corte de Pedra have been the focus of research, both clinical and scientific, for over 25 years (Marsden, 1994b). A virtual monotransmission of *L. V. braziliensis* occurs in the area; both visceral disease and Chagas' disease are absent (Marsden, 1994a).

Três Braços ("three arms") is situated at the junction of three rivers in one of the few surviving stands of littoral forest in Bahia State and undergoes mini-epidemics of CL and MCL. A field clinic and study site was set up by the late Philip Marsden, Cesar Cuba-Cuba and others in 1973 to treat infected individuals and to study the epidemiology of the disease. The initial study site encompassed 13 farms and 2 hamlets, comprising 276 families and 1956 individuals, within a 20km radius of the village of Três Braços.

Jones *et al.* (1987) reported results from a 5 year (1980 - 1984) study of leishmaniasis in Três Braços: the incidence of leishmaniasis was found to be 8 cases/1000 individuals, with a prevalence of 160 cases/1000 individuals in 1984. CL was predominantly observed in males and in individuals between the ages of 10 to 30 years (Jones *et al.*, 1987). Cutaneous lesions were usually rapidly-evolving (often reaching several centimetres diameter within a month), consisting of deep, delineated ulcers with raised borders (Jones *et al.*, 1987). More than 25% of patients presented with two or more active lesions; over 70% of individuals with active lesions presented primary lesions on the leg; other sites included arms (10%), trunk (10%) neck (5%) and nose (5%) (Jones *et al.*, 1987).

MCL was initially reported in up to 30% of cases from the field-clinic at Três Braços (Barreto *et al.*, 1981), however, this figure reflected a biased population sample consisting of the more severe cases. Jones *et al.* (1987) reported that 2.7% of CL cases developed MCL in Três Braços: MCL was found to be more common in individuals with large and / or multiple primary lesions and usually developed between 2 and 10 years after an episode of CL. MCL has also been reported in individuals never having had CL (Marsden, 1994b).

***Figure 4.2*: Map of region surrounding Três Braços, Bahia State, Brazil.** (from Cuba-Cuba *et al.*, 1985)

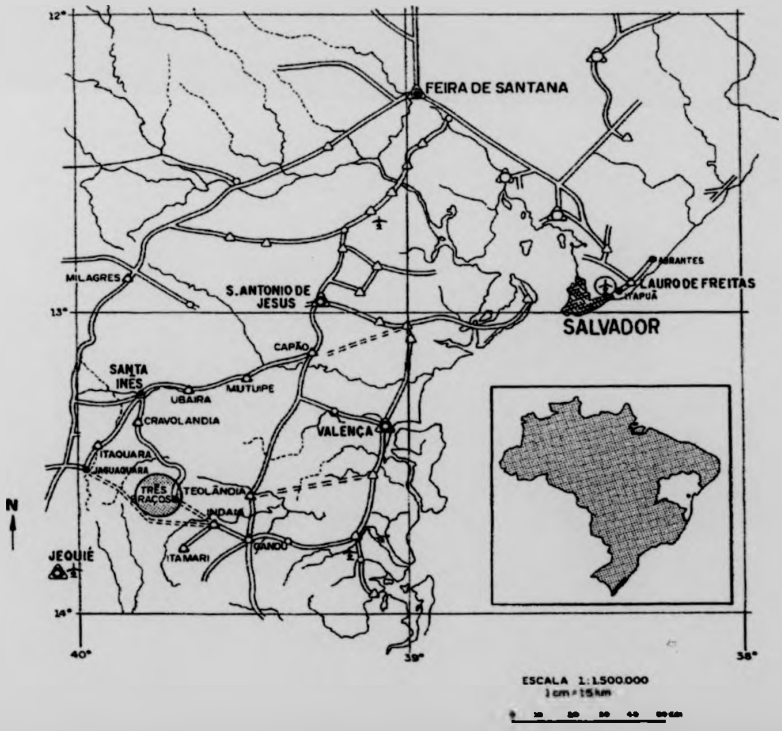


Figure 4.2: Map of region surrounding Três Braços, Bahia State, Brazil (from Cuba-Cuba *et al.*, 1985). Inset shows location of Bahia State (white) and Três Braços (dot) within a map of Brazil. Scale relates to regional map only.

Corte de Pedra is situated, 45km from Três Braços, in an area with virtually no virgin forest remaining, most having been cleared for planting with cocoa, banana and manioc. A major CL epidemic occurred between 1984 and 1986, with a recorded incidence of 83 cases /1000 individuals (Franca *et al.*, 1991).

Primary isolates were made from humans (both CL and MCL), dogs, equines and sand flies in the region using both *in vivo* (hamster inoculation) and *in vitro* (culture media inoculation) methods (Cuba-Cuba *et al.*, 1985; 1986). Almost all isolates were identified as *L. V. braziliensis*; two human cases have been reported in which parasites belonging to the *L. L. mexicana* complex were identified (Cuba-Cuba *et al.*, 1985).

There is evidence for both peridomestic and sylvatic transmission cycles of leishmaniasis in the region. Evidence for peri- and intradomestic transmission comes from observations of the disease both in very young and elderly individuals who do not enter forested areas (Jones *et al.*, 1987). Evidence for sylvatic transmission stems from observations that the disease is most common in male workers employed in plantation farming. Presumably, the predominance of the disease in these individuals is the result of greater exposure from the semi-forested and forested working environment.

Around 8% of individuals with no history of CL gave positive DTH responses, as revealed by skin-testing (Barreto *et al.*, 1981). Such inapparently-infected individuals may serve as disease reservoirs. Although humans and domestic animals are implicated as reservoirs for initiating peridomiciliary transmission in Três Braços / Corte de Pedra, the primary reservoir host is thought to be an as yet unidentified forest-dwelling mammal (Marsden, 1994a, b). More than 800 sylvatic mammals (sloths, anteaters, marsupials, rodents monkeys and rabbits) have been examined however no isolations have been made (Silva *et al.*, 1992).

The probable vector of leishmaniasis in Três Braços is *Lu. whitmani*, a species found in large numbers in and around houses in the region (Vexenat, 1986). Thirty-one other *Lutzomyia* species were recorded from the area, mostly from the remaining forest region. None of these however were found to be infected with *Leishmania* promastigotes (Vexenat, 1986). *Lu. flaviscutellata* is the probable vector of *L. L. amazonensis* in the Três Braços area: this vector species rarely bites man and has a

sylvatic cycle involving forest rodents, which may explain the rarity of infections caused by this species in humans and domestic animals in Três Braços.

In Três Braços today, transmission remains unchecked in the absence of control measures barring personal therapy (Vieira *et al.*, 1990). The cause of the mini epidemics occurring in Três Braços is still not clear but probably stem from an infected mammal (man, dog) initiating a peridomiciliary transmission cycle.

Três Braços stocks have been partially characterized using IEA and RAPD (Cuba-Cuba *et al.*, 1985, 1991; Larsen, 1994); one stock, LTB300, is a WHO-designated *L. V. braziliensis* reference strain.

4.3 *Leishmania* stocks

4.3.1 Brazilian stocks

Brazilian *Leishmania* stocks were selected from diverse geographical areas of Brazil, in seven States encompassing all Regions bar Central / West (see *table 4.1*). Stocks were isolates mostly from sand flies, but also from human and rodent sources. Stocks were obtained from the cryobank at the London School of Hygiene & Tropical Medicine (LSHTM)[a], from R. Naiff (Centro de Pesquisas Amazonas, Manaus, Amazonas, Brazil)[b], from Prof. J. Shaw (Instituto Evandro Chagas, Fundação Nacional de Saude, Belém, Pará, Brazil)[c] and from S. Brandão-Filho (Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Pernambuco, Brazil)[d]. The 19 stocks selected for study are listed in *table 4.3.1.*

4.3.2 Três Braços and Corte de Pedra stocks

Três Braços and Corte de Pedra *Leishmania* stocks (Bahia State) were selected to encompass those from human, sand fly, canine and equine sources. Stocks were obtained from the cryobank at the LSHTM and from Prof. J. Shaw (Instituto Evandro Chagas, Fundação Nacional de Saude, Belém, Pará, Brazil)[c]. The 18 stocks selected for study are listed in *table 4.3.2.*

***Table 4.3.1*: *Leishmania Viannia* stocks from Brazil (excluding stocks from Três Braços and Corte de Pedra)**

ISOLATE CODE NO.	ISOLATED FROM:	REGION / STATE	*LEISHMANIA* SPECIES	LESION TYPE*
MHOM/BR/75/M2903[a]	Human	Carajas / Pará	*L. V. braziliensis*	CL
MHOM/BR/75/M2904[a]	Human	Carajas / Pará	*L. V. braziliensis*	CL
MMES/BR/83/HM1B[c]	Rodent	Miracatu / São Paulo	*L. V. braziliensis*	-
MHOM/BR/81/IM403[c]	Human	Manaus / Amazonas	*L. V. shawi*	CL
ISQU/BR/86/IM2832[b]	Sand fly	Balbina / Amazonas	*L. V.* sp. n.†	-
IWEL/BR/84/M8522[c]	Sand fly	Carajas / Pará	*L. V. braziliensis*	-
IWHI/BR/87/M11367[c]	Sand fly	Carajas / Pará	*L. V. shawi*	-
MAKO/BR/87/BH325R[c]	Rodent	Caratinga / Minas Gerais	*L. V. braziliensis*	-
IWHI/BR/88/M12045[c]	Sand fly	Carajas / Pará	*L. V. shawi*	-
ICOM/BR/89/M12613[c]	Sand fly	Paragominas / Pará	*L. V. braziliensis*	-
ICOM/BR/89/M12620[c]	Sand fly	Paragominas / Pará	*L. V. braziliensis*	-
MHOM/BR/91/M14405[c]	Human	Flora / Paraná	*L. V. braziliensis*	CL
MHOM/BR/93/709-LOA[c]	Human	Peabiru / Paraná	*L. V. braziliensis*	CL
MHOM/BR/93/769-VU[c]	Human	S. Jorge do Ivai / Parana	*L. V. braziliensis*	CL
IWHI/BR/94/M15065[c]	Sand fly	Paragonimas / Parana	*L. V. shawi*	-
MHOM/BR/96/"Amaro"[d]	Human	Amariji / Pernambuco	*L. V. braziliensis*	CL
MHOM/BR/96/"Manoel"[d]	Human	Amariji / Pernambuco	*L. V. braziliensis*	CL
MHOM/BR/96/"Paulino"[d]	Human	Amariji / Pernambuco	*L. V. braziliensis*	CL
MHOM/BR/96/"Fragoso"[d]	Human	Amariji / Pernambuco	*L. V. braziliensis*	CL

Table 4.3.1: *Leishmania Viannia* stocks from Brazil (excluding stocks from Três Braços and Corte de Pedra). Stocks were obtained from the cryobank at the London School of Hygiene & Tropical Medicine (LSHTM)[a], from R. Naiff (Centro de Pesquisas Amazonas, Manaus, Amazonas, Brazil)[b], from Prof. J. Shaw (Instituto Evandro Chagas, Fundação Nacional de Saude, Belém, Pará, Brazil)[c] and from S. Brandão-Filho (Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Pernambuco, Brazil)[d]. † Grimaldi *et al.*, 1991. * Lesion type: CL - cutaneous leishmaniasis; MCL - mucocutaneous leishmaniasis.

***Table 4.3.2*: *Leishmania Viannia* stocks from Três Braços / Corte de Pedra**

ISOLATE CODE NO.	ISOLATED FROM:	REGION / STATE	*LEISHMANIA* SPECIES	LESION TYPE*
MCAN/BR/77/Dog-Lamprea	Dog	Três Braços / Bahia	*L. V. braziliensis*	CL
MCAN/BR/78/Dog-Rodrigo	Dog	Três Braços / Bahia	*L. V. braziliensis*	CL
MCAN/BR/81/Dog-Rico	Dog	Três Braços / Bahia	*L. V. braziliensis*	CL
MEQU/BR/86/JCP-184	Donkey	Corte de Pedra / Bahia	*L. V. braziliensis*	CL
MEQU/BR/86/JCP-225	Donkey	Corte de Pedra / Bahia	*L. V. braziliensis*	CL
vIWHI/BR/86/WHI-10187	Sand fly	Corte de Pedra / Bahia	*L. V. braziliensis*	-
IWHI/BR/86/WHI-CIPD	Sand fly	Corte de Pedra / Bahia	*L. V. braziliensis*	-
IWHI/BR/86/WHI-M11468[c]	Sand fly	Corte de Pedra / Bahia	*L. V. braziliensis*	-
IWHI/BR/86/WHI-EO1094	Sand fly	Três Braços / Bahia	*L. V. braziliensis*	-
IWHI/BR/86/WHI-EO1161	Sand fly	Três Braços / Bahia	*L. V. braziliensis*	-
ITUP/BR/86/TUP-EO252	Sand fly	Três Braços / Bahia	*L. V. braziliensis*	-
MHOM/BR/85/LTB12	Human	Três Braços / Bahia	*L. V. braziliensis*	MCL
MHOM/BR/76/LTB0014	Human	Três Braços / Bahia	*L. V. braziliensis*	MCL
MHOM/BR/00/LTB30	Human	Três Braços / Bahia	*L. V. braziliensis*	MCL
MHOM/BR/81/LTB179	Human	Três Braços / Bahia	*L. V. braziliensis*	CL
MHOM/BR/82/LTB206	Human	Três Braços / Bahia	*L. V. braziliensis*	CL
MHOM/BR/82/LTB253	Human	Três Braços / Bahia	*L. V. braziliensis*	CL
MHOM/BR/84/LTB300	Human	Três Braços / Bahia	*L. V. braziliensis*	MCL

Table 4.3.2: *Leishmania Viannia* stocks from from Três Braços and Corte de Pedra. Stocks were obtained from the cryobank at the London School of Hygiene & Tropical Medicine (LSHTM)[a] and from Prof. J. Shaw (Instituto Evandro Chagas, Fundação Nacional de Saude, Belém, Pará, Brazil)[c]. * Lesion type: CL - cutaneous leishmaniasis; MCL - mucocutaneous leishmaniasis.

4.4 Isoenzyme analysis

Three distinct groups were identified among the 37 stocks from Brazil (all stocks, including those from Três Braços / Corte de Pedra): *L. V. braziliensis* (32 stocks), *L. V. shawi* (4 stocks) and a potential new *Viannia* species (1 stock, *L. V.* sp. - IM2832, Grimaldi *et al.*, 1991, also used as a reference strain). *L. V. shawi* stocks (*guyanensis* species complex) could easily be differentiated from *L. V. braziliensis* stocks using 9 enzyme loci: MPI, NHi1, NHi2, PEPD, PGM, 6PGD, G6PD, ASAT and ALAT. IM2832 (*L. V.* sp. n.) could be differentiated from *L. V. braziliensis* stocks using 8 enzyme loci (MPI, ES, PGM, 6PGD, GPI, G6PD, ASAT and ALAT) and from *L. V. shawi* using 10 enzyme loci (MPI, NHi1, NHi2, ES, PEPD, PGM, GPI, G6PD, ASAT and ALAT). Examples of isoenzyme banding patterns observed in the stocks from Brazil are shown in *figure 4.4.1* for the enzymes MPI (polymorphic) and NHd (monomorphic).

4.4.1 Zymodemes

Ten zymodemes were observed among the stocks from Brazil: stocks identified as *L. V. shawi* (LON203) and *L. V.* sp. n. (LON205) accounted for two of these. Stocks identified as *L. V. braziliensis* could be divided into 8 zymodemes. *Table 4.4.1* (I. and II.) summarizes the IEA allele and zymodeme designations for the Brazilian (I.) and Três Braços / Corte de Pedra stocks (II.). Allele designations are the same as those given in *figure 3.2.2* (chapter 3). "Missing alleles" were scored (-) when no enzyme activity was repeatedly detected.

Of the 32 *L. V. braziliensis* stocks, 4 enzyme loci (MPI, PEPD, PGM AND ES) were shown to be polymorphic: 8 zymodemes were scored among stocks from combinations of polymorphisms at these loci. LON200 accounted for 21/32 (65.6%) of stocks examined: this figure is biased by the inclusion of the 18 stocks from Três Braços / Corte de Pedra since all isolates bar one from this locality were this zymodeme. Three zymodemes (LON210, LON212, LON215) contained a single stock and 4 (LON209, LON211, LON213, LON214) contained 2 stocks each. *L. V. braziliensis* stock zymodemes are summarized by geographical origin, host / vector source and clinical presentation in *table 4.4.2*.

***Figure 4.4.1*:** **Isoenzyme banding patterns of *Leishmania* stocks from Brazil.**

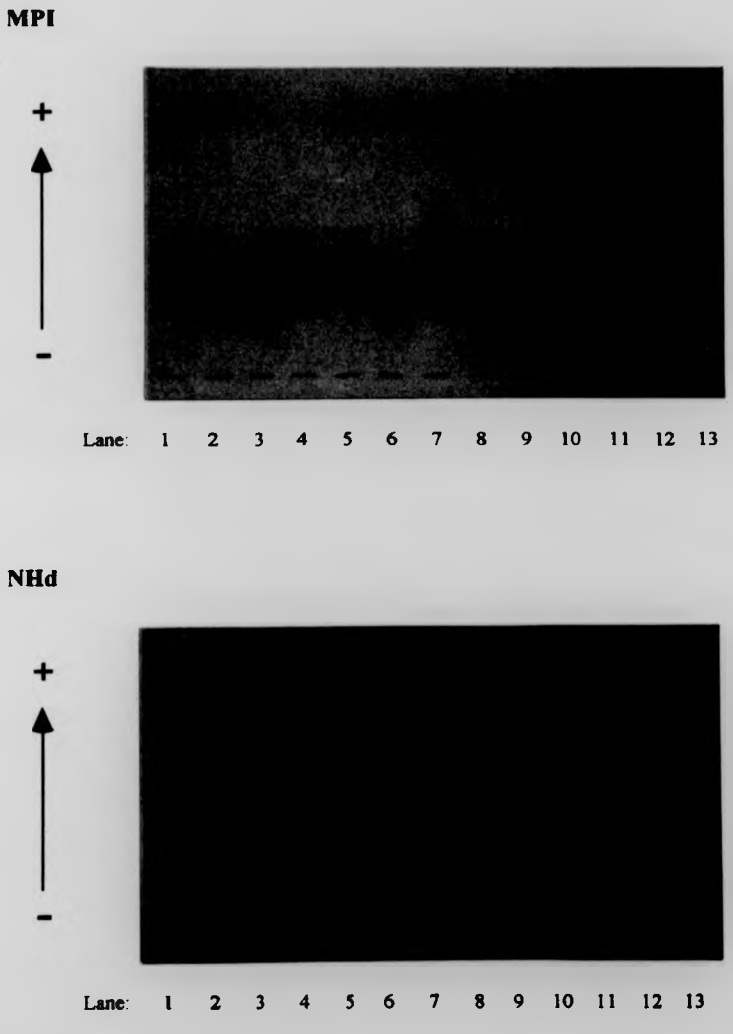


Figure 4.4.1: Isoenzyme variation in stocks from Brazil using MPI and NHd. Lane 1 - *L. V. braziliensis* reference strain LTB300; lane 2 - *L. V. guyanensis* reference strain M4147; lane 3 - M2903; lane 4 - M2904; lane 5 - HM1B; lane 6 - M11367; lane 7 - BH325R; lane 8 - M12613; lane 9 - M14916; lane 10 - M8522; lane 11 - M14913; lane 12 - M12045; lane 13 - *L. V. shawi* reference strain M15065. Using MPI, *L. V. braziliensis* variants are observed in lane 3 and 7. Stocks in lanes 6, 12 & 13 were identified (in combination with other enzyme loci) as *L. V. shawi* - using MPI they are identical to the *L. V. guyanensis* reference strain).

***Table 4.4.1*: I. Allele and zymodeme designations for Brazilian stocks.**

STOCK CODE NO.	* HOST SPECIES	ENZYME												Z*
		MPI	NHi 1	NHi 2	NHd	ES	PEPD	PGM	6PGD	GPI	G6PD	ASAT	ALAT	
Amaro, 709-LOA, 769-VU	MHOM	6/6	2/2	7/7	4/4	4/4	2/2	3/3	5/5	4/4	2/2	5/5	4/4	LON200
M8522	IWEL													
M15065, M12045, M11367	IWHI	8/8	3/3	5/5	4/4	4/4	7/7	5/5	6/6	4/4	1/1	2/2	3/3	LON203
IM403	MHOM													
IM2832	ISQU	5/6	2/2	7/7	4/4	5/5	4/4	2/2	6/6	2/2	3/3	4/4	5/5	LON205
Fragoso	MHOM	6/6	2/2	7/7	4/4	4/4	2/2	-	5/5	4/4	2/2	5/5	4/4	LON209
HM1B	MMES													
M2903, Manoel	MHOM	6/7	2/2	7/7	4/4	4/4	2/2	3/3	5/5	4/4	2/2	5/5	4/4	LON211
BH325R	MAKO	6/9	2/2	7/7	4/4	4/4	2/2	3/3	5/5	4/4	2/2	5/5	4/4	LON212
Paulino, M14405	MHOM	6/6	2/2	7/7	4/4	4/4	2/2	4/4	5/5	4/4	2/2	5/5	4/4	LON213
M2904	MHOM	6/6	2/2	7/7	4/4	4/4	4/4	3/3	5/5	4/4	2/2	5/5	4/4	LON214
M12613	ICOM													
M12620	ICOM	6/6	2/2	7/7	4/4	4/4	2/4	3/3	5/5	4/4	2/2	5/5	4/4	LON215

Notes to *table 4.4.1*: Allele and zymodeme designations for Brazilian stocks. Code names given in **bold** are reference strains. * host / vector species code: MHOM - mammal, human; MMES - mammal, rodent (*Mesocricetus* sp.); MAKO, mammal, rodent (*Akodon* sp.); IWHI - sand fly, *Lu. (Nys.) whitmani*, ICOM - sand fly, *Lu. complexus*; IWEL - sand fly, *Lu. (Psy.) wellcomei*; ISQU - sand fly, *Lu. (Psy.) squamiventris*.. Z = zymodeme, * LON numbers above 200 have been reassigned by the author as previous records have been lost (zymodemes are assigned based on the IEA conditions used, i.e. for *Leishmania Viannia* species). For full enzyme names, see chapter 2. See *figure 3.2.2* for allele scoring scheme. Data given assumes diploidy, see introduction.

***Table 4.4.1*:** **II. Allele and zymodeme designations for Três Braços / Corte de Pedra stocks.**

		ENZYME												
STOCK CODE NO.	*** HOST SPECIES**	**MPI**	**NHi 1**	**NHi 2**	**NHd**	**ES**	**PEPD**	**PGM**	**6PGD**	**GPI**	**G6PD**	**ASAT**	**ALAT**	**Z***
LTB300, LTB12, LTB14, LTB30, LTB179, LTB206, LTB253	MHOM	6/6	2/2	7/7	4/4	4/4	2/2	3/3	5/5	4/4	2/2	5/5	4/4	LON200
JCP-184, JCP-225	MEQQ													
Dog-Lamprea, Dog-Rico, Dog-Rodrigo,	MCAN													
WHI-M10187, M11468, EO1094, EO1161	IWHI													
TUP-EO252	ITUP													
WHI-C1PD	IWHI	6/6	2/2	7/7	4/4	-	2/2	-	5/5	4/4	2/2	5/5	4/4	LON210

Notes to *table 4.4.1*: Allele and zymodeme designations for Três Braços / Corte de Pedra stocks. Code names given in **bold** are reference strains. * host / vector species code: MHOM - mammal, human; MCAN - mammal, canid (dog); MEQU - mammal, equine (donkey); IWHI - sand fly, *Lu. (Nys.) whitmani*; ITUP - sand fly, *Lu. tupynambai*. Z = zymodeme; * LON numbers above 200 have been reassigned by the author as previous records have been lost (zymodemes are assigned based on the IEA conditions used, i.e. for *Leishmania Viannia* species). For full enzyme names, see chapter 2. See *figure 3.2.2* for allele scoring scheme. Data given assumes diploidy, see introduction.

***Table 4.4.2*: Summary of *L. V. braziliensis* stock zymodemes, by geographical origin, host / vector source and clinical presentation.**

STOCK CODE NO.	Z*	GEOGRAPHICAL ORIGIN		ISOLATED FROM:	CLINICAL TYPE
		REGION	STATE		
M12613	LON214	Paragominas	Pará	Sand fly	-
M12620	LON215	Paragominas	Pará	Sand fly	-
M8522	LON200	Carajas	Pará	Sand fly	-
M2903	LON211	Carajas	Pará	Human	CL
M2904	LON214	Carajas	Pará	Human	CL
HM1B	LON209	Miracatu	São Paulo	Rodent	-
BH325R	LON212	Caratinga	Minas Gerais	Rodent	-
"Amaro"	LON200	Amariji	Pernambuco	Human	CL
"Manoel"	LON211	Amariji	Pernambuco	Human	CL
"Paulino"	LON213	Amariji	Pernambuco	Human	CL
"Fragoso"	LON209	Amariji	Pernambuco	Human	CL
Dog-Lamprea	LON200	Três Braços	Bahia	Dog	CL
Dog-Rodrigo	LON200	Três Braços	Bahia	Dog	CL
Dog-Rico	LON200	Três Braços	Bahia	Dog	CL
JCP-184	LON200	Corte de Pedra	Bahia	Donkey	CL
JCP-225	LON200	Corte de Pedra	Bahia	Donkey	CL
WHI-10187	LON200	Corte de Pedra	Bahia	Sand fly	-
WHI-CIPD	LON210	Corte de Pedra	Bahia	Sand fly	-
WHI-M11468	LON200	Corte de Pedra	Bahia	Sand fly	-
WHI-EO1094	LON200	Três Braços	Bahia	Sand fly	-
WHI-EO1161	LON200	Três Braços	Bahia	Sand fly	-
TUP-EO252	LON200	Três Braços	Bahia	Sand fly	-
LTB12	LON200	Três Braços	Bahia	Human	MCL
LTB0014	LON200	Três Braços	Bahia	Human	MCL
LTB30	LON200	Três Braços	Bahia	Human	MCL
LTB179	LON200	Três Braços	Bahia	Human	CL
LTB206	LON200	Três Braços	Bahia	Human	CL
LTB253	LON200	Três Braços	Bahia	Human	CL
LTB300	LON200	Três Braços	Bahia	Human	MCL
M14405	LON213	Flora	Paraná	Human	CL
709-LOA	LON200	Peabiru	Paraná	Human	CL
769-VU	LON200	S. Jorge do Ivai	Paraná	Human	CL

Notes to *table 4.4.2*: (previous page) summary of *L. V. braziliensis* stock zymodemes, by geographical origin, host / vector source and clinical presentation. Code names given in **bold** are reference strains. Z = zymodeme (assigned based on the IEA conditions used, i.e. for *Leishmania Viannia* species). For full enzyme names, see chapter 2. See *figure 3.2.2* for allele scoring scheme. Data given assumes diploidy, see introduction.

The 18 *L. V. braziliensis* Três Braços / Corte de Pedra stocks were shown, in agreement with previous reports (Cuba-Cuba *et al.*, 1985; Rosa *et al.*, 1988), to be remarkably similar using IEA: all stocks bar one were zymodeme LON200, identical to the WHO reference strain LTB300 (see *figure 3.2.2*). The only stock among those from Três Braços / Corte de Pedra to show any variation was WHI-C1PD (LON210): this stock differed only in that it repeatedly showed a lack of detectable activity of the enzymes ES and PGM. Stocks from other areas of Brazil were more variable than those from Três Braços / Corte de Pedra, however too few stocks were examined from each locality to be able to associate zymodemes with geographical area. The four stocks from Amariji deserve further comment: these stocks were identified as *L. V. braziliensis*, however, each stock presented a different zymodeme (LON200, LON209, LON211 and LON213). Others groups have also reported *L. V. braziliensis* isoenzyme variants from the region (Felinto de Brito *et al.*, 1993).

Of the 17 stocks from humans, 13 were isolated from CL lesions and 4 from MCL lesions. Human stocks encompassed 5 zymodemes (LON200, 209, 211, 213, 214, see table 4.4.2). All stocks isolated from MCL lesions were LON200. No correlation was observed between zymodeme and clinical presentation. There was also no association between zymodeme and host / vector species.

4.5 Microsatellite analysis

4.5.1 PCR of microsatellite loci

4.5.1.1 Stocks with "null alleles"

Ten stocks from Brazil failed to generate PCR amplification products at one or more of the microsatellite loci. The amplification profiles of these stocks are summarized in *table 4.5.1.1*. PCR amplification failure (assumed to be due to mutation in the primer binding site) was observed most often for the AC01 and AC52 loci. Four stocks failed to amplify any of the three loci. The DNA from these stocks was shown to be intact

by electrophoretic techniques and by the successful PCR amplification of the 18S rRNA gene locus. Stocks failing to generate PCR products were scored as having "null alleles": mutation(s) in the binding site were not investigated further and it is therefore not possible to establish whether one or both priming sites were implicated. For reasons not determined, sand fly stocks from Três Braços / Corte de Pedra appeared to have the most null alleles (4/6 stocks, 66.6%) compared with stocks of non sand fly origin (1/14 stocks, 7.1%).

Table 4.5.1.1 **Summary of microsatellite locus PCR failures for stocks with null alleles (failing to amplify one or more loci.)**

Stock code no.	Species*	Geographical origin	Isolated from	AC01	AC16	AC52
IM2832	*L. V.* sp. n.	Balbina, AM	sand fly		X	X
IM403	*L. V. shawi*	Manaus, AM	human	X		X
M12613		Paragominas, PA	sand fly	X		X
709-LOA		Peabiru, PN	human	X		X
769-VU		S. Jorge do Ivai, PN	human	X	X	X
JCP-184	*L. V. braziliensis*	Corte de Pedra, BA	donkey	X		X
WHI-EO1161		Três Braços, BA	sand fly	X		
WHI-M11468		Corte de Pedra, BA	sand fly	X	X	X
TUP-EO252		Três Braços, BA	sand fly	X	X	X
WHI-EO1094		Três Braços, BA	sand fly	X	X	X
	PCR failures			9	5	9

Table 4.5.1.1: Summary of microsatellite locus PCR failures for stocks with null alleles (i.e. those failing to amplify one or more loci.). X indicates no amplification. * Species status was determined from isoenzyme analysis

4.5.1.2 PCR amplification of microsatellite loci

Analyzing PCR products using agarose gel electrophoresis and ethidium bromide staining, stocks from Brazil (excluding those with null alleles) generated PCR products for AC01 in the range 220 - 250 bp, in agreement with Russell *et al.* (1999). An additional faint band of approximately 450 bp was also observed in some stocks, which was probably a dimer since subsequent analyses (Genotyper® and sequencing) of these stocks revealed a single peak / sequence.

PCR products generated from Brazilian stocks for AC16 were in the range 240 - 270 bp, also in agreement with Russell *et al.* (1999). PCR products generated from Brazilian stocks for AC52 were in the range 280 - 310 bp, also in agreement with Russell *et al.* (1999). Diffuse bands were observed for several stocks; this suggested that more than one band was generated in these stocks which were not resolved using agarose gel electrophoresis.

4.5.2 Non-denaturing PAGE analysis

Agarose gel electrophoresis was used initially to determine the success of PCR amplification. Products were subsequently analyzed using PAGE gels, which permit greater resolution, and silver staining, which is highly sensitive. PAGE analysis of microsatellite locus products from all stocks revealed reproducible pattern types.

4.5.2.1 AC01

Figure 4.5.2.1 shows AC01 products from Brazilian stocks analyzed using non-denaturing PAGE and silver staining. Two intensely-staining bands were observed for most stocks at this locus: the band corresponding to the expected allele size was generally more intense. Another band , usually fainter, was also present in most stocks and always at a set distance from the allelic band. This additional band was not large enough to be a dimeric form of the allelic band. Several observations indicate that this additional band was a conformational variant of the allelic band: i) this band was not observed using agarose and ethidium bromide although it was intense and of a sufficiently different size from the allelic band to be detected within the limits of agarose gel electrophoresis / ethidium bromide staining; ii) this band "disappeared" after denaturing PAGE (Genescan® / Genotyper®) analysis (see section 4.5.3); iii) this band was too large to be considered as the second allele identified in some stocks by Genotyper® (see section 4.5.3); iv) sequencing analysis revealed that most products were composed of a single sequence type (see section 4.5.4). Similar additional bands have been described in reviews of microsatellite analysis as being PCR artefacts which are consistent between isolates giving a pattern of bands which is peculiar to the locus (Ashley & Dow, 1994; Schlötterer, 1998).

Figure 4.5.2.1 **AC01 products from Brazilian stocks**

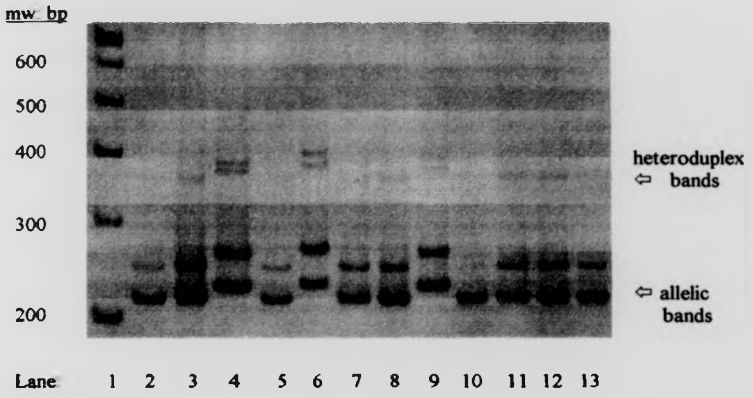


Figure 4.5.2.1: AC01 products from Brazilian stocks analyzed using non-denaturing 6% PAGE and silver staining. Lane 1 - 100 bp molecular weight marker (Gibco BRL, UK); lane 2 - *L. V. braziliensis* reference strain, LTB300; lane 3 - M8522; lane 4 - M11367; lane 5 - M11425; lane 6 - *L. V. shawi* reference strain, M15065; lane 7 - M12620; lane 8 - M14405; lane 9 - M12045; lane 10 - Amaro; lane 11 - Manoel; lane 12 - Fragoso; lane 13 - Paulino. The allelic band is the lowest intensely-staining band in each lane. Note the additional band (at set distance from the allelic band in each lane) and heteroduplex bands (indicated): see text for discussion. PCR products from *L. V. shawi* stocks are in lanes 4, 6 & 9.

In some stocks, heteroduplex bands were also observed. Heteroduplex molecules may arise from both homozygous and heterozygous individuals, and are formed during late PCR cycles (Schlötterer, 1998). *Figure 4.5.2.2* illustrates the formation of heteroduplex molecules during PCR amplification. Heteroduplex molecules arise when complementary strands fail to base-pair precisely when reannealing. Failure to base-pair correctly may be due to the reannealing of complementary strands from heterozygous alleles or occasionally due to an error during PCR amplification such as slippage (especially with microsatellite alleles) or due to the insertion of an incorrect base into the sequence of one strand. An apparently homozygous locus may have alleles which differ slightly in sequence but not size: reannealing of complementary strands from such different alleles may, therefore, also result in heteroduplex formation. Whatever the mechanism generating the mismatch, when the strands reanneal secondary structures occur in the double-stranded molecule at sites of mismatched bases.

***Figure 4.5.2.2*: Formation of heteroduplex molecules**

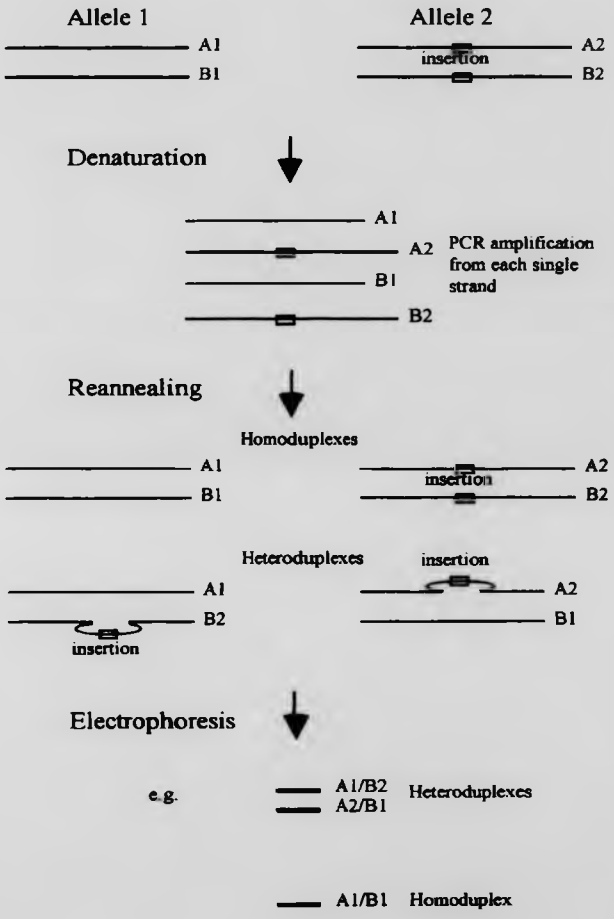


Figure 4.5.2.2: Formation of heteroduplex molecules. At a homozygous locus, assuming the sequence composition of alleles 1 and 2 are the same†, two complementary single strands will arise from denaturation: A1 (X 2) and B1 (X 2). Reannealing regenerates homoduplex molecules (A1 / B1). If an error occurs during PCR amplification such that, for example, there is a base change in one strand (say A1, with mutation called A1*), a heteroduplex molecule (A1* / B1) may be evident if the error results in a mismatch which causes secondary structure in the double-stranded, reannealed molecule. †(An apparently homozygous locus may also have alleles which differ slightly in sequence but not size: reannealing of complementary strands from different alleles at such a locus may also result in heteroduplex formation.) At a heterozygous locus, alleles 1 and 2 differ, for example by a dinucleotide insertion. Four distinct single strands will arise from denaturation: A1, A2, B1 and B2. Reannealing may regenerate both homoduplex molecules (A1 / B1 or A2 / B2) and heteroduplex molecules (A1 / B2 or A2 / B1). Further heteroduplex bands may also arise by the same processes described for homozygous alleles.

At homozygous loci, usually none or a single heteroduplex molecule is observed. At heterozygous loci, 2 or more bands are commonly present, resulting from combinations of strands from different alleles. Heteroduplex molecules appear much larger than homoduplex molecules when electrophoresed because the secondary structure retards their movement though the gel. The retardation of these molecules depends on the degree of secondary structure and the sequence composition of each molecule.

Among the Brazilian stocks, heteroduplex bands were particularly evident at the AC01 locus for the *L. V. shawi* stocks (lanes 4, 6 and 9 of *figure 4.5.2.1*). The heteroduplex bands for these stocks (2 in each) have slightly different mobilities, indicating the presence of different mismatches within the molecules (and therefore different secondary structures). Heteroduplex molecules (single) were also observed in *L. V. braziliensis* stocks (lanes 3, 7, 8, 11, 12): these exhibit an identical mobility suggesting that the sequence difference generating the mismatch is common to these stocks and was therefore unlikely to have been generated as a result of random error during PCR amplification.

4.5.2.2 AC16

Figure 4.5.2.3 shows the AC16 products from Brazilian stocks. At this locus, one or two bands were observed in each stock: a second band was less common than in AC01. Where a second band was present this band was, as for AC01, usually less intense and always at a set distance from the allelic band. Heteroduplex bands were rarely observed, even in stocks which were shown using Genotyper® to be heterozygous. Some size variation in the allelic bands was evident between stocks at this locus, e.g. lanes 4, 9, and 10. Heterozygote bands are evident in lanes 9 and 10 (weak) which reflect the Genotyper® results in these stocks (see section 4.5.3).

4.5.2.3 AC52

Figure 4.5.2.4 shows AC52 products from Brazilian stocks. The PCR products from this locus revealed complex banding patterns: even for stocks shown to be homozygous at AC52 using Genotyper® analysis (e.g. lanes 2-6, *figure 4.5.2.4*), several bands observed. As discussed previously, these bands were probably locus-specific PAGE artefacts as the larger bands did not correlate with allele peak sizes identified using Genotyper® analysis (see section 4.5.3). In addition, many less intense bands were observed which were most likely heteroduplex molecules.

Figure 4.5.2.3 **AC16 products from Brazilian stocks**

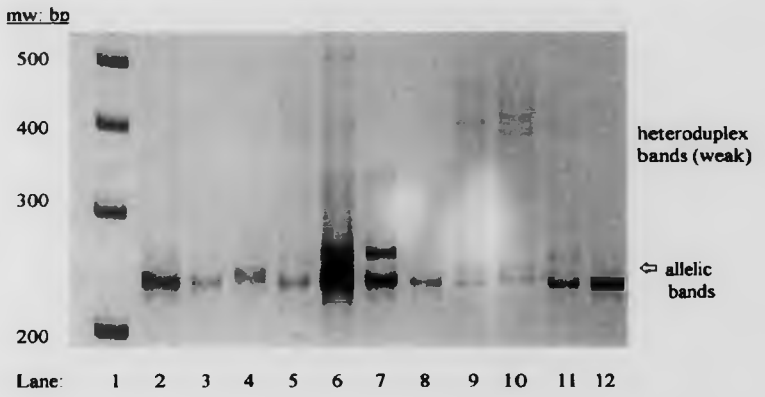


Figure 4.5.2.3: AC16 products from Brazilian stocks analyzed using non-denaturing 6% PAGE and silver staining. Lane 1 - 100 bp molecular weight marker (Gibco BRL, UK); lane 2 - *L. V. braziliensis* reference strain, LTB300; lane 3 - IM403; lane 4 - M8522; lane 5 - WHI-EO1611; lane 6 - M11367; lane 7 - BH325R; lane 8 - M12045; lane 9 - M12613; lane 10 - M12620, lane 11 - M14405; lane 12 - 709-LOA; lane 13 - *L. V. shawi* reference strain, M15065. Note the additional larger band (at set distance from the allelic band in several lanes) and heteroduplex bands (weak, indicated): see text for discussion.

Figure 4.5.2.4 **AC52 products from Brazilian stocks**

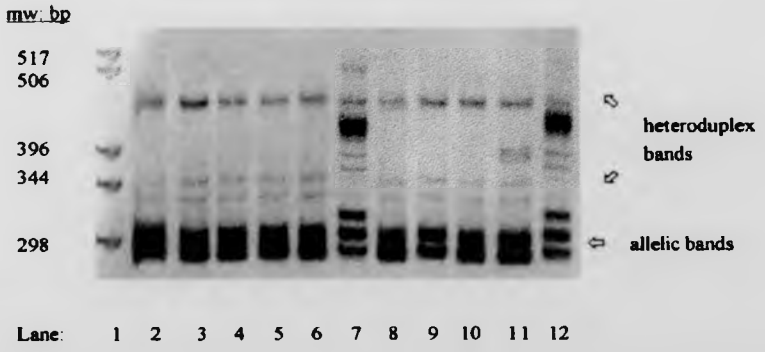


Figure 4.5.2.4: AC52 products from Brazilian stocks analyzed using non-denaturing 6% PAGE and silver staining. Lane 1 - 1kbp molecular weight marker (Gibco BRL, UK); lane 2 - LTB12; lane 3 - LTB14; lane 4 - LTB30; lane 5 - LTB179; lane 6 - LTB206; lane 7 - LTB253; lane 8 - *L. V. braziliensis* reference strain LTB300; lane 9 - WHI-10187; lane 10 - WHI-CIPD; lane 11 - JCP-225; lane 12 - Dog-Rico. Note the additional bands (at set distances from the allelic band(s) in each lane) and heteroduplex bands (indicated): see text for discussion.

In all stocks which were heterozygous for AC52 (e.g. lanes 7 and 12), 3 intense bands were observed as well as many heteroduplex bands. The origins of these additional bands are unclear, however the AC52 pattern in these stocks generally correlated with Genotyper® results.

Owing to the general lack of resolution and allele scoring power of non-denaturing PAGE and the availability of Genescan® and Genotyper® software, no further analyses were carried out from PAGE data, other than to note banding patterns and to attempt a correlation of these with Genotyper® data.

4.5.3 Genescan® and Genotyper® 2.0 analyses

The complexity of banding patterns observed using non-denaturing PAGE analysis was not easily interpreted: it was not always clear which band(s) to score and accurate sizing was not possible on the short PAGE gels used. To resolve these problems, Genescan® and Genotyper® analyses employing long-range, denaturing PAGE gels were used.

Using Genotyper® software, the PCR product(s) from each microsatellite locus were accurately sized for each stock. Heteroduplex bands and other additional bands "disappeared" such that accurate allele size could be assigned at all loci in almost all stocks.

Table 4.5.3.1 summarizes the allele sizes observed at each microsatellite locus in stocks from Brazil, listed by geographical origin. *Table 4.5.3.2* summarizes the data from *L. V. braziliensis* stocks by host and by clinical type. *Table 4.5.3.3* summarizes the data from *L. V. braziliensis* stocks isolated from sand flies. Results are discussed by each microsatellite locus and finally by multilocus genotype.

Table 4.5.3.1: (see next page) Summary of microsatellite allele sizes found in stocks from Brazil, listed by species then geographical area. Code names in **bold** type indicate stocks used as reference strains. * - isolate IM2832, an unnamed *Viannia* species (Grimaldi *et al.*, 1991). † - stocks identified as *L. V. shawi*. Assignment and scoring of alleles are explained in chapter 3. X indicates that no PCR product was generated. ND indicates not determined: Genescan® / Genotyper® analysis failed to generate a scorable peak. Allele sizes given in **bold** type indicate alleles different to the most common allele observed.

Table 4.5.3.1 **Summary of microsatellite allele sizes found in stocks from Brazil, by geographical area.**

		Microsatellite locus					
		AC01		AC16		AC52	
Stock code no.*†	Region / State	allele 1[a]	allele 2[b]	allele 1	allele 2	allele 1	allele 2
IM2832*	Balbina / Amazonas	227	245	X	X	X	X
IM403†	Manaus / Amazonas	X	X	246	248	X	X
M12045†	Carajas / Pará	231	231	246	246	293	293
M11367†	Carajas / Pará	231	231	244	248	291	291
M15065†	Paragominas / Pará	241	241	246	248	297	299
M2903	Carajas / Pará	227	227	**250**	**266**	**297**	**297**
M2904	Carajas / Pará	227	227	**246**	**254**	**293**	**295**
M8522	Carajas / Pará	227	227	**248**	**254**	ND	ND
M12613	Paragominas / Pará	X	X	244	**256**	X	X
M12620	Paragominas / Pará	**223**	**223**	**254**	**262**	**285**	**293**
Manoel	Amariji / Pernambuco	227	**231**	244	**252**	283	283
Paulino	Amariji / Pernambuco	227	**231**	244	**250**	**285**	**303**
Fragoso	Amariji / Pernambuco	227	**231**	**248**	**248**	283	**301**
Amaro	Amariji / Pernambuco	**231**	**231**	244	244	283	**301**
HM1B	Miracatu / São Paulo	227	227	244	244	283	**301**
BH325R	Caratinga /Minas Gerais	227	227	244	**246**	283	283
Dog-Lamprea	Três Braços / Bahia	227	227	244	244	283	**305**
Dog-Rodrigo	Três Braços / Bahia	227	227	244	244	283	283
Dog-Rico	Três Braços / Bahia	227	227	244	244	283	**305**
WHI-EO1094	Três Braços / Bahia	X	X	X	X	X	X
WHI-EO1161	Três Braços / Bahia	X	X	244	244	283	283
TUP-EO252	Três Braços / Bahia	X	X	X	X	X	X
LTB12	Três Braços / Bahia	227	227	244	244	283	283
LTB14	Três Braços / Bahia	227	227	244	244	283	283
LTB30	Três Braços / Bahia	227	227	244	244	283	283
LTB179	Três Braços / Bahia	227	227	244	244	283	283
LTB206	Três Braços / Bahia	227	227	244	244	283	283
LTB253	Três Braços / Bahia	227	227	244	244	283	**303**
LTB300	Três Braços / Bahia	227	227	244	244	283	283
JCP-184	Corte de Pedra / Bahia	X	X	244	**246**	X	X
JCP-225	Corte de Pedra / Bahia	227	227	244	**246**	283	283
WHI-10187	Corte de Pedra / Bahia	227	227	244	244	283	283
WHI-CIPD	Corte de Pedra / Bahia	227	227	244	244	283	283
M11468	Corte de Pedra / Bahia	X	X	X	X	X	X
M14405	Flora / Paraná	227	227	**242**	**242**	283	**301**
709-LOA	Peabiru / Paraná	X	X	244	244	X	X
769-VU	S. Jorge do Ivai / Parana	X	X	X	X	X	X

Table 4.5.3.2 **Summary of microsatellite allele sizes of *L. V. braziliensis* stocks from Brazil, by host and by clinical type.**

			Microsatellite locus					
			AC01		AC16		AC52	
Stock code no.	Host	Clinical type	allele 1[a]	allele 2[b]	allele 1	allele 2	allele 1	allele 2
M2903	Human	CL	227	227	**250**	**266**	**297**	**297**
M2904	Human	CL	227	227	**246**	**254**	**293**	**295**
Manoel	Human	CL	227	**231**	244	**252**	283	283
Paulino	Human	CL	227	**231**	244	**250**	**285**	**303**
Fragoso	Human	CL	227	**231**	**248**	**248**	283	**301**
Amaro	Human	CL	**231**	**231**	244	244	283	**301**
M14405	Human	CL	227	227	**242**	**242**	283	**301**
709-LOA	Human	CL	X	X	244	244	X	X
769-VU	Human	CL	X	X	X	X	X	X
LTB30	Human	CL	227	227	244	244	283	283
LTB206	Human	CL	227	227	244	244	283	283
LTB253	Human	CL	227	227	244	244	283	**303**
LTB12	Human	MCL	227	227	244	244	283	283
LTB14	Human	MCL	227	227	244	244	283	283
LTB179	Human	MCL	227	227	244	244	283	283
LTB300	Human	MCL	227	227	244	244	283	283
Dog-Lamprea	Dog	CL	227	227	244	244	283	**305**
Dog-Rodrigo	Dog	CL	227	227	244	244	283	283
Dog-Rico	Dog	CL	227	227	244	244	283	**305**
JCP-184	Donkey	CL	X	X	244	**246**	X	X
JCP-225	Donkey	CL	227	227	244	**246**	283	283
HM1B	Rodent	-	227	227	244	244	283	**301**
BH325R	Rodent	-	227	227	244	**246**	283	283

Table 4.5.3.2: Summary of microsatellite allele sizes of *L. V. braziliensis* stocks from Brazil, by host and by clinical type. Code names in **bold** type indicate stocks used as reference strains. Assignment and scoring of alleles are explained in chapter 3. X indicates that no PCR product was generated. Allele sizes given in **bold** type indicate alleles different to the most common allele observed.

Table 4.5.3.3 **Summary of microsatellite allele sizes of *L. V. braziliensis* stocks isolated from sand flies from Brazil.**

		Microsatellite locus					
		AC01		AC16		AC52	
Stock code no.	Sand fly vector species	allele 1[a]	allele 2[b]	allele 1	allele 2	allele 1	allele 2
M8522	*Lu. wellcomei*	227	227	**248**	**254**	ND	ND
M12613	*Lu. complexus*	X	X	244	**256**	X	X
M12620	*Lu. complexus*	**223**	**223**	**254**	**262**	**285**	**293**
WHI-EO1094	*Lu. whitmani*	X	X	X	X	X	X
WHI-EO1161	*Lu. whitmani*	X	X	244	244	283	283
TUP-EO252	*Lu. tupynambai*	X	X	X	X	X	X
WHO-10187	*Lu. whitmani*	227	227	244	244	283	283
WHI-CIPD	*Lu. whitmani*	227	227	244	244	283	283
WHI-M11468	*Lu. whitmani*	X	X	X	X	X	X

Table 4.5.3.3: Summary of microsatellite allele sizes of *L. V. braziliensis* stocks isolated from sand flies from Brazil.. Assignment and scoring of alleles are explained in chapter 3. X indicates that no PCR product was generated. ND indicates not determined: Genescan® / Genotyper® analysis failed to generate a scorable peak. Allele sizes given in **bold** type indicate alleles different to the most common allele observed.

4.5.3.1 AC01

AC01 genotypes identified in Brazilian stocks are summarized in *table 4.5.3.4*. The most common allele size identified among *L. V. braziliensis* stocks was 227: 4 genotypes were scored. Most *L. V. braziliensis* stocks were homozygous (i.e. generated a single peak), with a presumed genotype of 227/227 (19/24 stocks).

All stocks from Três Braços / Corte de Pedra, excluding those having "null alleles", were genotyped as 227/227 (see *table 4.5.3.1*). Among *L. V. braziliensis* stocks from Pará, one (M12620) was genotype 223/223; all others were 227/227. In Brazilian *L. V. braziliensis* stocks, the 231 allele was found only in Amariji (Pernambuco State). Three Amariji stocks were heterozygous at AC01 (genotype 227/231) and a fourth stock was homozygous (genotype 231/231). The 231 allele was identified originally in the *L. V. peruviana* reference strain LC39: among these Brazilian stocks it has also been found to be associated with *L. V. braziliensis* and 2 *L. V. shawi* stocks. *L. V. shawi* stocks had genotypes of 231/231 or 241/241. The stock IM2832 was

heterozygous, as indicated by the presence of 2 peaks, with genotype 227/245. All stocks isolated from MCL lesions were AC01 genotype 227/227.

Two AC01 alleles, 227 and 231, were identified in *L. V. braziliensis* stocks from host species: 231 was found only in stocks isolated from human hosts. This allele was also observed in sand fly isolates, but these stocks were identified as *L. V. shawi*. Two genotypes, 227/227 and 223/223, were observed in *L. V. braziliensis* stocks from sand fly vectors: 223 was found only in 1 sand fly stock. Null alleles were commonly observed among sand fly stocks from Três Braços / Corte de Pedra (4/6 stocks).

Table 4.5.3.4 **AC01 genotypes found in stocks from Brazil**

Species§	Inferred genotype*	No. stocks	Stocks
L. V. sp. n.	227 / 245	1	**IM2832***
L. V. shawi	231 / 231	2	M12045, M11367
	241 / 241	1	**M15065**
L. V. braziliensis	223 / 223	1	M12620
	227 / 227	19	M2903, M2904, M8522, HM1B, BH325R, Dog-Lamprea, Dog-Rodrigo, Dog-Rico, LTB12, LTB14, LTB30, LTB179, LTB206, LTB253, **LTB300**, JCP-225, WHI-10187, WHI-C1PD, M14405
	227 / 231	3	Manoel, Paulino, Fragoso
	231 / 231	1	Amaro

Table 4.5.3.4: AC01 genotypes found in stocks from Brazil. § Species status assigned from IEA. * Genotype inferred from scoring Genotyper® peaks. Code names in **bold** type indicate stocks used as reference strains. * - isolate IM2832, an unnamed *Viannia* species (Grimaldi *et al.*, 1991). Allele assignment is explained in chapter 3.

4.5.3.2 AC16

AC16 genotypes found in stocks from Brazil are summarized in *table 4.5.3.5*. Among *L. V. braziliensis* stocks, the most common genotype (of the 11 genotypes scored) at the AC16 locus was 244/244. Many more alleles (10) and genotypes (11) were scored among *L. V. braziliensis* stocks at this locus than at AC01, and many of the stocks (10/28) were heterozygous.

Table 4.5.3.5 **AC16 genotypes found in stocks from Brazil**

Species§	Inferred genotype*	No. stocks	Stocks
L. V. shawi	244 / 248	1	M11367
	246 / 246	1	M12045,
	246 / 248	2	**M15065**, IM403
L. V. braziliensis	242 / 242	1	M14405
	244 / 244	16	Amaro, HM1B, Dog-Lamprea, Dog-Rodrigo, Dog-Rico, LTB12, LTB14, LTB30, LTB179, LTB206, LTB253, **LTB300**, WHI-EO1161, WHI-10187, WHI-C1PD, M14405, 709-LOA
	244 / 246	3	BH325R, JCP-184, JCP-225
	244 / 250	1	Paulino
	244 / 252	1	Manoel
	244 / 256	1	M12613
	246 / 254	1	M2904
	248 / 248	1	Fragoso
	248 / 254	1	M8522
	250 / 266	1	M2903
	254 / 262	1	M12620

Table 4.5.3.5: AC16 genotypes found in stocks from Brazil. § Species status assigned from IEA. * Genotype inferred from scoring Genotyper® peaks. Code names in **bold** type indicate stocks used as reference strains. Allele assignment is explained in chapter 3.

Most stocks from Três Braços / Corte de Pedra were genotyped as 244/244: the 2 donkey stocks were genotyped as 244/246. The 246 allele was also observed in two other stocks: BH325R, a rodent isolate from Minas Gerais and M2904, a human stock from Carajas (Pará). Stocks from Pará State were highly variable at this locus: 5 stocks from 2 regions each had a distinct heterozygous genotype. *L. V. braziliensis* allele sizes in stocks from more northern Brazilian states (Para, Pernambuco) were commonly larger than those from other states. The four stocks from Amariji (Pernambuco) each had a different genotype at this locus, 2 of which were heterozygous. All alleles identified in the *L. V. shawi* stocks were also found in the *L. V. braziliensis* stocks, however in combinations giving rise to different genotypes. All stocks isolated from MCL lesions were AC16 genotype 244/244.

The AC16 genotype 244/246 was identified only in non-human host species: the 246 allele was also observed in 1 human stock, M2904, but in a different genotype (246/254). Among the AC16 genotypes observed from sand fly stocks, those from Pará State were, similarly to host species stocks, more variable than those originating from Três Braços / Corte de Pedra. As with AC01, null alleles were commonly observed among sand fly stocks from Três Braços / Corte de Pedra (3/6 stocks).

4.5.3.3 AC52

AC52 genotypes found in stocks from Brazil are summarized in *table 4.5.3.6.* Among *L. V. braziliensis* stocks, the most common genotype (of the 8 genotypes scored) was 283/283 (13/24). As with AC16, many *L. V. braziliensis* stocks (11/24) were heterozygous.

Table 4.5.3.6 **AC52 genotypes found in stocks from Brazil**

Species§	Inferred genotype*	No. stocks	Stocks
L. V. shawi	293 / 293	1	M12045
	291 / 291	1	M11367
	297 / 299	1	**M15065**
L. V. braziliensis	283 / 283	13	Manoel, BH325R, Dog-Rodrigo, LTB12, LTB14, LTB30, LTB179, LTB206, **LTB300**, JCP-225, WHI-EO1161, WHI-10187, WHI-C1PD
	283 / 301	4	Fragoso, Amaro, HM1B, M14405
	283 / 303	1	LTB253
	283 / 305	2	Dog-Lamprea, Dog-Rico
	285 / 293	1	M12620
	285 / 303	1	Paulino
	293 / 295	1	M2904
	297 / 297	1	M2903
	ND	1	M8522

Table 4.5.3.6: AC52 genotypes found in stocks from Brazil. § Species status assigned from IEA. * Genotype inferred from scoring Genotyper® peaks. Code names in **bold** type indicate stocks used as reference strains. Allele assignment is explained in chapter 3.

Three genotypes were scored among the 14 stocks from Três Braços / Corte de Pedra (excluding stocks with null alleles). Most stocks were genotype 283/283, 2 dog stocks were 283/305 and a single human stock was 283/303. Three genotypes were scored from the 4 stocks from Amariji: 2 genotypes (3 stocks) were heterozygous. Of the 3 stocks scorable at AC52 from Pará State, each had a distinct genotype. Of these, two stocks from Carajas had genotypes which were more similar to those of *L. V. shawi* stocks (also from Pará). Each *L. V. shawi* stock had a different AC52 genotype: four alleles were observed in these stocks of which two (293 and 297) were also found in the *L. V. braziliensis* stocks from Pará State. As with AC16, however, these alleles were observed in different genotypes. All stocks isolated from MCL lesions were AC52 genotype 283/283. The AC52 genotype 283/305 was identified only in 2/3 stocks isolated from dogs: the 305 allele was not found in any other stocks. As with AC01 and AC16, null alleles were commonly observed among sand fly *L. V. braziliensis* stocks (4/9 stocks).

4.5.3.4 Multilocus analysis of microsatellite data

I propose here to use the term "microdeme" to describe *Leishmania* sharing the same microsatellite multilocus genotype (i.e. combined genotype data from the 3 microsatellite loci: AC01, AC16 and AC52). "LMS" numbers ("London microsatellite") have been used to identify different microdemes (i.e. LMS1, 2, 3 etc.). Microdemes observed in stocks from Brazil are summarized in *table 4.5.3.7*.

Stocks which failed to generate PCR products at any of the three loci were not assigned a microdeme number because the genetic basis of failure to amplify could not be determined. Stocks which had null alleles at one or two loci were assigned LMS numbers: each of these stocks belonged to a different microdeme owing mostly to variation at the AC16 locus. Including these stocks, 23 microdemes were scored among the 33 Brazilian stocks: *L. V. braziliensis* stocks (28) were separated into 18 microdemes and *L. V. shawi* stocks (4) into 4 microdemes. LMS1 was the most common microdeme observed, primarily because of the relatively large number of stocks from Três Braços / Corte de Pedra, most of which were LMS1. Most microdemes contained a single stock, testimony to the discriminatory power of this technique.

Table 4.5.3.7: **Summary of microdemes† (multilocus microsatellite genotypes) observed in Brazilian stocks.**

Microdeme †	Microsatellite locus genotype						Geographical origin		Stock(s)	*Viannia* species§
	AC01		AC16		AC52		State/Dept	Region/Area◊		
LMS1	227	227	244	244	283	283	Bahia	Três Braços / CDP	*LTB300, LTB12, LTB14, LTB30*, LTB179, LTB206, Dog-Rodrigo, WHI-10187, WHI-CIPD	b
LMS4	241	241	246	248	297	299	Pará	Paragominas	**M15065**	s
LMS7	227	245	X	X	X	X	Amazonas	Balbina	**IM2832***	*
LMS8	227	227	244	246	283	283	Bahia Minas Gerais	Três Braços / CDP Caratinga	JCP-225 BH325R	b
LMS9	227	227	244	244	283	301	São Paulo	Miracatu	HM1B	b
LMS10	227	227	244	244	283	303	Bahia	Três Braços / CDP	LTB253	b
LMS11	227	227	244	244	283	305	Bahia	Três Braços / CDP	Dog-Rico, Dog-Lamprea	b
LMS12	227	227	242	242	283	301	Paraná	Flora	M14405	b
LMS13	227	227	246	254	293	295	Pará	Carajas	M2904	b
LMS14	227	227	248	254	F	F	Pará	Carajas	M8522	b
LMS15	227	227	250	266	297	297	Pará	Carajas	M2903	b
LMS16	227	231	244	250	285	303	Pernambuco	Amaraji	Paulino	b
LMS17	227	231	244	252	283	283	Pernambuco	Amaraji	Manoel	b
LMS18	227	231	248	248	283	301	Pernambuco	Amaraji	Fragoso	b
LMS19	231	231	244	244	283	301	Pernambuco	Amaraji	Amaro	b
LMS20	231	231	244	248	291	291	Pará	Carajas	M11367	s
LMS21	231	231	246	246	293	293	Pará	Carajas	M12045	s
LMS22	223	223	254	262	285	293	Pará	Paragominas	M12620	b
LMS23	X	X	244	244	283	283	Bahia	Três Braços / CDP	WHI-EO1161	b
LMS24	X	X	244	244	X	X	Paraná	Peabiru	709-LOA	b
LMS25	X	X	244	246	X	X	Bahia	Três Braços / CDP	JCP-184	b
LMS26	X	X	244	256	X	X	Pará	Paragominas	M12613	b
LMS27	X	X	246	248	X	X	Amazonas	Manaus	IM403	s
not assigned±	X	X	X	X	X	X	Bahia Parana	Três Braços / CDP S. Jorge do Ivai	WHI-EO1094, TUP-EO252, WHI-M11468 769-VU	b

Notes to *table 4.5.3.7:* (see previous page) summary of microdemes observed in Brazilian stocks. † microdeme is the term coined here to describe *Leishmania* sharing the same microsatellite multilocus genotype (i.e. combined genotype data from the 3 microsatellite loci: AC01, AC16 and AC52). "LMS" numbers have been used to identify different microdemes (i.e. LMS1, 2, 3 etc.). Stocks which failed to generate PCR products at any of the three loci were not assigned a microdeme number because the genetic basis of failure to amplify could not be determined. ◊ CdP - abbreviation for Corte de Pedra. §*Viannia* species codes: b - *braziliensis*; s - *shawi*; * - unnamed *Viannia* species (*L. V.* sp. n., see Grimaldi *et al.* 1991). Code names in **bold** type indicate reference strains. Stocks which are in *italics* are from MCL lesions. F - failed to generate scorable Genotyper® peak.

Microdemes observed in *L. V. braziliensis* stocks from Brazil are summarized by geographical region in *table 4.5.3.8* and by host / vector group in *table 4.5.3.9*.

***Table 4.5.3.8*: Microdemes † observed in *L. V. braziliensis* stocks from Brazil, summarized by geographical region.**

Geographical region	Number of stocks	Number of microdemes† observed	Microdemes† observed (LMS No.)
Três Braços / Corte de Pedra	15	6	1, 8, 10, 11, 23, 25
Pará	5	5	13, 14, 15, 22, 26
Pernambuco	4	4	16, 17, 18, 19
Other	4	4	8, 9, 12, 24

***Table 4.5.3.9*: Microdemes† observed in *L. V. braziliensis* stocks from Brazil, summarized by host / vector group.**

Host / vector group	Number of stocks	Number of microdemes† observed	Microdemes† observed (LMS No.)
Human	15	10	1, 10, 12, 13, 15, 16, 17, 18, 19, 24, 27
Dog	3	2	1, 11
Donkey	2	2	8, 25
Rodent	2	2	8, 9
Sand fly	6	5	1, 14, 22, 23, 26

Tables 4.5.3.8 and *4.5.3.9*: microdemes observed in *L. V. braziliensis* stocks from Brazil, summarized by geographical region (*4.5.3.8*) and by host / vector group (*4.5.3.9*). † "microdeme" is the term coined here to describe *Leishmania* sharing the same microsatellite multilocus genotype. LMS numbers have been used to identify different microdemes. Stocks which failed to generate PCR products at any of the three loci are not included in this multilocus analysis because the genetic basis of their null alleles could not be determined.

Of the 15 *L. V. braziliensis* stocks assigned from Três Braços / Corte de Pedra, 6 microdemes were scored. This is in contrast to the single zymodeme scored in these stocks using isoenzyme analysis. All stocks isolated from MCL lesions (all from Três Braços / Corte de Pedra) were LMS1. Every stock from Pará (Carajas and Paragominas) and Pernambuco (Amariji) States belonged to a different microdeme. Only one microdeme (LMS8) contained stocks isolated from different geographical areas. Microdemes 1 and 8 encompassed stocks from different host / vector groups. LMS1 was observed in human, dog and sand fly stocks (all Três Braços / Corte de Pedra); LMS 8 was observed in 1 stock each from the dog and rodent groups.

4.5.4 AC01 Sequencing

This method of microsatellite analysis is the most sensitive of all the approaches used, allowing visualization of the microsatellite region itself and identification of any diversity in the flanking regions. Sequencing analysis was carried out for the AC01 locus only. Of the 28 Brazilian stocks which generated a PCR product for AC01, all presented readable AC01 sequence data except the reference strain IM2832 (*L. V.* sp., see section 3.8.4). Several stocks were sequenced at least twice and the sequence was found to be reproducible. *Figure 4.5.4.1* shows the alignment of sequences from the AC01 microsatellite locus for Brazilian stocks.

Notes to *figure 4.5.4.1*: (see next page) sequence alignment of the AC01 locus from Brazilian stocks and *L. V. guyanensis*. §*Viannia* species abbreviation: b - *braziliensis*; s - *shawi*; g - *guyanensis*. * Stock type: LTB300 type (LTB300, LTB12, Dog-Rico, Dog-Lamprea, WHI-10187, M2904); LTB14 type (LTB14, LTB30, LTB179, LTB206, LTB253, Dog-Rodrigo, JCP-225, WHI-C1PD, HM1B, BH325R, Fragoso, Paulino); M2903 type (M2903, M8522, M12620). Reference strains are indicated in **bold** type. Bases in blue type form the microsatellite repeat region; bases in red type illustrate the TCCGC end motif; bases in green type are invariant downstream in all stocks. Numbers above sequence data indicate the base position used for alignment purposes. Only 165 bases of the total AC01 product were aligned and used for phylogenetic analysis (see chapter 3). See Appendix IV for base coding system.

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Figure 4.5.4.1 **Sequence alignment of the AC01 locus from Brazilian stocks and the *L. V. guyanensis* reference strain.**

Species §	Strain /stock* code no.	10	20	30	40	50	60	70	80
b	**LTB300** type	ATGTGCCTCT	CCCACCCTTA	GTGCTTGTCT	TCTTCCTGCT	TTGCCTCTCT	GTC----TGT	GTGTGTGTGT	GTG-------
b	LTB14 type	....	....	....	....	....	...----...	....	...-------
b	M14405	....	....	....	....	....	...----...	....	...-------
b	M2903 type	....	....	....	....	....	A..----...	....	...-------
b	Manoel	....	....	....	....	....	A..----...	....	...-------
b	Amaro	....	....	....	....	....	A..----...	..C.......	...-------
s	**M15065**	....	....	.......C..	....	....	...TGTC...	....	...TGTGCGT
s	M11367	....	....	....C..C..	....	....	...----...	....	...TGTGTGT
s	M12045	....	....	.......C..	....	....	...TGTC...	....	...TGTGTGY
g	**M4147**	....	....	.......C..	....	C	...----...	....	...TGYGYGT

Species §	Strain /stock* code no.	90	100	110	120	130	140	150	160	165
b	**LTB300** type	----------	-TCCGCTTCA	GTGGGCCGAT	CCGTTTCACT	TTTTGCCGGT	GACGTTTGTG	TGCTGACGTG	TCTGGTGGCC	TCWCA
b	LTB14 type	----------	-.........	....	....	....	....	....	....	..A..
b	M14405	----------	-.........	....	....	....	....	...N......	....	..A..
b	M2903 type	----------	-.........	....	....	....	....	....	....	..A..
b	Manoel	----------	-.........	....	....	....	....	....	....	.....
b	Amaro	----------	-.........	....	....	....	....	....	....	.....
s	**M15065**	GCG-------	-.........	....	....	..K.......	....CK....	...W......	....	..A..
s	M11367	GTGTG-----	-.....C...	......A...	....CW....	..G......A	....CG....	...A......	..G.......	W.A..
s	M12045	GYGYG-----	-..Y..GC..	CW...SS..G	A...G....A	......G..W	...ACGC...	..YA..SA..	.S........	A.A..
g	**M4147**	GTGYGYS---	-.S...CA..	.K...SA..Y	S...CW....	..GY.SS..A	S...CG....	..SA..Y...	.SG..K....	W.A..

4.5.4.1 AC01 sequence variation among Brazilian stocks

L. V. braziliensis and *L. V. shawi* stocks could easily be separated based on the size of the repeat (8 : 13/14 respectively) and the base at position 28 ([T : C] respectively). The sequence upstream of the repeat itself was highly conserved in all stocks: a single (different) base change was identified in stocks of both *L. V. braziliensis* ([G to A] at position 51 in 5 stocks) and *L. V. shawi* ([T to C] at position 25 in 1 stock).

Among *L. V. braziliensis* stocks, 6 sequence types were scored compared to the 4 AC01 genotypes scored using Genotyper®. Six stocks had AC01 sequences identical to that of the *L. V. braziliensis* reference strain LTB300: all bar one (M2904) came from Três Braços / Corte de Pedra. *L. V. braziliensis* stocks could be divided into 2 groups based on comparison of the upstream flanking region. Five stocks (M2903-type stocks [3], Amaro and Manoel) had [C] at position 51 instead of the [G] present in all other (19) stocks. The base change in the sequence of these 5 stocks (originating from 2 northern States, Pará and Pernambuco) was very clear. Stocks from Três Braços / Corte de Pedra could be split into 2 groups: LTB300-type stocks were heterozygous at position 163 [W, i.e. A or T] whereas all LTB14-type stocks had [A] at this position. Permutations of bases present at positions 51 and 163 generated 4 of the sequence types in *L. V. braziliensis* stocks from Brazil. Two further sequence types were observed involving a single base change ([T to C] at position 63, within the repeat) and a failed base (Amaro and M14405 stocks respectively).

The *L. V. shawi* stocks, all homozygous at AC01 using Genotyper®, each had unique AC01 sequence. In addition to the *guyanensis* complex-specific [C] at nucleotide position 28, two of the 3 *L. V. shawi* stocks (reference strain M15065 and M11367) had a 4 base pair insertion immediately before the repeat motif. The third *L. V. shawi* stock had a mutation [T to C] at position 25. All *L. V. shawi* stocks were found to have [CG] or [YG] insertions in the repeat motif ([Y] indicates [C] or [T]) . The *L. V. shawi* reference strain M15065 had a dinucleotide repeat [(TG + CG)]n, where n = 13; both of the other *L. V. shawi* stocks had n = 14. The stock M12045 appeared to have heterozygous sites [Y] within the repeat suggesting that the AC01 alleles varied at these positions. The sequence downstream of the repeat for all *L. V. shawi* stocks was more diverse than those of *L. V. braziliensis* stocks. Many of these downstream sites in *L. V. shawi* stocks were scored as heterozygous, i.e. had split peaks. This variability is likely due to the fact that the repeat is longer in these stocks resulting in more

replication errors (i.e. DNA slippage and subsequent inaccurate DNA mismatch repair, see chapters 1 and 3). Overall, *L. V. braziliensis* stock repeats are shorter and the entire locus sequence appears to be much more stable than those of *L. V. shawi*.

4.5.4.2 Comparison of AC01 data from sequencing and Genotyper® analyses

Sequence data sometimes failed to correlate with Genotyper® data. Among *L. V. braziliensis* stocks, the majority of alleles identified as 227 using Genotyper® had virtually identical sequence lengths where the number of dinucleotide repeats (n) was 8. However, 2 other alleles (223 and 231) were scored in three genotypes: 223/223 (1 stock), 227/231 (3 stocks) and 231/231 (1 stock) in these stocks. AC01 sequence analysis did not account for these differences in allele size: the number of dinucleotide repeats scored in these stocks was identical to other *L. V. braziliensis* stocks (i.e. n = 8) and no insertions or deletions were revealed. It is possible that a 4 base pair insertion (to give 231) or deletion (to give 223) occurred in the extreme 5' sequence which was not used for alignment. Stocks scored as 227/231 using Genotyper® generated a single sequence which was compatible, in length, to the *L. V. braziliensis* reference strain. If heterozygous, two sequences should have been produced which, if changes in repeat number were the cause of the allele size difference, should have resulted in the sequences overlapping after the repeat. It is possible that Genotyper® analysis was incorrect or that cycle sequencing greatly favoured the amplification of the shorter allele: the latter seems more likely of the two although both Genotyper® and sequence data for these stocks appeared unequivocal. Heteroduplex bands were observed in PAGE analysis of these stocks (see *figure 4.5.2.1*) but, as discussed previously, these are not always evidence of heterozygous loci as they can occur at both heterozygous and homozygous loci.

Sequence analysis of AC01 for the *L. V. shawi* reference strain M15065 reveals, in comparison with LTB300 (allele size 227), the presence of a 4 base insert plus 5 additional dinucleotide repeats. This gives a difference in bases of 14 (4 + 5x2): the expected allele size is therefore 241 (227 + 14). The Genotyper® data from M15065 (observed allele size 241) thus correlates with sequence data. The Genotyper® data from M11367 and M12045 (231/231) however, do not correlate with sequence data. M12045 has the 4 base insertion and 6 additional dinucleotides which should give a size difference of 16 bases (4 + 12), i.e. expected allele size 243. M11367 does not have the 4 base insert but has 6 additional dinucleotide repeats, giving a size

difference of 12 bases, i.e. expected allele size 239. Genotyper® analysis of both of these stocks reveals allele sizes of 231. The discrepancy between the two methods may be due to insertions (12 and 8 bases respectively) outside the sequence aligned for phylogenetic analysis, or due to an error in Genotyper® analysis. The ambiguities observed in some stocks between sequencing analysis and Genotyper® allele size scoring need to be resolved. Ambiguities were more commonly encountered in stocks which were identified as heterozygous by Genotyper® (i.e. double peaks scored) but which produced sequence data which appeared to be homozygous.

Sequencing analysis is not an option for the multilocus analysis of many stocks, or for the analysis of populations containing large numbers of heterozygote individuals. The use of microsatellite allele sizes for population and phylogenetic analyses is increasingly common (Rongnoparut *et al.*, 1996; Lehmann *et al.*, 1997; Oliveira *et al.*, 1998), despite observations that allele size variation may also result from insertions and deletions in the flanking regions (here; Valdes *et al.*, 1993; Orti *et al.*, 1997; Grimaldi & Cronau-Roy, 1997; Viard *et al.*, 1998). Over all stocks examined, a good correlation was observed between sequencing and Genotyper® results, suggesting that population and phylogenetic analyses based on Genotyper® data (i.e. allele size) alone is a valid approach, giving rise to only a few errors. Of course, without sequence analysis, any errors would remain unidentified and could seriously affect later analyses. Even for those stocks for which an exact correlation between sequence data and allele size was found, subsequent population and phylogenetic analyses based on Genotyper® data would underestimate the true level of diversity (as revealed by sequence analysis). As discussed in chapters 1 and 3, Genotyper® analysis lends itself very well for population genetic purposes because large amounts of data can be generated using a multiplex approach. Another advantage of Genotyper® analysis is that heterozygote stocks may be easily identified: heterozygote detection is paramount to stocks from Nicaragua and Huánuco, Peru, and is thus discussed in more detail in chapters 5 and 6.

4.6 Phylogenetic analysis of Brazilian stocks

Phylogenetic analysis of isoenzyme data (multilocus) included data from all *Leishmania* reference strains. Phylogenetic analysis of sequences from the microsatellite locus AC01 included data from all reference strains of the *Viannia* subgenus, with the exception of *L. V.* sp. n. reference strain IM2832 which failed to produce readable sequence. The AC01 microsatellite locus is specific to the *Viannia* subgenus hence it was not possible to include reference strains from the subgenus *Leishmania* in this phylogenetic analysis.

4.6.1 Phylogenetic analysis of isoenzyme data

Isoenzyme phylogenies were constructed, as described in chapter 2, using a maximum-likelihood approach, quartet puzzling (Strimmer & von Haeseler, 1996). *Figure 4.6.1.1* depicts a cladogram showing the results of quartet puzzling of zymodeme data for the Brazilian stocks and reference strains. The corresponding phylogram is not shown because the outgroup branch lengths (i.e. subgenus *Leishmania* species) therein were, by comparison, very long thus making the shorter branch lengths (i.e. between stocks of the subgenus *Viannia*) difficult to see.

The cladogram was generated from analysis of data spanning 8 of *L. V. braziliensis* zymodemes and 9 zymodemes from other *Leishmania* taxa. Two of the *Leishmania* subgenus reference strains (PP75, *L. L. chagasi* and M2269, *L. L. amazonensis*) were clearly distinct (55% quartet support) from species of the *Viannia* subgenus. The *L. L. mexicana* reference strain BEL21 however, clustered amongst the more distantly related species of the *Viannia* subgenus. The reasons for this disparity are not immediately evident, but most likely due to shared characters observed between these taxa using this particular set of enzyme loci. The phylogenetic positions of the reference strains IM2832 (*L. V.* sp. n.) and M6426 (*L. V. lainsoni*) as members of the subgenus *Viannia* were not clearly defined by this analysis as both of these strains fell outside of the 2 main *Viannia* species complexes.

Members of the *guyanensis* complex (cluster **I**) were distinct from outlying species. Strains and stocks examined were separated into species groups (*L. V. panamensis*, LS94, *L. V. guyanensis*, M4147 and *L. V. shawi*, M15065) which were highly supported (91%, 94% and 98% quartet support). All Brazilian *L. V. shawi* stocks clustered together (cluster **Ia**, *figure 4.6.1.1*) indicating the validity of this species group.

***Figure 4.6.1.1*: Cladogram showing the results of quartet puzzling for Brazilian stocks and reference strains based on IEA data.**

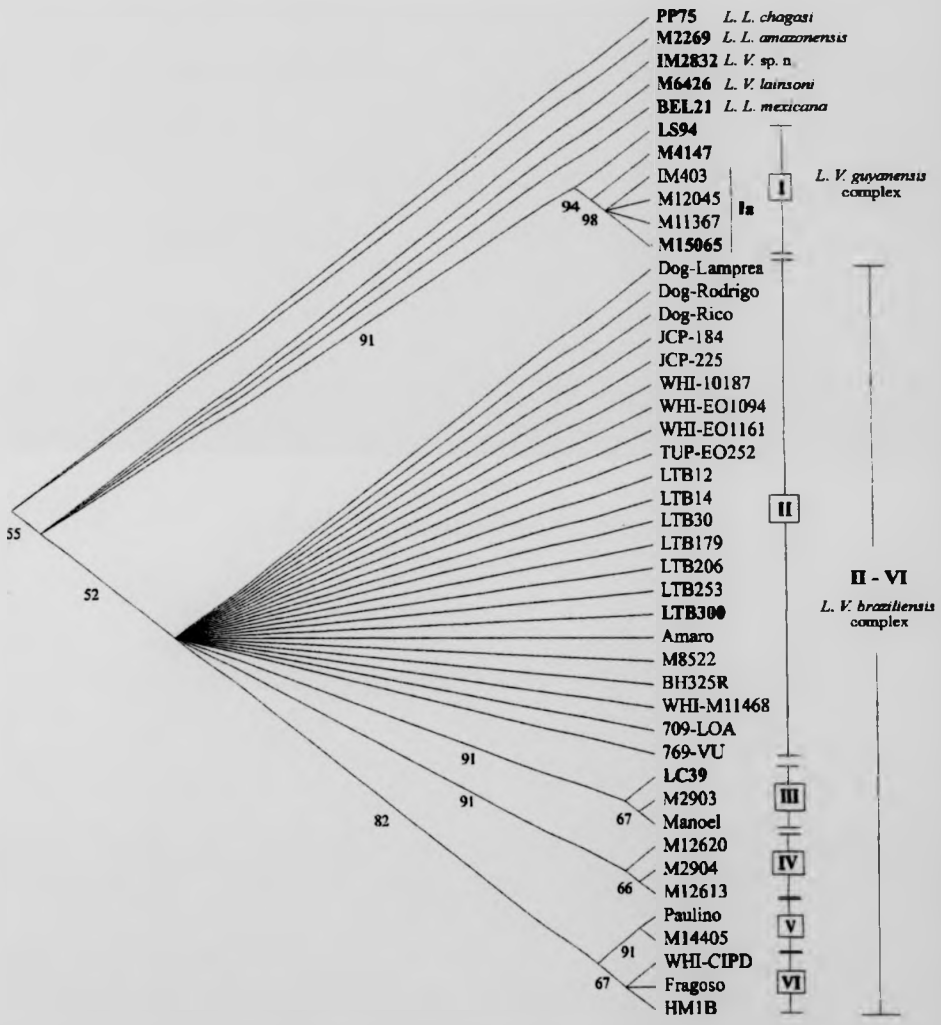


Figure 4.6.1.1: Cladogram showing the results of quartet puzzling for Brazilian stocks and reference strains based on zymoderne data. Values on branches are "quartet supports", figures similar to bootstraps (Strimmer & von Hassler, 1996). Values below 50% are not shown. Quartet supports indicate the percentage number of quartets (differing in allele frequencies) supporting each given branching order. Branch lengths are arbitrary. Cluster I contains strains and stocks belonging to the *L. V. guyanensis* complex and includes all *L. V. shawi* stocks (cluster **Ia**, 1 zymodeme, LON203). Clusters **II - VI** contains strains and stocks of the *L. V. braziliensis* complex: **II** contains 2 zymodemes (LON200 and LON212); **III** contains 2 zymodemes (LON202 and LON211), one of which is the *L. V. peruviana* reference strain LC39; **IV** contains 2 zymodemes (LON214 and LON215); **V** contains 1 zymodeme, LON213 and **VI** contains 2 zymodemes (LON200 and LON210).

Strains and stocks of the *L. V. braziliensis* complex were also separated from other taxa (52% quartet support). The heterogeneous nature of this taxon is illustrated by the division into clades (**II - VI**, *figure 4.6.1.1*) which were all highly supported by quartet supports (range 82 - 91% quartet support). *L. V. braziliensis* stocks were divided into 5 clades: the largest clade (**II**) contained 22 stocks from 2 zymodemes including the *L. V. braziliensis* reference strain LTB300 and all bar one of the Três Braços / Corte de Pedra stocks. Clade **III** contains 2 *L. V. braziliensis* stocks (both LON211) and the *L. V. peruviana* reference strain LC39 (LON201), testimony to the close relationship of *L. V. peruviana* and *L. V. braziliensis* stocks. Clades **IV** and **VI** each contain 3 stocks from 2 zymodemes; clade V contains 2 stocks from a single zymodeme.

Owing to the difficulty of weighting binary data for maximum likelihood analysis, all alleles were equally weighted in this analysis. Equal weighting of alleles, as opposed to enzymes, results in highly polymorphic enzyme loci assuming more importance than less polymorphic loci because, by nature, more alleles can be scored. Equal weighting of enzymes may have been more appropriate since mutation rates of alleles at a given enzyme locus may differ.

Analysis of these stocks using a similarity approach (based on a similarity coefficient matrix, SCM, produced according to Jaccard's method, 1908) was also carried out. In this approach, equal weighting is also given to all alleles, but the "over-weighting" of polymorphic loci is removed. Dendrograms were constructed by UPGMA (the unweighted pair-group method using arithmetic averages) using the SYN-TAX-pc package (Podani, 1993). *Figure 4.6.1.2* shows the dendrogram constructed for these stocks from a similarity coefficient matrix. Stocks are classified according to similarity (range 0 - 1).

The clustering observed using this method is in overall agreement with results from quartet puzzling, however individual zymodemes are identified. The *Viannia* and *Leishmania* subgenera are clearly defined with the exception of the *L. V. lainsoni* reference strain M6426, which groups more closely with members of the *Leishmania* subgenus.

Figure 4.6.1.2 **UPGMA dendrogram constructed for Brazilian stocks and reference strains based on isoenzyme data.**

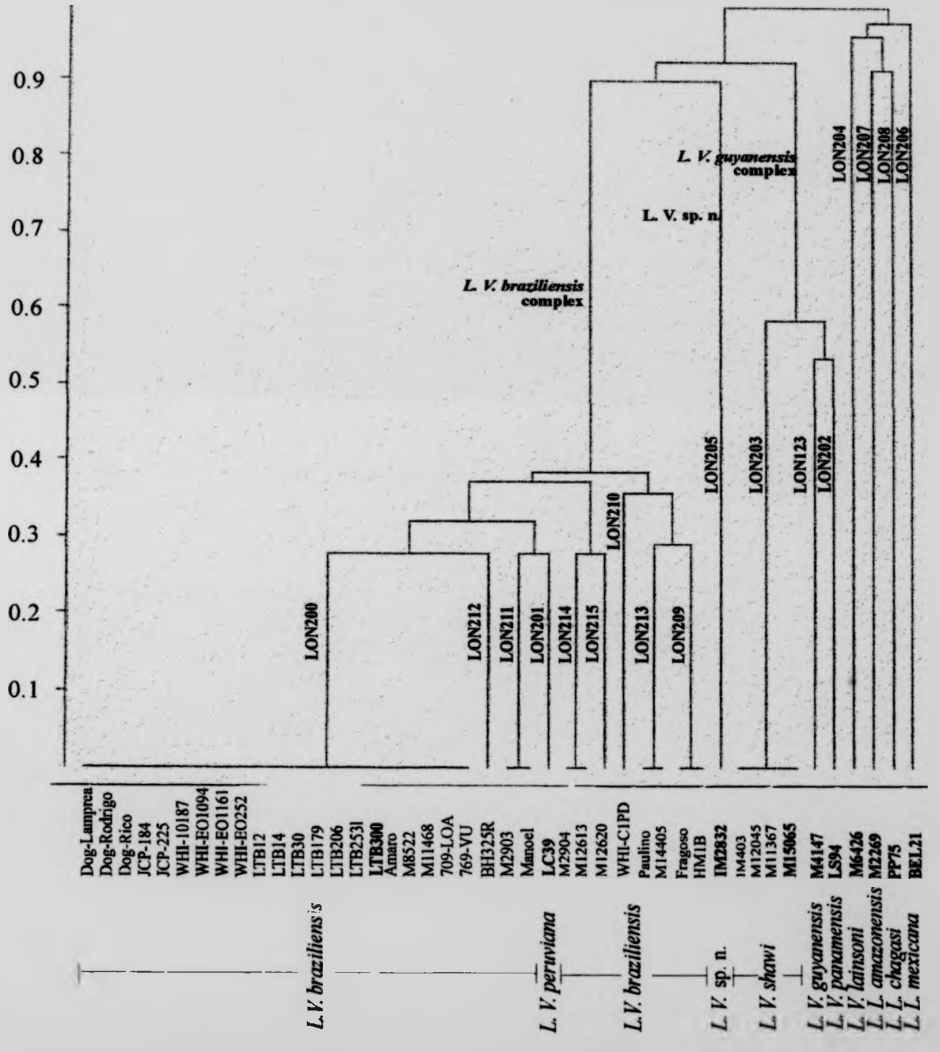


Figure 4.6.1.2: UPGMA dendrogram constructed from the similarity coefficient matrix for Brazilian stocks and reference strains based on isoenzyme data. Similarities between zymodemes (y-axis, range 0-1) were calculated, using SYN-TAX-pc (Podani, 1993), by averaging the Jaccard coefficients calculated from data from twelve enzyme loci. Stocks / strains in **bold** type are reference strains. Zymodemes (LON numbers) are indicated on branches.

The *L. L. mexicana* reference strain BEL21 clearly groups in the *Leishmania* subgenus using this approach, however it is still separated *from L. L. amazonensis* (*L. L. mexicana* complex) which again shows more similarity to *L. L. chagasi* (*L. L. donovani* complex).

Using this approach, the *L. V.* sp. n. reference strain IM2832 lies between the *braziliensis* and *guyanensis* complexes, but clearly within the *Viannia* subgenus. This parasite, as a potential new *Viannia* species (Grimaldi *et al.*, 1991) will be discussed further in chapter 6.

Within the *L. V. guyanensis* complex, each species was identified, sharing 50-60% divergence. Stocks in the *L. V. braziliensis* complex were more closely related with only 25 – 40% divergence. The close relationship of strains and stocks in this complex has been previously reported based on analyses of isoenzyme data (Cupolillo *et al.*, 1994), IEA and RFLP analysis of the gp63 gene locus (Victoir *et al.*, 1998) and RAPD markers (Dujardin *et al.*, 1995b). As found in the previous analysis, the *L. V. peruviana* reference strain LC39 nested within the *L. V. braziliensis* complex; the *L. V. braziliensis* reference strain LTB300 was more similar to *L. V. peruviana* than to many other *L. V. braziliensis* stocks.

4.6.2 Phylogenetic analysis of sequence data from microsatellite locus AC01

Molecular phylogenies for sequence data from the AC01 microsatellite locus were constructed using the neighbor-joining method using the Kimura 3-parameter model in PAUP* 4.0 beta version (Swofford, 1997). *Figure 4.6.2.1* shows a phylogram of the neighbour-joining consensus tree for the Brazilian stocks and reference strains based on sequence data from the microsatellite locus AC01. The reliability of tree topologies was assessed by the bootstrap method (n = 1000). Only branches with greater than 50% bootstrap support are indicated.

The authors who developed microsatellite analysis for *Viannia* subgenus parasites (Russell *et al.*, 1999) acknowledge that alleles identified using single microsatellite loci did not correlate with designated species and that multilocus data was required to separate stocks into correct species groups. These authors did not attempt phylogenetic analysis, despite having sequencing data from three microsatellite loci.

***Figure 4.6.2.1*: Phylogram based on sequence data from the microsatellite locus AC01 for the Brazilian stocks and reference strains.**

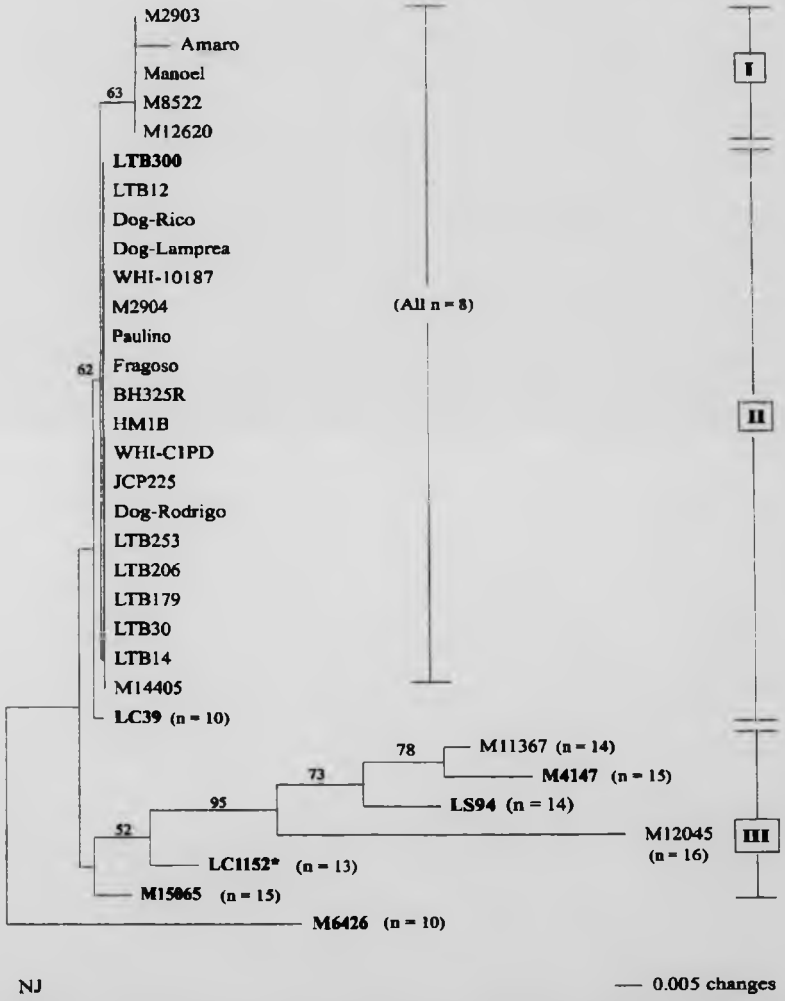


Figure 4.6.2.1: unrooted phylogram of the neighbour-joining consensus tree for the Brazilian stocks and reference strains based on sequence data from the microsatellite locus AC01. The reliability of tree topology was assessed by the bootstrap method (n = 1000). Numbers indicate the percentage of bootstrap support for each branch. Values are given only for branches with more than 50% support (50% majority rule). The scale bar represents 0.005 (0.5%) nucleotide changes per sequence position. Numbers in parentheses indicate the number of dinucleotide repeats (n) scored (for *L. V. guyanensis* and *L. V. shawi* stocks the repeats are not perfect). Strains / stocks in clusters **I** and **II** belong to the *L. V. braziliensis* complex. Stocks / strains in cluster **III** belong, with the exception of LC1152, to the *L. V. guyanensis* complex: *LC1152 is an additional *L. V. peruviana* reference strain. M6426 is the *L. V. lainsoni* reference strain, here used as the outgroup (see text).

It proved difficult to select an outgroup for this data because only *Viannia* strains could be amplified. The *L. V. lainsoni* reference strain M6426 was selected as the outgroup on the basis that this species is agreed to be distantly related to other *Viannia* species. Depicted as such (*figure 4.6.2.1*), the bifurcation occurring from M6426 should not be taken as indicative of the ancestral status of this species. A trifurcating layout with 3 major branches separating *L. V. lainsoni*, the *L. V. braziliensis* complex and the *L. V. guyanensis* complex would be more correct here.

Less diversity was evident among *L. V. braziliensis* stocks based on AC01 sequence data compared to IEA data: this is not surprising since the latter comprises multilocus data. Nevertheless, the phylogram highlights the close relationship between these *L. V. braziliensis* stocks, in particular those stocks from Três Braços / Corte de Pedra. Among *L. V. braziliensis* stocks, two clusters were evident (**I** and **II**), supported by 63% and 62% bootstrap values, respectively. All stocks from Três Braços / Corte de Pedra, including the reference strain LTB300, grouped in cluster **II**. Since all *L. V. braziliensis* stocks had n = 8, the clusters were generated from sequence diversity in the flanking regions. Stocks in clusters **I** and **II** could be separated only by a single base difference in the upstream flanking region. Cluster **II** contained stocks from 3 AC01 sequence groups separated, using visual examination of the data, by single base differences.

As with analyses of isoenzyme data, the *L. V. peruviana* reference strain LC39 grouped with *L. V. braziliensis* stocks in cluster **II**: this strain differed from the *L. V. braziliensis* reference strain LTB300 by having 2 additional dinucleotide repeats (data not shown): the relationship between *L. V. braziliensis* and *L. V. peruviana* will be discussed further in Chapter 6.

Stocks in cluster **III**, with the exception of LC1152, belonged to the *L. V. guyanensis* complex. These stocks were highly variable in AC01 sequence (see *figure 4.5.4.1*), both in the microsatellite repeat and downstream flanking region. It is therefore not surprising that these stocks were separated by relatively long branches and supported by high bootstrap values. It is not clear why the *L. V. peruviana* stock LC1152 groups with *the L. V. guyanensis* complex members. This stock does not have the upstream sequence changes shown by all *guyanensis* complex members, nor the diverse downstream flanking sequence shown by *L. V. guyanensis* and *L. V. shawi stocks*. In

fact, LC1152 was identical to the *L. V. peruviana* reference strain LC39 in both upstream and downstream flanking regions: sequence variation between these stocks was limited to LC1152 having 3 additional dinucleotide repeats (data not shown, see chapter 6). The grouping of this stock in the *guyanensis* complex therefore appears to be solely the result of this longer repeat region.

Ambiguous clustering could be due to an incorrect sequence alignment. Aligning these sequences was subject to interpretation owing to insertions and deletions in and around the dinucleotide repeat region. The upstream flanking region was easily aligned because it was generally highly conserved. The repeat region was identified by a "TG/ATC" motif at the start and by a "TCCGC" motif at the end (see *figure 4.5.4.1*). In 2 *L. V. shawi* stocks (M15065 and M12045), however, the start motif appeared to have been duplicated (bases 54-57 in *figure 4.5.4.1*) and these 4 bases were not counted as part of the repeat. It is possible however, that these bases did form part of the repeat (giving 2 additional dinucleotide repeats for these stocks) which may have led to a different tree topology. The position of LC1152 is more likely the result of limitations of the phylogenetic analysis used.

Phylogenetic reconstruction programs specifically written for the analysis of microsatellite data are still in their infancy. The difficulty in writing these programs stems from the lack of knowledge regarding the creation of microsatellite arrays. As discussed in chapter 1, although it is not possible to determine whether a particular microsatellite allele arose from a longer or shorter allele, alleles closely related in size are thought to share a more common recent ancestry than alleles of much different size (Di Rienzo *et al.*, 1994).

The analysis used here was the best available at the time of analysis (Michael Gaunt, pers. comm). The neighbor-joining (NJ) method constructed topologies from pairwise comparisons of 4 character states using the Kimura 3-parameter model. The latter distinguishes between transitions and transversions, splits transversion types and takes into account reverse mutation. In this analysis, pairwise comparisons were made between 4 character states only and although ambiguity codes were scored, gaps (which in microsatellite arrays are highly informative) were ignored.

Several authors have suggested that, in the absence of knowledge regarding the creation of the microsatellite array, more accurate phylogenies may be derived from microsatellite data with the repeat region excluded (Ashley & Dow, 1994; Orti *et al.*, 1997). Hence phylogenetic analyses would take into account mutations in the flanking regions only. Only very recently however, a 5–state model has been developed which scores gaps as informative characters (MATRIX, David Posado, http://bioag.byu.edu/zoology/crandall-lab/programs.htm.), and which may prove applicable to the analysis of microsatellite sequences including the repeat region.

4.7 Discussion of diversity among stocks from Brazil.

Microsatellite analysis revealed greater diversity than IEA which is probably not surprising considering that these markers appear not to be functionally constrained (Ashley & Dow, 1994; Schlötterer, 1998). Among Brazilian stocks, Genotyper® analysis revealed 23 microdemes from 33 stocks compared to 10 zymodemes from 37 stocks using IEA. *L. V. braziliensis* stocks were separated into 18 microdemes and 6 AC01 sequence types (28 stocks scored) compared to 8 zymodemes (32 stocks scored). *L. V. shawi* stocks were separated into 4 microdemes and 3 AC01 sequence types compared to a single zymodeme (4 stocks scored by both methods). Notwithstanding the fact that the multilocus Genotyper® results from these microsatellite loci need to be verified, this technique was highly discriminatory and has great potential.

In agreement with previous reports (Cuba-Cuba *et al.*, 1985; Rosa *et al.*, 1988), stocks from Três Braços / Corte de Pedra were shown to be remarkably similar using IEA. Microsatellite analysis, using both Genotyper® and sequencing analyses, has revealed previously undetected diversity among these stocks: 6 microdemes and 6 AC01 sequence types were scored in these 18 stocks which also presented null alleles indicating further, undefined diversity. Many more stocks are available from this area: it would be very interesting to compare data from a larger population set from this region to see if any more evidence is forthcoming regarding *Leishmania* population structure. Isoenzyme-based studies from other localized areas in Brazil have also reported a lack of heterogeneity among *L. V. braziliensis* stocks: no heterogeneity was observed among 15 *L. V. braziliensis* stocks from Ceará State (NE Brazil, de Queiroz *et al.*, 1994) or among 13 stocks from São Paulo State (SE Brazil, Yoshida *et al*, 1990). The observations of the same zymodeme, microdeme and AC01 sequence type

occurring in different stocks, seen here among stocks from Três Braços / Corte de Pedra, supports the proposed hypothesis of clonal propagation (Tibayrenc *et al.*, 1991).

L. V. braziliensis heterogeneity has, however, been widely reported from many Latin American countries (Kreutzer & Christensen, 1980; Evans *et al.*, 1984; Saravia *et al.*, 1985; Revollo *et al.*, 1992; Felinto de Brito *et al.*, 1993; Kreutzer, 1996; Cupolillo *et al.*, 1994; 1995; Chouicha *et al.*, 1997; Delgado *et al.*, 1997; Lucas *et al.*, 1998; Saravia *et al.*, 1998). *L. V. braziliensis* stocks from a single area (Amariji, Pernambuco State), were shown to very heterogeneous, with 4 zymodemes, 4 microdemes and 3 AC01 sequence types scored from just 4 stocks. This region has an ongoing CL epidemic and it would be extremely interesting to examine more stocks by both IEA and microsatellite analysis. Isoenzyme and microdeme heterogeneity among *L. V. braziliensis* stocks was more common among stocks originating from different geographical areas. Heterogeneity may arise from mutational or recombinational processes, the latter of which may be asexual or sexual. The application of microsatellite analysis to larger numbers of localized *Leishmania* populations containing heterogeneous stocks will likely provide further evidence regarding the mechanisms(s) generating this diversity and may identify alleles which are associated with geographical origin, clinical presentation etc.

CHAPTER 5 - NICARAGUA: SYMPATRIC TRANSMISSION OF *L. V. BRAZILIENSIS* AND *L. V. PANAMENSIS*

5.1 Introduction

Nicaragua, the largest country in Central America, is bordered between Honduras (north) and Costa Rica (south) and between the Pacific Ocean and Caribbean Sea on the east and west respectively. Half of Nicaragua is covered by forests, lakes and rivers. The low, fertile Pacific coast region, which includes most of the country's major cities, produces cotton, cattle, sugarcane, and basic food crops (rice, corn, and beans). Coffee is grown in the cooler central highlands bordering Honduras. The forested Caribbean (Atlantic) lowlands are sparsely populated.

CL and MCL are endemic in Nicaragua, notably in northern, central and eastern regions (Darce *et al.*, 1991). Between 1980 and 1987, more than 9500 cases of leishmaniasis were notified (Desjeux, 1992b). Visceral disease has not been reported from Nicaragua but is thought likely to occur in northern regions along the border with Honduras (WHO, 1990). *L. V. braziliensis* and *L. V. panamensis* occur sympatrically in the mountainous regions of northern Nicaragua, whereas *L. V. panamensis* is the major species present in the Atlantic rainforest regions of southern Nicaragua (Belli *et al.*, 1994). Putative *L. V. braziliensis / L. V. panamensis* hybrids have also been reported from Nicaragua (Missoni *et al.*, 1986; Darce *et al.*, 1991; Belli *et al.*, 1994), all from northern foci (Belli *et al.*, 1994).

5.2 Nicaraguan *Leishmania* stocks

Stocks representative of the observed isoenzyme and RFLP patterns from the Belli study (Belli *et al.*, 1994) were selected for further analysis: these are listed in *table 5.2. Figure 5.2* shows a map of Nicaragua indicating the geographical distribution of stocks characterized.

These stocks, including putative *L. V. braziliensis* and *L. V. panamensis* parental and *L. V. braziliensis /L. V. panamensis* hybrid stocks, have been previously characterized by IEA, serodeme and RFLP analysis (Missoni *et al.*, 1986; Darce *et al.*, 1991; Belli *et al.*, 1994); several have recently been further analyzed using RAPD analysis (Noyes *et al.*, 1996).

***Table 5.2*: *Leishmania* stocks from Nicaragua.**

ISOLATE CODE NO.	HOST SPECIES	DEPARTMENT / AREA	*LEISHMANIA* SPECIES ∞†§±	LESION TYPE*
MHOM/NI/88/XD28	Human	Jinotega / Bocay	*L. V. braziliensis*†	CL
MHOM/NI/88/XD09	Human	Jinotega / Bocay	*Lb /Lpa* hybrid†	CL
MHOM/NI/90/HN196	Human	Jinotega / Bocay	*L. V. braziliensis*§	MCL
MHOM/NI/88/XD05	Human	Jinotega / Bocay	*L. V. panamensis*†	CL
MHOM/NI/88/XD42	Human	Jinotega / Pantasma	*Lb /Lpa* hybrid†	CL
MHOM/NI/88/XD36	Human	Jinotega / Pantasma	*Lb /Lpa* hybrid†	CL
MHOM/NI/92/WSL03	Human	Zelaya Norte / Guayabo	*Lb /Lpa* hybrid§	CL
MHOM/NI/88/XD13	Human	Zelaya Norte / Wamblan	*Lb /Lpa* hybrid†	CL
MHOM/NI/89/XD19	Human	Zelaya Norte / Leymus	*L. V. braziliensis*§	MCL
MHOM/NI/89/XD20	Human	Zelaya Norte / Waslala	*Lb /Lpa* hybrid§	CL
MHOM/NI/92/WSL06	Human	Zelaya Norte / Waslala	*Lb /Lpa* hybrid§	CL
MHOM/NI/84/NICA2	Human	Zelaya Norte / Waslala	*Lb /Lpa* hybrid±	CL
MHOM/NI/87/ZE09	Human	Zelaya Sur/ Nva Guinea	*L. V. panamensis*†	CL
MHOM/NI/89/XD17	Human	Ocotal / Murra	*L. V. braziliensis*†	CL
MHOM/NI/89/AZ01	Human	Ocotal / Murra	*L. V. braziliensis*§	MCL
MHOM/NI/88/XD39	Human	Matagalpa / Paiwas	*L. V. panamensis*§	MCL
MHOM/NI/89/XD41	Human	Matagalpa / Rio Blanco	*Lb /Lpa* hybrid§	CL
MHOM/NI/91/ZF01	Human	Matagalpa / El Coyolar	*L. V. panamensis*§	CL
MHOM/NI/88/XD45	Human	Chontales / Los Chiles	*L. V. panamensis*†	CL
MHOM/NI/89/XD03	Human	Rio Sn Juan / Azucenas	*L. V. panamensis*§	CL

Table 5.2: *Leishmania* stocks from Nicaragua. ∞- *Lb /Lpa* hybrid indicates a putative *L. V. braziliensis / L. V. panamensis* hybrid. All stocks were obtained from the cryobank at LSHTM. § - stocks examined by Belli *et al.* (1994); † - stocks examined by Darce *et al.* (1991); ± - stocks examined by Missoni *et al.* (1986). * Lesion type: CL, cutaneous lesion; MCL, mucocutaneous lesion. Note comments on page173-4: 2 stocks previously identified as putative hybrids (MHOM/NI/92/WSL03 and MHOM/NI/92/WSL06) were identified here as *L. V. panamensis*; 1 stock previously identified as *L. V. panamensis* (MHOM/NI/88/XD05) was identified here as a putative hybrid (possible mis-labeling in LSHTM cryobank ?).

***Figure 5.2*: Map of Nicaragua showing the geographical distribution of stocks.**

Figure 5.2: Map of Nicaragua showing the geographical distribution of stocks examined (from Belli *et al.*, 1994). (●) - *L. V. braziliensis*; (○) - *L. V. panamensis*; (*) - putative *L. V. braziliensis* / *L. V. panamensis* hybrid.

5.3 Isoenzyme analysis

L. V. panamensis stocks could be differentiated from *L. V. braziliensis* stocks using 8 enzyme loci: MPI, NHi1, NHi2, 6PGD, PGM, PEPD, ASAT and ALAT. Examples of isoenzyme banding patterns observed in the stocks from Nicaragua are shown in *figure 5.3.1.1* for the enzymes MPI (monomeric), 6PGD (dimeric) and NHi 1/2 (1 monomeric; 2 tetrameric). Banding patterns observed in the Nicaraguan stocks are shown diagramatically alongside reference strain patterns, in *figure 5.3.1.2*, for all enzymes studied.

In agreement with previous work involving these Nicaraguan stocks (Darce *et al.*, 1991; Belli *et al.*, 1994), three distinct groups were identified: among the 20 stocks studied here, 5 were identified as *L. V. braziliensis*, 7 as *L. V. panamensis* and 8 as putative *L. V. braziliensis* / *L. V. panamensis* hybrids. However, three discrepancies were noted. Two stocks (WSL03 and WSL06) reported as hybrids by Belli *et al.*

(1994) were characterized as *L. V. panamensis*: in their report, the IEA pattern shown photographically for one of these stocks (WSL03) at one of the loci showing hybrid phenotypes (6PGD) is not conclusive. Another stock (XD05) reported as *L. V. panamensis* by Belli *et al.* (1994) was characterized here as a hybrid. The XD05 stock used here (two separate stocks with the same name but different year of isolation are listed in Belli's report) was from Bocay / Jinotega and was also studied by Darce *et al.* (1991). These authors reported, although not giving results by individual stock names, that stocks examined from this area were either *L. V. braziliensis* or hybrids.

***Figure 5.3.1.1*: Isoenzyme banding patterns of Nicaraguan stocks**

Figure 5.3.1.1: Isoenzyme variation in stocks from Nicaragua using MPI. Lane 1 - *L. V. braziliensis* reference strain LTB300; lane 2 - *L. V. panamensis* reference strain LS94; lane 3 - XD05; lane 4 - XD09; lane 5 - XD13; lane 6 - XD17; lane 7 - XD19; lane 8 - XD20; lane 9 - XD28; lane 10 - XD36; lane 11 - XD39. Note the hybrid pattern of stock XD05, see text.

Figure 5.3.1.1: **Isoenzyme banding patterns of Nicaraguan stocks (continued)**

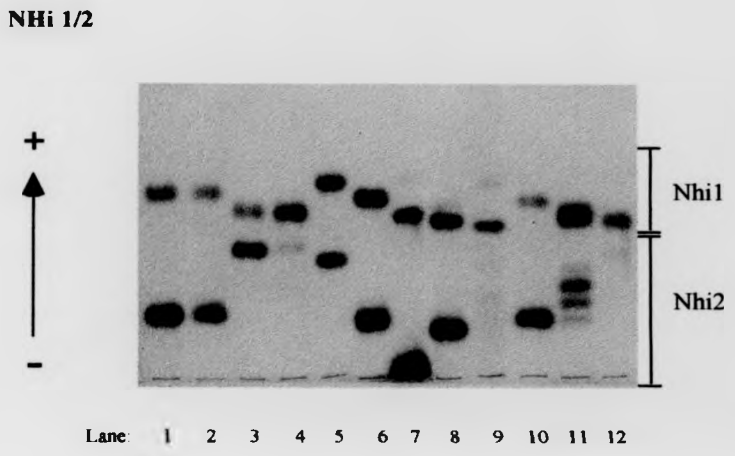


Figure 5.3.1.1: Isoenzyme variation in stocks from Nicaragua using NHi and 6PGD. Lane 1 - *L. V. braziliensis* reference strain LTB300; lane 2 - *L. V. peruviana* reference strain LC39; lane 3 - *L. V. guyanensis* reference strain M4147; lane 4 - *L. V. panamensis* reference strain LS94; lane 5 - *L. V. lainsoni* reference strain M6426; lane 6 - *L. V.* sp. n. reference strain IM2832; lane 7 - *L. L. chagasi* reference strain PP75; lane 8 - *L. L. mexicana* reference strain BEL21; lane 9 - *L. L. amazonensis* reference strain M2269; lane 10 - XD28; lane 11 - XD09; lane 12 - ZF01.

Figure 5.3.1.2: **Diagrammatic representation of IEA banding patterns observed in reference strains and Nicaraguan stocks.**

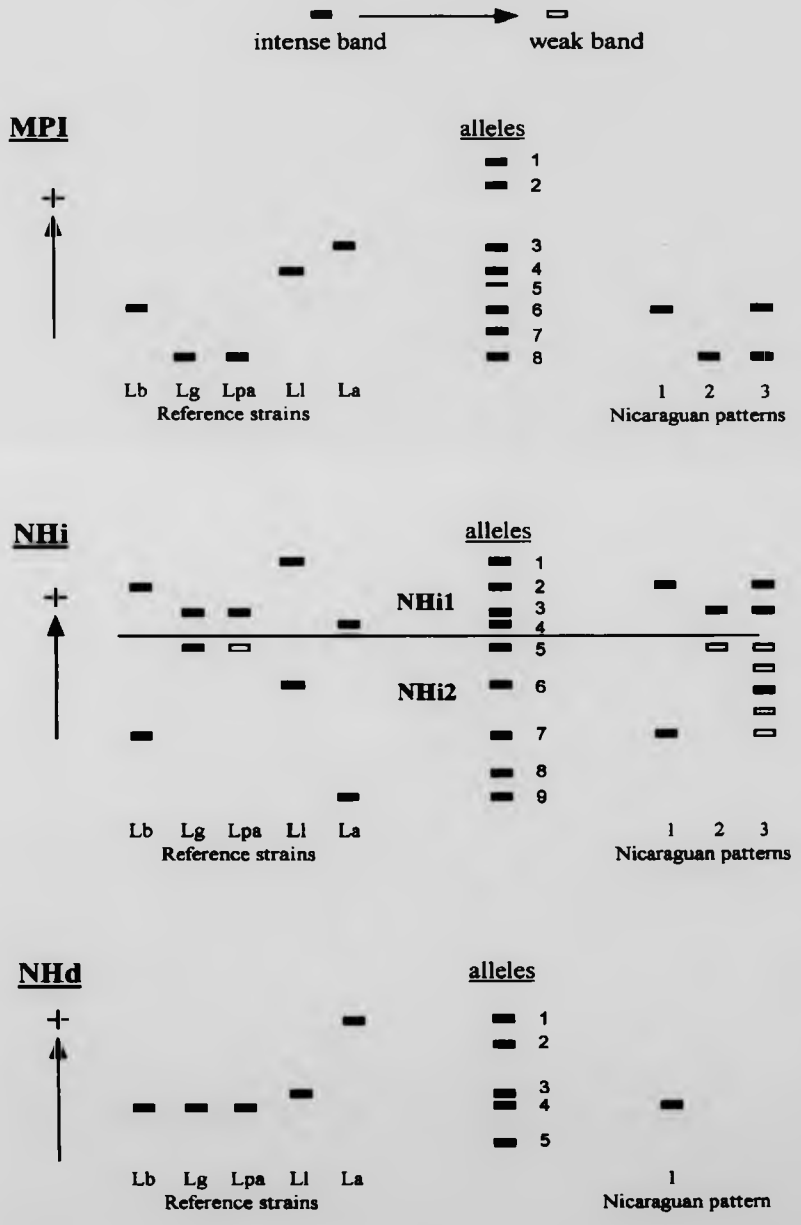


Figure 5.3.1.2: **Diagrammatic representation of IEA banding patterns observed in reference strains and Nicaraguan stocks (c'td).**

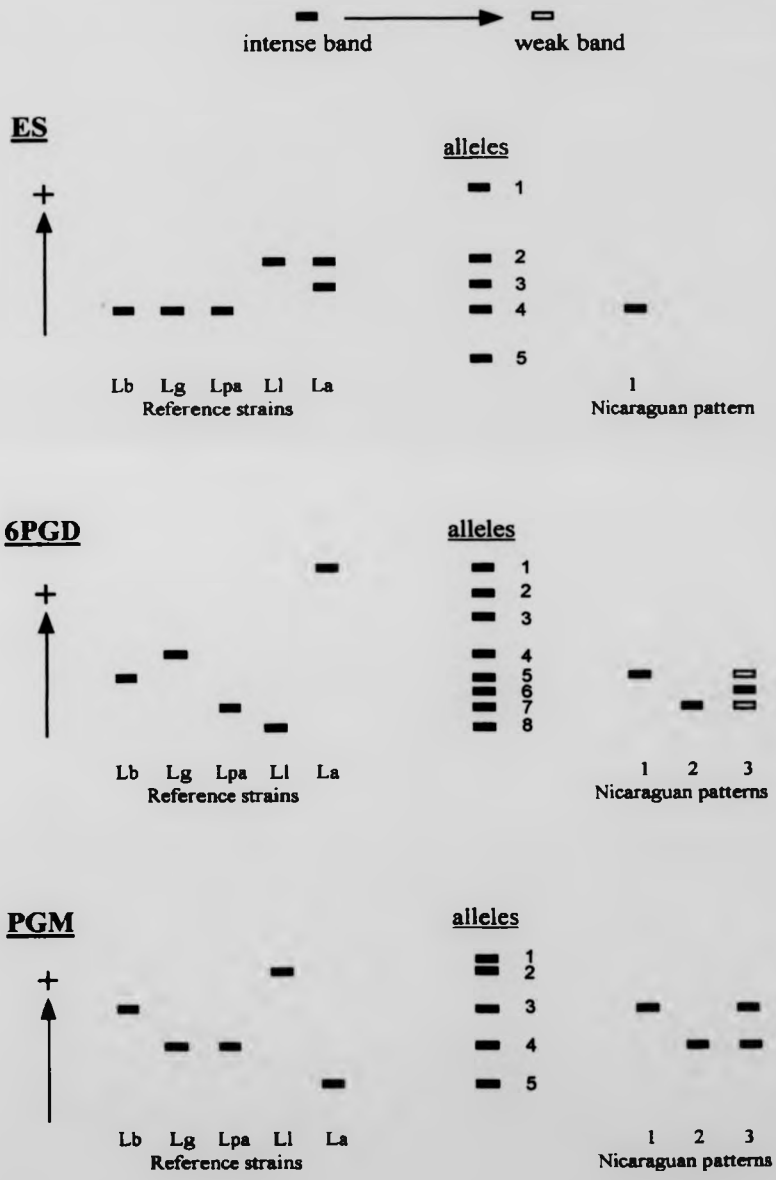


***Figure 5.3.1.2*:** **Diagrammatic representation of IEA banding patterns observed in reference strains and Nicaraguan stocks (c'td).**

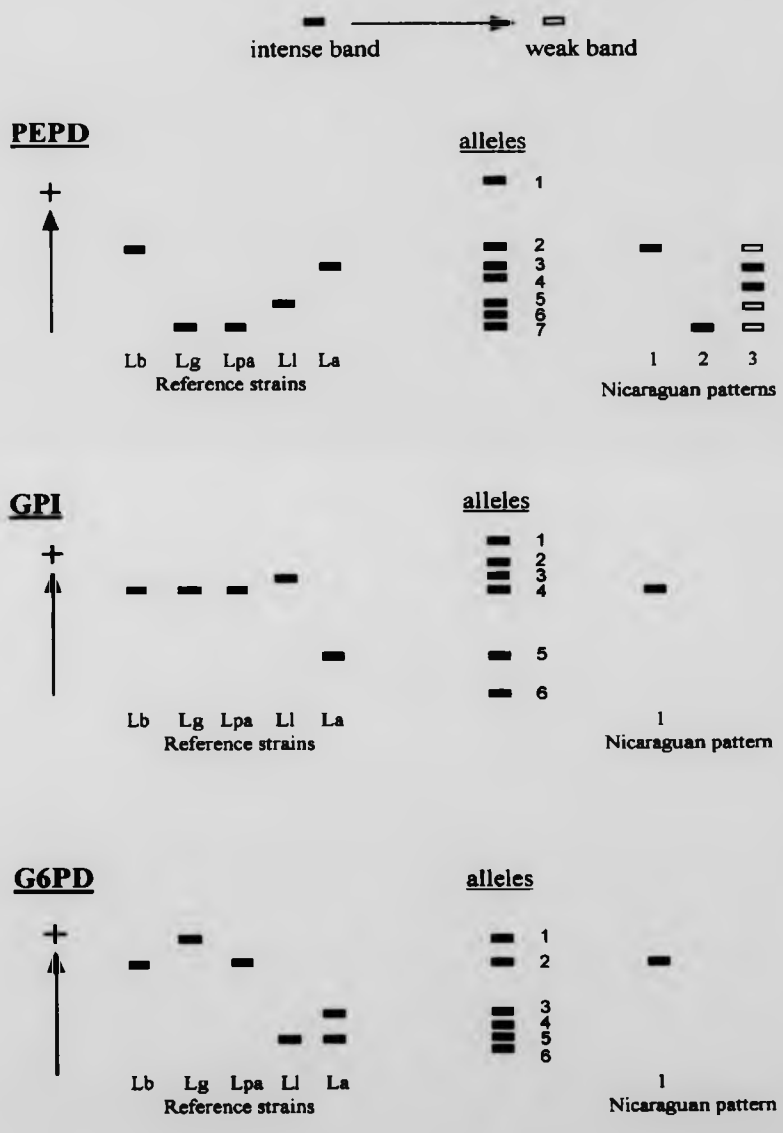
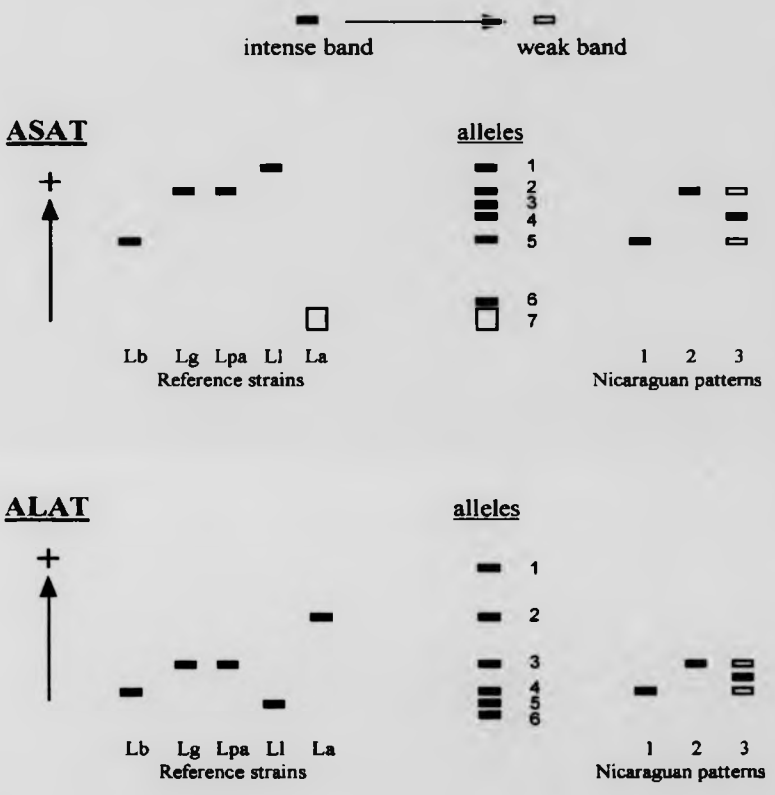


Figure 5.3.1.2: **Diagrammatic representation of IEA banding patterns observed in reference strains and Nicaraguan stocks (c'td).**



5.3.1 Zymodemes

Only three zymodemes were scored from the Nicaraguan stocks using these eleven enzymes. A single zymodeme was scored for both *L. V. braziliensis* (LON200) and *L. V. panamensis* (LON202) stocks: these zymodemes are identical to the reference strains for *L. V. braziliensis* (LTB300) and *L. V. panamensis* (LS94) respectively. The zymodeme pattern of the *L. V. braziliensis / L. V. panamensis* hybrid (LON 216) appears to be an exact juxtaposition of the two parental type zymodemes, with one allele from each parental type evident at each variable enzyme locus. *Table 5.3.1.1* summarizes the IEA allele and zymodeme designations for the Nicaraguan stocks.

***Table 5.3.1.1*:** **Allele and zymodeme designations for Nicaraguan stocks.**

	ENZYME												
STOCK CODE NO.	**MPI**	**NHi 1**	**NHi 2**	**NHd**	**ES**	**PEPD**	**PGM**	**6PGD**	**GPI**	**G6PD**	**ASAT**	**ALAT**	**Z***
XD28, XD17, XD19 HN196, AZ01	6 / 6	2 / 2	7 / 7	4 / 4	4 / 4	2 / 2	3 / 3	5 / 5	4 / 4	2 / 2	5 / 5	4 / 4	LON200
XD42, XD05, XD09 XD13, XD20, XD36 XD41, NICA2	6 / 8	2 / 3	5 / 7	4 / 4	4 / 4	2 / 7	3 / 4	5 / 7	4 / 4	2 / 2	2 / 5	3 / 4	LON216
ZF01, ZE09, XD03 XD39, XD45, WSL03 WSL06	8 / 8	3 / 3	5 / 5	4 / 4	4 / 4	7 / 7	4 / 4	7 / 7	4 / 4	2 / 2	2 / 2	3 / 3	LON202

Table 5.3.1.1: allele and zymodeme designations for Nicaraguan stocks. Z = zymodeme; * LON numbers above 200 have been reassigned by the author as previous records have been lost (zymodemes are assigned based on the IEA conditions used, i.e. for *Leishmania Viannia* species). For full enzyme names, see chapter 2. See *figure 3.2.2* for allele scoring scheme. Data given assumes diploidy, see introduction.

Nicaraguan stock zymodemes are summarized by geographical origin and clinical presentation in *table 5.3.1.2.* Stocks within each group were shown to be identical using IEA, irrespective of geographical origin. Four stocks were isolated from MCL lesions: 3 of these stocks were identified as *L. V. braziliensis* and 1 as *L. V. panamensis*. In agreement with Darce *et al.* (1991), the hybrid isoenzyme genotype was not found to be associated with MCL (0/4 stocks examined).

Table 5.3.1.2 **Summary of Nicaraguan stock zymodemes, by geographical origin and clinical presentation.**

STOCK CODE NO.	Z*	GEOGRAPHICAL ORIGIN		ISOLATED FROM:	CLINICAL TYPE
		AREA	DEPARTMENT		
XD28	LON200	Bocay	Jinotega	Human	CL
XD09	LON216	Bocay	Jinotega	Human	CL
HN196	LON200	Bocay	Jinotega	Human	MCL
XD05	LON216	Bocay	Jinotega	Human	CL
XD42	LON216	Pantasma	Jinotega	Human	CL
XD36	LON216	Pantasma	Jinotega	Human	CL
WSL03	LON202	Guayabo	Zelaya Norte	Human	CL
XD13	LON216	Wamblan	Zelaya Norte	Human	CL
XD19	LON200	Leymus	Zelaya Norte	Human	MCL
XD20	LON216	Waslala	Zelaya Norte	Human	CL
WSL06	LON202	Waslala	Zelaya Norte	Human	CL
NICA2	LON216	Waslala	Zelaya Norte	Human	CL
ZE09	LON202	Nova Guinea	Zelaya Sur	Human	CL
XD17	LON200	Murra	Ocotal	Human	CL
AZ01	LON200	Murra	Ocotal	Human	MCL
XD39	LON202	Paiwas	Matagalpa	Human	MCL
XD41	LON216	Rio Blanco	Matagalpa	Human	CL
ZF01	LON202	El Coyolar	Matagalpa	Human	CL
XD45	LON202	Los Chiles	Chontales	Human	CL
XD03	LON202	Azucenas	Rio San Juan	Human	CL

Table 5.3.1.2: summary of Nicaraguan stock zymodemes, by geographical origin and clinical presentation. Z = zymodeme (assigned based on the IEA conditions used, i.e. for *Leishmania Viannia* species): LON200 – *L. V. braziliensis*; LON202 – *L. V. panamensis*; LON216 – *L. V. braziliensis* / *L. V. panamensis* hybrids. For full enzyme names, see chapter 2. See *figure 3.2.2* for allele scoring scheme. Data given assumes diploidy, see introduction. Note that WSL03 and WSL06 have been characterized as *L. V. panamensis*: andXD05 as a hybrid, in contrast to Belli *et al.* (1994): see text.

5.4 RAPD analysis

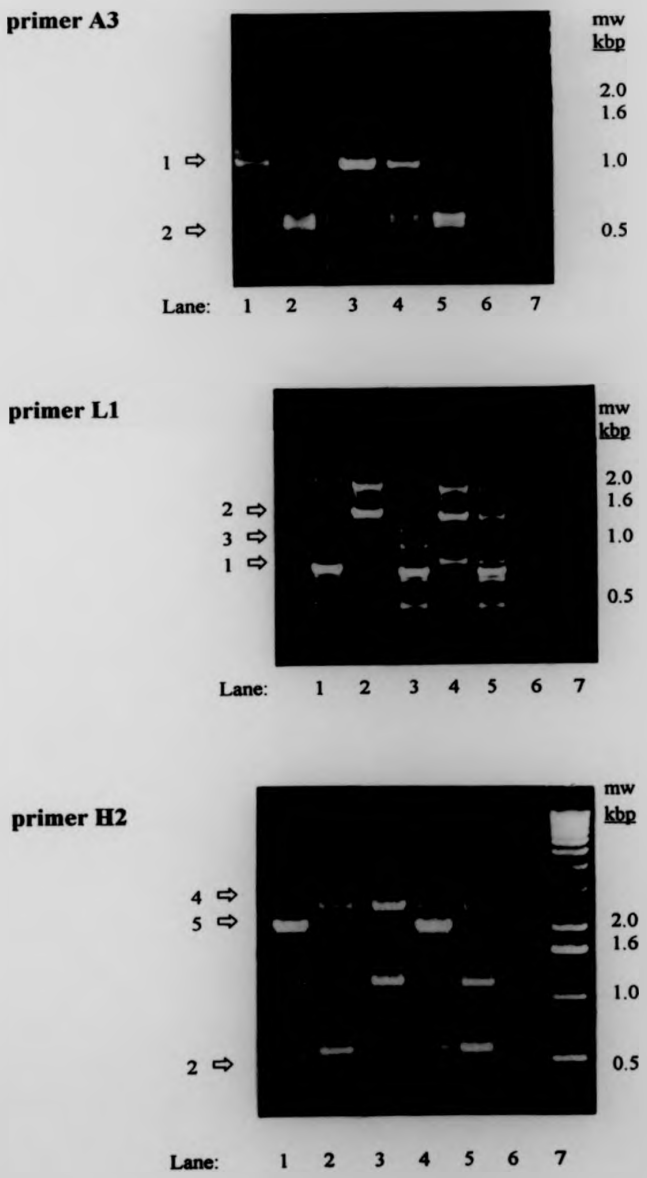
Notwithstanding the fact that this technique was discontinued, for reasons discussed in chapter 3, several observations regarding stocks from Nicaragua deserve comment. Three stocks, one from each of the three zymodemes observed among the Nicaraguan sample, were examined using RAPD. *Figure 5.4.1* shows RAPD profiles generated from reference strains and these three Nicaraguan stocks (XD28, *L. V. braziliensis*; ZF01, *L. V. panamensis*; XD09, putative hybrid), using 3 primers (A3, L1 and H2).

Fourteen RAPD primers generated profiles which discriminated between the reference strains of *L. V. braziliensis* and *L. V. panamensis*. Stocks identified as *L. V. braziliensis* or *L. V. panamensis* from Nicaragua, although generating patterns which were similar to their counterpart reference strains, often differed by the presence and/or absence of minor bands (e.g. arrow 3, *figure 5.4.1*). This observation was not unexpected since the reference strains originated from geographically distant locations (Brazil and Panama respectively). Eight of the 14 primers found to separate *L. V. braziliensis* and *L. V. panamensis* also generated profiles for the putative hybrid stock which were heterozygous between those of the parental type profiles, i.e. contained bands "inherited" from both (e.g. arrows 1 and 2, *figure 5.4.1*). Two hybrid profiles (D1 and H2) included bands which were not present in either putative parent (e.g. arrow 5, *figure 5.4.1*). This band is, however, present in the profile of the *L. V. braziliensis* reference strain. The origin of such bands, as with all RAPD bands, is unknown but not unexpected since the putative parental types are unlikely to be the actual parents of the putative hybrid.

These observations illustrate the potential usefulness of this technique for inferring relationships between organisms in the absence of information from other sources, as discussed in chapters 1 and 3.

Notes to *figure 5.4.1.* (see next page) RAPD profiles generated from parental and hybrid type Nicaraguan stocks using primers A3, L1 and H2, visualized after electrophoresis through 1.2% agarose gel and ethidium bromide staining. Lane 1 - *L. V. braziliensis* reference strain, LTB300, lane 2 - *L. V. panamensis* reference strain, LS94. Lanes 3 - 5, Nicaraguan stocks: lane 3 - XD28 (*L. V. braziliensis*), lane 4 - XD09 (putative hybrid *L. V. braziliensis* / *L. V. panamensis*); lane 5 - ZF01 (*L. V. panamensis*). Lane 6 - negative control, lane 7 - 1kb molecular weight (mw) marker (Gibco BRL, UK). Stocks in lanes 4 and 5 are reversed in primer L1 picture. Arrows (numbered 1 - 5) illustrate the following: arrow 1 - band from *L. V. braziliensis* "parent"; arrow 2 - band from *L. V. panamensis* "parent"; arrow 3 - band found in *L. V. braziliensis* stock from Nicaragua but not in the reference strain; arrow 4 - band found in all subgenus *Viannia* stocks; arrow 5 - band found in putative hybrid but not present in Nicaraguan parental strains (present in *L. V. braziliensis* reference strain).

Figure 5.4.1: **RAPD profiles generated from parental and hybrid type Nicaraguan stocks using primers A3, L1 and H2.**



5.5 PCR-RFLP analysis of rRNA gene ITS region

PCR-RFLP analysis of the rRNA gene ITS region was used to examine all stocks from the Nicaraguan sample. *L. V. braziliensis* and *L. V. panamensis* stocks could be distinguished by RFLP analysis using the enzymes *Bst*UI, *Scr*FI, *Fsp*I, *Taq*I and *Sph*I. Hybrid stocks were shown, using each of these enzymes, to generate fragments of the same apparent size as the *L. V. panamensis* stocks. *Figure 5.5.1* shows the RFLPs of Nicaraguan stocks from digestion using *Bst*UI. Using several enzymes, RFLP profiles of hybrid stocks contained multiple heteroduplex bands which were not seen in *L. V. braziliensis* or *L. V. panamensis* stocks (arrow* *figure 5.5.1*).

***Figure 5.5.1*:** **RFLP analysis of rRNA ITS amplification products from Nicaraguan stocks using *Bst*UI.**

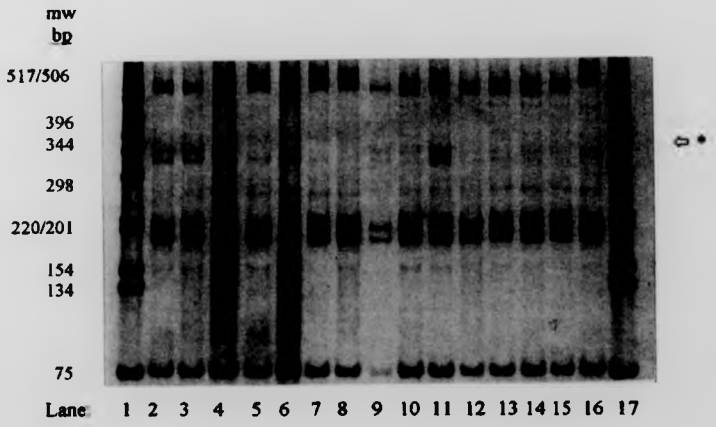


Figure 5.5.1: RFLP analysis of ribosomal RNA (rRNA) internal transcribed spacer (ITS) region amplification products from Nicaraguan stocks, visualized after electrophoresis through 6% polyacrylamide gel and silver staining. Lanes 1 & 17: 1kb molecular weight (mw) marker (Gibco BRL). *L. V. braziliensis* stocks: lanes 5, 7, 8, 16 (XD19, XD28, XD28, HN196). *L. V. panamensis* stocks: lanes 10, 12, 13, 14, 15 (XD39, WSL03, WSL06, ZE09, ZF01). Hybrid stocks: lanes 2, 3, 4, 6, 9, 11 (XD05, XD09, XD13, XD20, XD36, XD42). *L. V. braziliensis* and *L. V. panamensis* stocks can be differentiated by the smaller size of the upper fragment at approx. 200bp in *L. V. braziliensis* stocks. Hybrid stocks appear identical to the *L. V. panamensis* stocks except for the presence of 2 heteroduplex bands (arrow *) at approx. 340/360bp. Intra-specific variation can be seen for *L. V. braziliensis* stock XD28 (lanes 7 & 8, additional fragments at approx. 220bp) and for the hybrid XD42 (lane 11, doublet upper band at approx. 200bp)

Diversity between stocks identified, using IEA, as the same type (species or hybrid) was rare, but is illustrated here for two stocks, XD28 (*L. V. braziliensis*) and XD42 (hybrid), using *Bst*UI (lanes 7/8 and 11, *figure 5.5.1*). Greater diversity may have been undetected since the PAGE system used did not resolve fragments with small differences in size which made scoring difficult. PCR-RFLP analysis is, like microsatellite analysis, amenable to multiplexing. PCR products from different loci labeled with different fluorophores could be pooled for RFLP analysis. To ensure that all RFLP fragments were labeled, the PCR reaction mix would have to include labeled dNTPs (as opposed to the labeled primers used for microsatellite analysis). Fragments from different loci could be identified from the myriad of fragments generated by using a different label for each locus. Multiplexed RFLP products could thus be analyzed using the Genescan® and Genotyper® approach as described previously. PCR-RFLP analyses of *Viannia* parasites based on the gene loci and ITS regions of the *gp63* and rRNA arrays have recently been reported (Cupolillo *et al.*, 1995; Victoir *et al.*, 1998). Other gene families such as the mini-exon array (Hassan *et al.*, 1993; Fernandes *et al.*, 1994) have also been characterized in *Viannia* species and could be included in this approach: combined results from several such targets will provide multilocus data for population and/or phylogenetic analyses.

5.6 Microsatellite analysis

5.6.1 PCR of microsatellite loci

As observed among the Brazilian stocks, several Nicaraguan stocks had null alleles at one or more of the microsatellite loci. Two stocks (XD17 and XD41) failed to amplify any of the 3 loci and one stock (XD45) failed to amplify AC01. These stocks originated from different geographical areas. PCR products generated from Nicaraguan stocks for all loci were within the expected size range.

5.6.2 Non-denaturing PAGE analysis

5.6.2.1 AC01

Figure 5.6.2.1 shows AC01 products from Nicaraguan stocks analyzed using non-denaturing PAGE and silver staining.

As for Brazilian stocks, 2 intensely-staining bands were observed for Nicaraguan stocks at this locus. Using PAGE, products from *L. V. braziliensis* and *L. V. panamensis* stocks could be separated by size (e.g. lanes 2 and 3, respectively, *figure 5.6.2.1*), with the exception of the *L. V. braziliensis* stock AZ01, the product of which was similar to *L. V. panamensis* stocks (data not shown, discussed in sections 5.6.3.1 and 5.7). Products of two different sizes were evident among the *L. V. panamensis stocks* (compare product sizes in lanes 3-5 with those in 6 & 8, *figure 5.6.2.1*). The putative hybrid stocks generated profiles that were an exact combination of those from one of the *L. V. panamensis* types (smaller allele) and *L. V. braziliensis*. In these hybrid profiles, the products correlating to *L. V. panamensis* were weak by comparison with those correlating to *L. V. braziliensis*. It is not clear why this should be so: it is possible that there is a mutation in the primer binding site of this allele in hybrid stocks such that the *L. V. panamensis* allele is amplified with less efficiency. Another possibility is that microsatellite alleles containing significantly different numbers of repeats may be amplified with different efficiencies (alleles with smaller numbers of repeats would be expected to be amplified more readily than alleles with large numbers of repeats). PCR amplification of a locus at which the alleles did vary significantly in repeat number would be biased towards the allele which was amplified more readily. This allele would dominate the reaction resulting in the generation of substantially more product.

Figure 5.6.2.1 **AC01 products from Nicaraguan stocks (PAGE)**

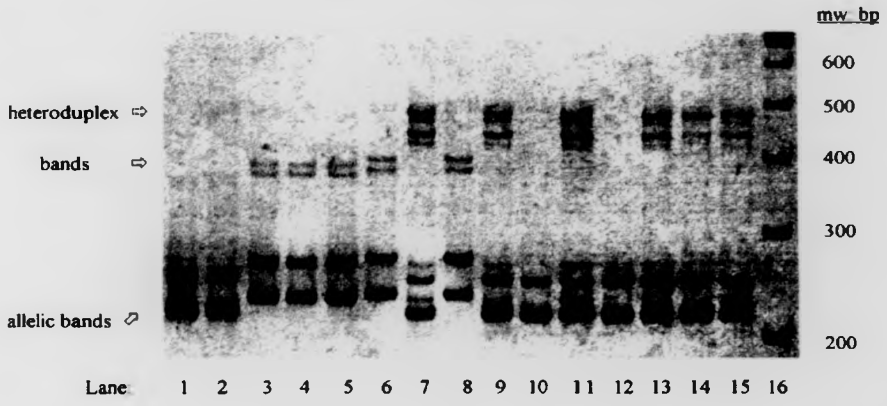


Figure 5.6.2.1: AC01 products from Nicaraguan stocks analyzed using non-denaturing 6% PAGE and silver staining. Lane 1 - *L. V. braziliensis* reference strain, LTB300; lane 2 - HN196; lane 3 - WSLO6; lane 4 - WSL03, lane 5 - ZF01, lane 6 - ZE09; lane 7 - XD42; lane 8 - XD39; lane 9 - XD36; lane 10 - XD28; lane 11 - XD20; lane 12 - XD19, lane 13 - XD13; lane 14 - XD09; lane 15 - XD05; lane 16 - 100 bp molecular weight marker (Gibco BRL, UK). PCR products from *L. V. braziliensis* stocks are in lanes 2, 10 and 12; *L. V. panamensis* stocks are in lanes 3 - 6 & 8, hybrids stocks are in lanes 7, 9, 11 & 13 - 15. The allelic band is the lowest intensely-staining band in each lane. Note the additional band (at set distance from the allelic band in each lane) and heteroduplex bands (indicated): see text for discussion.

Heteroduplex bands were also observed in Nicaraguan stocks: as with Brazilian stocks, Nicaraguan stocks designated as both homozygous and heterozygous for AC01 generated these molecules (designated as such using Genotyper® data, section 5.6.3). Heteroduplex bands are particularly evident in profiles from hybrid stocks, wherein 4 heteroduplex bands are present. Diploidy is a working hypothesis for *Leishmania*: whilst four heteroduplex bands could be generated by the reannealing of heterozygote allelic strands with products resulting from slippage or incorrect base insertion during PCR amplification, aneuploidy cannot be ruled out. Ploidy will be discussed further in chapter 7. Additional bands present in AC01 profiles have been discussed previously. AC01 PAGE patterns for these stocks correlate with, and are further discussed under, Genotyper® results (section 5.6.3.1).

5.6.2.2 AC16

Figure 5.6.2.2 shows the AC16 products from Nicaraguan stocks. All Nicaraguan stocks exhibited heteroduplex molecules at approx. 350bp. *L. V. panamensis* stocks could be grouped by the presence of three additional heteroduplex bands at approx. 370 - 400bp. Genotyper® results score the majority of these stocks as homozygous at this locus so the origin of these heteroduplex molecules is not clear. The *L. V. panamensis* stock XD45 did not generate these heteroduplex molecules but had an additional band, the origin of which was not determined from Genotyper® results (see section 5.6.3). Comparisons between XD45 and other *L. V. panamensis* stocks at the other two loci was not possible since XD45 had null alleles for both. *L. V. braziliensis* and hybrid stock profiles appeared similar: slight mobility differences were observed between the allelic bands of different stocks (e.g. between lanes 7 and 8) in these two groups. The overall profiles generated with Nicaraguan stocks at the AC16 locus correlate well with Genotyper® results.

Figure 5.6.2.2 **AC16 products from Nicaraguan stocks (PAGE)**

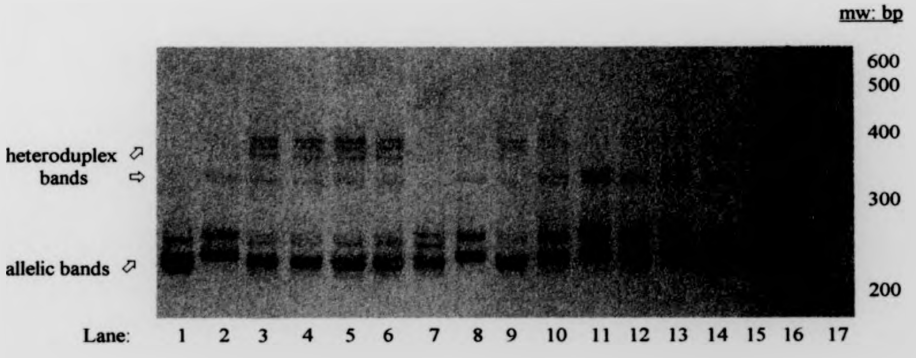


Figure 5.6.2.2: AC16 products from Nicaraguan stocks analyzed using non-denaturing 6% PAGE and silver staining. Lane 1 - *L. V. braziliensis* reference strain, LTB300; lane 2 - HN196, lane 3 - WSLO6; lane 4 - WSL03; lane 5 - ZF01, lane 6 - ZE09; lane 7 - XD45; lane 8 - XD42; lane 9 - XD39; lane 10 - XD36, lane 11 - XD28; lane 12 - XD20; lane 13 - XD19, lane 14 - XD13; lane 15 - XD09; lane 16 - XD05; lane 17 - 100 bp molecular weight marker (Gibco BRL, UK). *L. V. braziliensis* stocks lanes 2, 11 and 13, *L. V. panamensis* stocks lanes 3 - 7 & 9, hybrids stocks lanes 8, 10, 12 & 14 - 16. Note the additional band (at set distance from the allelic band in each lane) and heteroduplex bands (indicated): see text for discussion.

5.6.2.3 AC52

Figure 5.6.2.3 shows AC52 products from Nicaraguan stocks. Allele size differences were clearly evident at this locus, but as with all PAGE analyses, these could not be quantified with any precision: stocks were grouped, as previously, by comparison of overall profiles. Two distinct allelic band sizes were observed in *L. V. braziliensis* stocks (lanes 5, 7 & 15): XD28 and HN196 (lanes 7 & 15) bands were larger than those of XD19 (lane 5). Products from XD19 appeared to be the same size as *L. V. panamensis* stocks (lanes 9, 11 - 14). *L. V. panamensis* products were generally less intense than those of *L. V. braziliensis*. Most *L. V. braziliensis* and hybrid stocks had prominent heteroduplex molecules which were not present in *L. V. panamensis* stocks. Size variation was also observed between products from *L. V. panamensis* stocks (see lanes 12 - 14). In the profiles of several hybrid stocks, faint bands corresponding to allelic *L. V. panamensis* (or XD19) bands were visible (lanes 6, 8 & 10). These results generally concur with those of Genotyper® and are discussed further in section 5.6.3.

Figure 5.6.2.3 **AC52 products from Nicaraguan stocks (PAGE)**

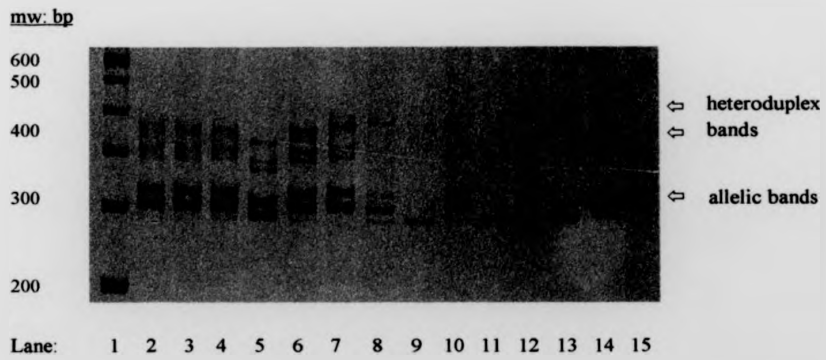


Figure 5.6.2.3 AC52 products from Nicaraguan stocks analyzed using non-denaturing 6% PAGE and silver staining. Lane 1 - 100bp molecular weight marker (Gibco BRL, UK), lane 2 - XD05; lane 3 - XD09; lane 4 - XD13; lane 5 - XD19; lane 6 - XD20; lane 7 - XD28; lane 8 - XD36; lane 9 - XD39; lane 10 - XD42; lane 11 - ZE09; lane 12 - ZF01; lane 13 - WSL03; lane 14 - WSL06; lane 15 - HN196. *L. V. braziliensis* stocks lanes 5, 7 & 15; *L. V panamensis* stocks lanes 9, 11 - 14; hybrid stocks lanes 2 - 4, 6, 8 & 10. Note the additional bands (at set distances from the allelic band(s) in each lane) and heteroduplex bands (indicated): see text for discussion.

5.6.3 Genescan® and Genotyper® 2.0 analyses

Table 5.6.3 (page 192) summarizes the allele sizes scored, using Genotyper®, at each microsatellite locus in stocks from Nicaragua, listed by species type, geographical origin and by clinical presentation (all stocks were isolated from humans). Results are discussed for each microsatellite locus and by multilocus genotype.

5.6.3.1 AC01

In agreement with PAGE results, 2 different AC01 allele sizes were observed in Nicaraguan *L. V. panamensis* stocks: 241 (4 stocks, genotype 241/241) and 245 (2 stocks, genotype 245/245). The size of the AC01 allele observed in all Nicaraguan *L. V. braziliensis* stocks bar one was 227 (3 stocks, genotype 227/227): this is the same genotype scored amongst the majority of Brazilian *L. V. braziliensis* stocks. Also in agreement with PAGE results, one *L. V. braziliensis* stock, AZ01, generated a peak of 245 which is associated here with *L. V. panamensis* stocks. This stock will be discussed further in section 5.7.

Two peaks (227 and 241) were observed in putative hybrid stocks: these corresponded to alleles from *L. V. braziliensis* and *L. V. panamensis*, respectively, providing further evidence of the hybrid status of these stocks. In agreement with observations from PAGE analysis, the *L. V. panamensis* allele was much less intense than that of *L. V. braziliensis*: possible reasons for this difference have been discussed previously.

A single hybrid stock appeared homozygous, genotype 227/227: it is possible that the *L. V. panamensis* allele in this stock was so weakly amplified that it was not detected; it is also possible that this stock was indeed homozygous at this locus and did not have the 241 allele. *Figure 5.6.3.1* illustrates the Genotyper® peaks obtained for each of the genotypes 227/227 (*L. V. braziliensis* stock XD19), 241/241 (*L. V. panamensis* stock WSL03) and 227/241 (hybrid stock XD13).

There was no apparent association between geographical origin and AC01 genotype. Stocks isolated from MCL lesions (3 *L. V. braziliensis*, 1 *L. V. panamensis*) were genotype 227/227 (as in Brazil) or 245/245 (2 stocks including AZ01). The 241 and 245 alleles are not exclusive to *L. V. panamensis*: 241 was also observed in the *L. V. shawi* reference strain (genotype 241/241) and 245 was observed in the *L. V.* sp. n. stock IM2832 (genotype 227/245).

AC01 Genotyper® results for these stocks can be explained by variation of the number of dinucleotide repeats as revealed by sequencing (see section 5.6.4).

Figure 5.6.3.1 **Genotyper® output from the AC01 locus for selected Nicaraguan stocks, illustrating the peaks observed in putative parental and hybrid profiles.**

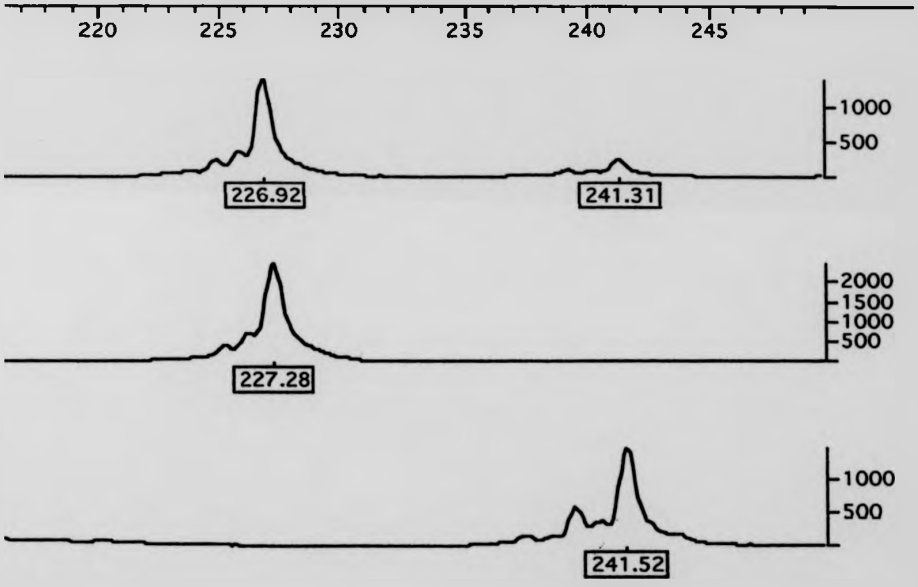


Figure 5.6.3.1: Genotyper® output from the AC01 locus for selected Nicaraguan stocks, illustrating the peaks observed in putative parental and hybrid profiles. Numbers across the top of the figure represent peak size, in bases. The exact sizes of individual peaks were scored automatically by the software. The scale to the right of each trace indicates the peak intensity. See chapter 3 for discussion of allele scoring. Top - hybrid stock XD13 (genotype 227/241); middle - *L. V. braziliensis* stock XD19; bottom - *L. V. panamensis* stock WSL03 (genotype 242/241). Note the lower intensity of the 241 allele in the hybrid stock: see text for discussion.

Table 5.6.3 **Microsatellite allele sizes of stocks from Nicaragua by species type, geographical origin and clinical presentation.**

Stock code no. *†	Species ∞†§±	Area / Department	Lesion type*	Microsatellite locus AC01 allele 1[a]	AC01 allele 2[b]	AC16 allele 1	AC16 allele 2	AC52 allele 1	AC52 allele 2
LTB300	b	Brazil	MCL	**227**	**227**	**244**	**244**	**283**	**283**
XD28	b†	Jinotega / Bocay	CL	227	227	250	252	313	313
HN196	b§	Jinotega / Bocay	MCL	227	227	250	250	307	307
XD19	b§	Zelaya Norte / Leymus	MCL	227	227	254	254	297	299
XD17	b†	Ocotal / Murra	CL	X	X	X	X	X	X
AZ01	b§	Ocotal / Murra	MCL	245	245	250	252	291	291
XD09	b/pa†	Jinotega / Bocay	CL	227	241	250	250	291	309
XD05[2]	b/pa†	Jinotega / Bocay	CL	227	241	250	250	291	309
XD42	b/pa†	Jinotega / Pantasma	CL	227	241	250	250	291	313
XD36	b/pa†	Jinotega / Pantasma	CL	227	241	250	250	291	307
XD13	b/pa†	Zelaya Norte / Wamblan	CL	227	241	250	250	291	309
XD20	b/pa§	Zelaya Norte / Waslala	CL	227	241	250	250	291	309
NICA2	b/pa±	Zelaya Norte / Waslala	CL	227	227	250	250	291	309
XD41	b/pa§	Matagalpa / Rio Blanco	CL	X	X	X	X	X	X
WSL03[1]	pa§	Zelaya Norte / Guayabo	CL	241	241	244	244	291	291
WSL06[1]	pa§	Zelaya Norte / Waslala	CL	241	241	244	244	299	299
ZE09	pa†	Zelaya Sur/ Nva Guinea	CL	245	245	244	244	291	291
XD39	pa§	Matagalpa / Paiwas	MCL	245	245	244	244	291	291
ZF01	pa§	Matagalpa / El Coyolar	CL	241	241	244	244	295	295
XD45	pa†	Chontales / Los Chiles	CL	X	X	250	252	F	F
XD03	pa§	Rio Sn Juan / Azucenas	CL	227	241	250	250	291	309
LS94	pa	Panama	CL	**239**	**239**	**244**	**244**	**299**	**299**

Table 5.6.3: Summary of microsatellite allele sizes of stocks from Nicaragua by species type, geographical area and by clinical type. Code names in **bold** type indicate reference strains which, although not from Nicaragua, are included for comparison. All stocks were of human origin. ∞Species: b - *braziliensis*; pa - *panamensis*; b/pa - putative *braziliensis/panamensis* hybrid; [1]WSL03 and WSL06 were identified using IEA as *L. V. panamensis*, not putative *braziliensis/panamensis* hybrids as reported by Belli *et al.* (1994). [2]XD05 (MHOM/NI/88/XD05) was identified using IEA as putative *braziliensis/panamensis* hybrid, not *L. V. panamensis* as reported by Belli *et al.* (1994). § - stocks examined by Belli *et al.* (1994); † - stocks examined by Darce *et al.* (1991); ± - stocks examined by Missoni *et al.* (1986). * Lesion type: CL, cutaneous lesion; MCL, mucocutaneous lesion. Assignment and scoring of alleles are explained in chapter 3. X indicates that no PCR product was generated. F - failed to generate scorable Genotyper® peak.

5.6.3.2 AC16

Three genotypes were scored amongst the 4 Nicaraguan *L. V. braziliensis* stocks: 250/250; 250/252 and 254/254. All hybrid stocks were genotype 250/250. The genotype 244/244 was the most common genotype observed in Nicaraguan *L. V. panamensis* stocks. Two other genotypes were scored in *L. V. panamensis* stocks, 250/250 and 250/252: these were also seen in *L. V. braziliensis* stocks. AC16 genotype 244/244, here associated only with *L. V. panamensis* stocks, was scored in many *L. V. braziliensis* stocks from Brazil. One *L. V. panamensis* stock, XD45, presented an additional band using PAGE (see *figure 5.6.2.2*): this stock was scored as 250/252 using Genotyper®. Other stocks also genotyped as 250/252 did not have this PAGE band, the origin of which is thus not clear. Four genotypes were observed among stocks isolated from MCL lesions: 250/250, 250/252, 254/254 and 244/244 (as in Brazilian *L. V. braziliensis* stocks, here a *L. V. panamensis* stock).

5.6.3.3 AC52

Many AC52 genotypes were scored among Nicaraguan stocks. *L. V. braziliensis* stocks presented 4 genotypes: three were homozygous with allele sizes of 291*, 307 and 313; a fourth stock was heterozygous with alleles of 297 and 299 (this AC52 genotype was also scored in the *L. V. shawi* reference strain M15065. Four genotypes were scored from 5 *L. V. panamensis* stocks: four were homozygous with allele sizes of 291 (2 stocks), 295 and 299; the fifth stock was heterozygous with alleles of 291 and 309. In agreement with AC01 results, one *L. V. braziliensis* stock, AZ01*, had a genotype which was found in *L. V. panamensis* stocks.

Three genotypes, all heterozygous, were scored amongst the hybrid stocks: 291/307 (1 stock), 291/309 (5 stocks) and 291/313 (1 stock). These AC52 genotypes, as do AC01 genotypes, support the status of these stocks as hybrids between *L. V. braziliensis* and *L. V. panamensis*. As observed in PAGE analysis at the AC01 locus, peaks equivalent to *L. V. panamensis* alleles in these hybrid stocks were weaker than peaks equivalent to alleles of *L. V. braziliensis*. For this locus however, the hypothesis that alleles with longer repeat regions have lower amplification efficiencies (section 5.6.2.1) is not applicable because it is the *L. V. braziliensis* allele which contains the larger number of dinucleotide repeats. It is more likely here that there is a mutation in the primer binding site at this locus in alleles of *L. V. panamensis* stocks: this could also explain

why the products of *L. V. panamensis* stocks were generally less intense than those of *L. V. braziliensis*. (see *figure 5.6.2.3*).

Many Brazilian *L. V. braziliensis* stocks were homozygous for the AC52 allele 283: the alleles observed in several Nicaraguan *L. V. braziliensis* stocks are considerably larger. This is in agreement with Russell *et al.* (1999) who, from sequence data, scored AC52 repeat numbers in Nicaraguan and Brazilian *L. V. braziliensis* (2 and 7 examined) stocks as 21/23 and 9 (& rarely 15 and 16) respectively. These differences would, assuming size differences equate to changes in the number of repeats, result in allele sizes of 307 (283 + 24) and 311 (283 + 28), sizes in the range observed from Nicaraguan stocks (one exact match). These size differences illustrate an association between geographical origin and allele size. The correlation observed between Genotyper® (here) and sequencing data (Russell *et al.*, 1999) indicates that the Genotyper® technique is generally suitable for the analysis of microsatellite loci.

5.6.3.4 Multilocus analysis of microsatellite data

Microsatellite genotype data is compared with species type, as determined using IEA, in *table 5.6.3.4*. This presentation highlights 3 stocks (AZ01, XD03 and NICA2) for which microsatellite and IEA data appear contradictory.

AZ01 was identified as *L. V. braziliensis* using IEA. Microsatellite analysis using AC01 and AC52 however, identifies this stock as *L. V. panamensis* (AC16 results are ambiguous). XD03 was identified as *L. V. panamensis* using IEA. Microsatellite analysis shows that this stock is identical to the majority of hybrid stocks at all 3 loci (but, see section 5.6.4.2). Microsatellite results for NICA2 (hybrid stock) differed from IEA results at just one locus, AC01, where NICA2 failed to generate a *L. V. panamensis* peak: for reasons discussed previously regarding the amplification of *L. V. panamensis* alleles at this locus, this ambiguity is probably not significant.

Microdemes observed in stocks from Nicaragua are summarized in *table 5.6.3.5*. Fourteen microdemes were scored among the 18 Nicaraguan stocks assigned: each *L. V. braziliensis* stock (4) had a different microdeme, *L. V.* panamensis stocks (7) could be separated into 6 microdemes. Putative hybrid stocks (7) were separated into 4 microdemes; three hybrid stocks had identical multilocus genotypes, microdeme LMS34. In agreement with results for the Brazilian stocks, most microdemes contained a single stock. MCL stocks were LMS29, LMS30, LMS31 and LMS39.

Table 5.6.3.4: **Comparison of microsatellite (Genotyper®) and isoenzyme data, by genotypes observed at each microsatellite locus**

Locus	Observed genotype	Stocks: species status assigned using IEA data		
		L. V. braziliensis	*L. V. braziliensis* / *L. V. panamensis* hybrid	*L. V. panamensis*
AC01	227 / 227	**LTB300**, XD28, HN196, XD19	NICA2	
	227 / 241		XD09, XD42, XD36, XD13, XD20, XD05	XD03
	241 / 241			WSL03, WSL06, ZF01
	245 / 245	AC01		ZE09, XD39
AC16	244 / 244	**LTB300**		**LS94**, WSL03, WSLO6, ZE09, XD39, ZF01
	250 / 250	HN196	XD09, XD42, XD36, XD13, XD20, NICA2, XD05	XD03
	250 / 252	XD28, AZ01		XD45
	254 / 254	XD19		
AC52	283 / 283	**LTB300**		
	291 / 291	AZ01		**LS94**, WSL03, ZE09, XD39
	291 / 307		XD36	
	291 / 309		XD09, XD13, XD20, NICA2, XD05	XD03
	295 / 295			ZF01
	299 / 299			WSL06
	297 / 299	XD19		
	307 / 307	HN196		
	313 / 313	XD28		

Table 5.6.3.4 comparison of microsatellite (Genotyper®) and isoenzyme data, by genotypes observed at each microsatellite locus, Code names in **bold** indicate reference strains, not of Nicaraguan origin. Stocks which are underlined have conflicting microsatellite and IEA results, see text.

Notes to *table 5.6.3.5* (next page): summary of microdemes observed in Nicaraguan stocks. † microdeme is the term coined here to describe *Leishmania* sharing the same microsatellite multilocus genotype (i.e. combined genotype data from the 3 microsatellite loci: AC01, AC16 and AC52). "LMS" numbers have been used to identify different microdemes (i.e. LMS1, 2, 3 etc.). Stocks which failed to generate PCR products at any of the three loci were not assigned a microdeme number because the genetic basis of failure to amplify could not be determined. §*Viannia* species codes: b - *braziliensis*; pe - *peruviana*; g - *guyanensis*; s - *shawi*; pa - *panamensis*, l - *lainsoni*; * *L. V.* sp. n. (reference strain IM2832, Grimaldi et al., 1991); h - putative *L. V. braziliensis / L. V. panamensis* hybrid. Stocks which are in *italics* are from MCL lesions. F - failed to generate scorable Genotyper® peak.

Table 5.6.3.5: **Summary of microdemes† observed in Nicaraguan stocks.**

Microdeme	Microsatellite locus genotype						Geographical origin		Stock(s)	*Viannia*
†	AC01		AC16		AC52		State/Dept	Region/Area◊		species§
LMS1	227	227	244	244	283	283	BRAZIL		*LTB300*	**b**
LMS2	231	231	ND	ND	ND	ND	PERU		LC39	**pe**
LMS3	225	241	242	242	ND	ND	BRAZIL		M4147	**g**
LMS4	241	241	246	248	297	299	BRAZIL		M15065	**s**
LMS5	239	239	244	244	291	291	PANAMA		LS94	**pa**
LMS6	231	231	240	270	281	283	BRAZIL		M6426	**l**
LMS7	227	245	X	X	X	X	BRAZIL		IM2832	*
LMS28	227	227	250	252	313	313	Jinotega	Bocay	XD28	**b**
LMS29	227	227	250	250	307	307	Jinotega	Bocay	*HN196*	
LMS30	227	227	254	254	297	299	Zelaya Norte	Leymus	*XD19*	
LMS31	245	245	250	252	291	291	Ocotal	Murra	*AZ01*	
LMS32	227	241	250	250	291	307	Jinotega	Pantasma	XD36	**h**
LMS33	227	227	250	250	291	309	Zelaya Norte	Waslala	NICA2	
LMS34	227	241	250	250	291	309	Jinotega	Bocay	XD05, XD09	
							Zelaya Norte	Wamblan	XD13	
								Waslala	XD20	
LMS35	227	241	250	250	291	313	Jinotega	Pantasma	XD42	
LMS36	241	241	244	244	291	291	Zelaya Norte	Guayabo	WSL03	**pa**
LMS37	241	241	244	244	295	295	Matagalpa	El Coyolar	ZF01	
LMS38	241	241	244	244	299	299	Zelaya Norte	Waslala	WSL06	
LMS39	245	245	244	244	291	291	Zelaya Sur Matagalpa	Nova Guinea Paiwas	ZE09 *XD39*	
LMS40	227	241	250	250	291	309	Rio San Juan	Azucenas	XD03	
LMS41	X	X	250	252	F	F	Chontales	Los Chiles	XD45	
not assigned±	X	X	X	X	X	X	Ocotal	Murra	XD17	**b**
							Matagalpa	Rio Blanco	XD41	**h**

5.6.4 AC01 Sequencing

Of the 17 Nicaraguan stocks which generated a PCR product for AC01, all produced readable AC01 sequence data. *Figure 5.6.4.1* shows the alignment of sequences from the AC01 microsatellite locus for Nicaraguan stocks and reference strains.

5.6.4.1 AC01 sequence variation among Nicaraguan stocks

L. V. braziliensis and *L. V. panamensis* stocks could be separated based on the size of the repeat (8 : 14-17 repeats respectively), by two bases changes at positions 25 and 28 ([TTGT : CTGC]), by two bases changes at positions 135/6 ([TT : CG]) and by a single base change at position 144 ([T : A]), respectively. These differences appear to be species or complex specific. The [C] at position 25 was observed in all *L. V. panamensis* stocks examined but not in the *L. V. guyanensis* or *L. V. shawi* reference strains. *L. V. panamensis*, like *L. V. guyanensis*, did not have the 4 base insert immediately prior to the repeat seen in *L. V. shawi* stocks. One of the Brazilian stocks identified as *L. V. shawi* (M11367) did not have this insert either and, comparing sequence data (see *figure 4.5.4.1*), appears to be more similar to *L. V. panamensis* stocks (see section 5.7).

Among *L. V. panamensis* stocks, 6 sequence types were scored. Differences in the number of repeats were evident in these stocks: 3 stocks (ZF01, WSL06 and WSL03) had n = 15 and 3 (ZE09, XD03 and XD39) had n = 17. Stocks sharing the same number of repeats appeared also to have shared base types at positions 115 and 123. Four AC01 sequence types were scored among Nicaraguan *L. V. braziliensis* stocks: all bar one derived from very minor differences (single base changes or bases scored as heterozygous) in the downstream flanking region. One *L. V. braziliensis* stock (AZ01) was identical to *L. V. panamensis* at all the discriminatory positions noted above and in the number of repeats (n = 17): this stock will be discussed further in section 5.7. As discussed in chapters 3 and 4, the sequence upstream of the repeat itself, excluding the differences noted above, was conserved. All *L. V. braziliensis* stocks (bar AZ01) were identical in this region, as were *L. V. panamensis* stocks.

Figure 5.6.4.1 **Sequence alignment of the AC01 locus from Nicaraguan stocks and *Viannia* reference strains.**

Species §	Strain/stock* code no.	10	20	30	40	50	60	70	80
b	**LTB300 type**	ATGTGCCTCT	CCCACCCTTA	GTGCTTGTCT	TCTTCCTGCT	TTGCCTCTCT	GTC----TGT	GTGTGTGTGT	GTG-------
b	XD19	.....	.....	.....	.....	.....	...----...	.....	...-------
b	HN196	.....	.....	.....	.....	.....	...----...	.....	...-------
h	NICA2 type	.....	.....	.....	.....	.....	...----...	.....	...-------
h	XD13	.....	.....	.....	.....	.....	...----...	.....	...-------
h	XD36	.....	.....	.....	.....	.....	...----...	.....	...-------
h	XD09	.....	.....	.....	.....	.....	...----...	.....	...-------
h	XD42	.....	.....	.....	.....	.....	...----...	.....	...-------
h	XD20	.....	.....	.....	.....	.....	...----...	.....	...-------
pa	**LS94**	.....	.....	....C..C..	.....	.....	...----...	.....	...TGTGTGT
pa	ZF01	.....	.....	....C..C..	.....	.....	...----...	.....	...TGTGTGT
pa	WSL06	.....	.....	....C..C..	.....	.....	...----...	.....	...TGTGTGT
pa	WSL03	.....	.....	....C..C..	.....	.....	...----...	.....	...TGTGTGT
pa	ZE09	.....	.....	....C..C..	.....	.....	...----...	.....	...TGTGTGT
b	AZ01	.....	.....	....C..C..	.....	.....	...----...	.....	...TGTGTGT
pa	XD03	.....	.....	....C..C..	.....	.....	...----...	.....	...TGTGTGT
pa	XD39	.....	.....	....C..C..	.....	.....	...----...	.....	...TGTGTGT
g	**M4147**	.....	.....	.....C..	.....	C	...----...	.....	...TGTGTGT
s	**M15065**	.....	.....	.....C..	.....	.....	...TGTC...	.....	...TGTGTCT
pe	**LC39**	.....	.....	.....	.....	.....	...----...	.....	...TGTG---
l	**M6426**	.....	.....	..A....C..	C	......T.GC	C..----...	.....	...TGTG ---

Notes to *figure 5.6.4.1*: sequence alignment of the AC01 locus from Nicaraguan stocks and *Viannia* reference strains. §Species abbreviation: b - *braziliensis*; pa – panamensis, h - putative *braziliensis* / *panamensis* hybrid, g - *guyanensis*, s - *shawi*, pe - *peruviana*, l - *lainsoni*. Species designation determined using IEA. Stock type: LTB300 type (LTB300 and XD28); NICA2 type (NICA2 and XD05). Reference strains are indicated in **bold** type. Bases in blue type form the microsatellite repeat region; bases in red type illustrate the TCCGC end motif; bases in green type are invariant downstream in all stocks. Numbers above sequence data indicate the base position used for alignment purposes. Only 165 bases of the total AC01 product were aligned and used for phylogenetic analysis (see chapter 3). See *Appendix IV* for base coding system.

Figure 5.6.4.1 **Sequence alignment of the AC01 locus from Nicaraguan stocks and *Viannia* reference strains (continued).**

Species §	Strain/stock* code no	90 100 110 120 130 140 150 160 165
		| | | | | | | | |
b	**LTB300 type**	---------- -TCCGCTTCA GTGGGCCGAT CCGTTTCACT TTTTGCCGGT GACGTTTGTG TGCTGACGTG TCTGGTGGCC TCWCA
b	XD19	---------- -......... ....A..
b	HN196	---------- -.....Y... ....Y..... ....W...... ....
h	NICA2 type	---------- -......... .... A......... ....A..
h	XD13	---------- -......... .... A......... ....
h	XD36	---------- -......... ....W....... A......... ....
h	XD09	---------- -.....Y... ....W....... A......... ....
h	XD42	---------- -.....Y..W ....K..... ..W....... M......... .... .S........ ..T..
h	XD20	---------- -........W ....S. ..W....... M......... .... .S.......M ..T..
pa	**LS94**	GTGTG----- -.....YW.. ......M... ....Y..... ..K..SS..W ....CG.... ...A..Y... ..K..K.... ..A..
pa	ZF01	GTGTGTG--- -.....Y... ......M... ....Y..... ..K......W ....CG.... ...A...... ..K..K.... W.A..
pa	WSL06	GTGTGTG--- -.....Y... ......M... ....Y..... ..K......W ....CG.... ...A...... ..K..K.... W.A..
pa	WSL03	GTGTGTG--- -.....CW.. ......M... ....Y..... ..K...S..W ....CG.... ...A...... ..G..K.... W.A..
pa	ZE09	GTGTGTGTGT G.....CW.. ......M... ....C..... ..G...S..W ....CG.... ...A..Y... ..K..K.... A.A..
b	AZ01	GTGTGTGTGT G.....CW.. ......M..Y ....CW..... .G..SG..A ....CG.... ...A..Y... .SG..K.... A.A..
pa	XD03	GTGTGTGTGT G.....YW.. ......M... ....CW.... ..G...S..W S...CG.... ...A..Y... ..G....... A.A..
pa	XD39	GTGTGTGTGT G.....YW.. .K...SA..Y ....C..... ..G..SS..W S...CG.... ...A..Y... .SG..K.S.. A.A..
g	**M4147**	GTGYGYS--- -.S...CA.. .K...SA..Y S...CW.... ..GY.SS..A S...CG.... ..SA..Y... .SG..K.... W.A..
s	**M15065**	GCG------- -......... ....K....... ....CK.... ...W...... ....A..
pe	**LC39**	---------- -......... ....K.... ...W...... ..K....... W.A..
l	**M6426**	---------- -......... ....T. ..A....... ...C...... ....W...... ....A..

Notes to *figure 5.6.4.1*: sequence alignment of the AC01 locus from Nicaraguan stocks and *Viannia* reference strains. §Species abbreviation: b - *braziliensis*, pa – panamensis, h - putative *braziliensis* / *panamensis* hybrid, g - *guyanensis*, s - *shawi*, pe - *peruviana*, l - *lainsoni*. Species designation determined using IEA. Stock type: LTB300 type (LTB300 and XD28); NICA2 type (NICA2 and XD05). Reference strains are indicated in **bold** type. Bases in blue type form the microsatellite repeat region; bases in red type illustrate the TCCGC end motif; bases in green type are invariant downstream in all stocks. Numbers above sequence data indicate the base position used for alignment purposes. Only 165 bases of the total AC01 product were aligned and used for phylogenetic analysis (see chapter 3).
See *Appendix IV* for base coding system.

Genotyper® data for the putative *L. V. braziliensis* / *L. V. panamensis* hybrid stocks at this locus indicated that they were heterozygous. Sequencing analysis showed however, that they were identical to *L. V. braziliensis* stocks in the upstream flanking region and in the number of dinucleotide repeats scored (n = 8). The downstream flanking region was generally more variable in hybrid stocks than in *L. V. braziliensis* stocks, with 6 sequence types scored from 7 stocks. As discussed previously, it is likely that sequencing analysis only detected the *L. V. braziliensis* allele in these stocks because of the relatively poor amplification of the *L. V. panamensis* allele.

Relationships among the Nicaraguan stocks based on AC01 sequence comparisons will be discussed in section 5.7.

5.6.4.2 Comparison of AC01 data from sequencing and Genotyper®

Sequence data correlated exactly with Genotyper® data for all homozygous *L. V. braziliensis* and *L. V. panamensis* stocks, and for the stock AZ01 which appears to have isoenzyme loci corresponding to *L. V. braziliensis* and microsatellite loci corresponding to *L. V. panamensis* (see section 5.7). Data from putative hybrid stocks (genotype 227/241) did not concur for reasons discussed previously.

The Genotyper® result for the stock XD03 at AC01 was identical to most other hybrid stocks i.e. having 2 alleles, genotype 227 / 241. The AC01 sequence of this stock, however, indicated that this stock was equivalent to *L. V. panamensis* stocks with the AC01 allele 245 (i.e. n = 17). XD03 was identified as *L. V. panamensis* using IEA. Sequencing and Genotyper® analyses of all Nicaraguan stocks were repeated and generated the same results. The observed discrepancy between sequencing and Genotyper® results is difficult to explain. All other stocks of AC01 genotype 227/241 had n = 8 (the *L. V. panamensis* AC01 allele 241 is assumed to have been "out-amplified" during cycle sequencing, see previous sections). Here, only sequence from the *L. V. panamensis* allele is scored and this is not of the expected size (245 instead of 241). The most plausible explanation is that DNA samples were repeatedly mixed up, either for Genotyper® or sequencing analysis: the phylogenetic position of this stock using microsatellite analysis (section 5.7) has been disregarded here.

5.7 Phylogenetic analysis of Nicaraguan stocks

Phylogenetic analysis of isoenzyme data (multilocus data) included data from all *Leishmania* reference strains. Phylogenetic analysis of sequence data from the microsatellite locus AC01 included data from all reference strains of the *Viannia* subgenus, with the exception of *L. V.* sp. n. reference strain IM2832 which failed to produce readable sequence data. The AC01 microsatellite locus is specific to the *Viannia* subgenus hence it was not possible to include reference strains from the subgenus *Leishmania* in this phylogenetic analysis.

5.7.1 Phylogenetic analysis of isoenzyme data

Isoenzyme phylogenies were constructed, as described in chapter 2, using a maximum-likelihood approach, quartet puzzling (Strimmer & von Hassler, 1996). *Figure 5.7.1.1* shows the consensus cladogram constructed after quartet puzzling analysis of isoenzyme data from *Leishmania* reference strains and from Nicaraguan stocks. The latter spanned 2 species (*L. V. braziliensis* and *L. V. panamensis*) of the 2 main *Viannia* subgenus complexes (*L. V. braziliensis* and *L. V. guyanensis* complexes, respectively), and included putative *L. V. braziliensis* / *L. V. panamensis* (i.e. species-complex) hybrids.

The *Leishmania* subgenus reference strains (PP75, *L. L. chagasi*, BEL21, *L. L. mexicana* and M2269, *L. L. amazonensis*) plus the two outlying *Viannia* reference strains M6426 (*L. V. lainsoni*) and IM2832 (*L. V.* sp. n., Grimaldi *et al.*, 1991) were clearly distinct (78% quartet support) from species in the 2 main species complexes of the *Viannia* subgenus.

The 2 main *Viannia* species complexes (*L. V. braziliensis* and *L. V. guyanensis* complexes) were clearly separated from the other reference strains (63% and 88% quartet support, respectively). Three clades are evident in *figure 5.7.1.1*. The first clade (88% quartet support) contains reference strains and Nicaraguan stocks belonging to the *L. V. guyanensis* complex (3 zymodemes: LON203 - *L. V. shawi*; LON 123 - *L. V. guyanensis* and LON202 - *L. V. panamensis*). Within *the L. V. guyanensis* complex, *L. V. panamensis* stocks were distinct from *both L. V. guyanensis* and *L. V. shawi* reference strains (53% quartet support), forming a discrete cluster. The second clade (100% quartet support) contains all Nicaraguan

***Figure 5.7.1.1*: Cladogram showing the results of quartet puzzling for Nicaraguan stocks and reference strains based on zymodemes.**

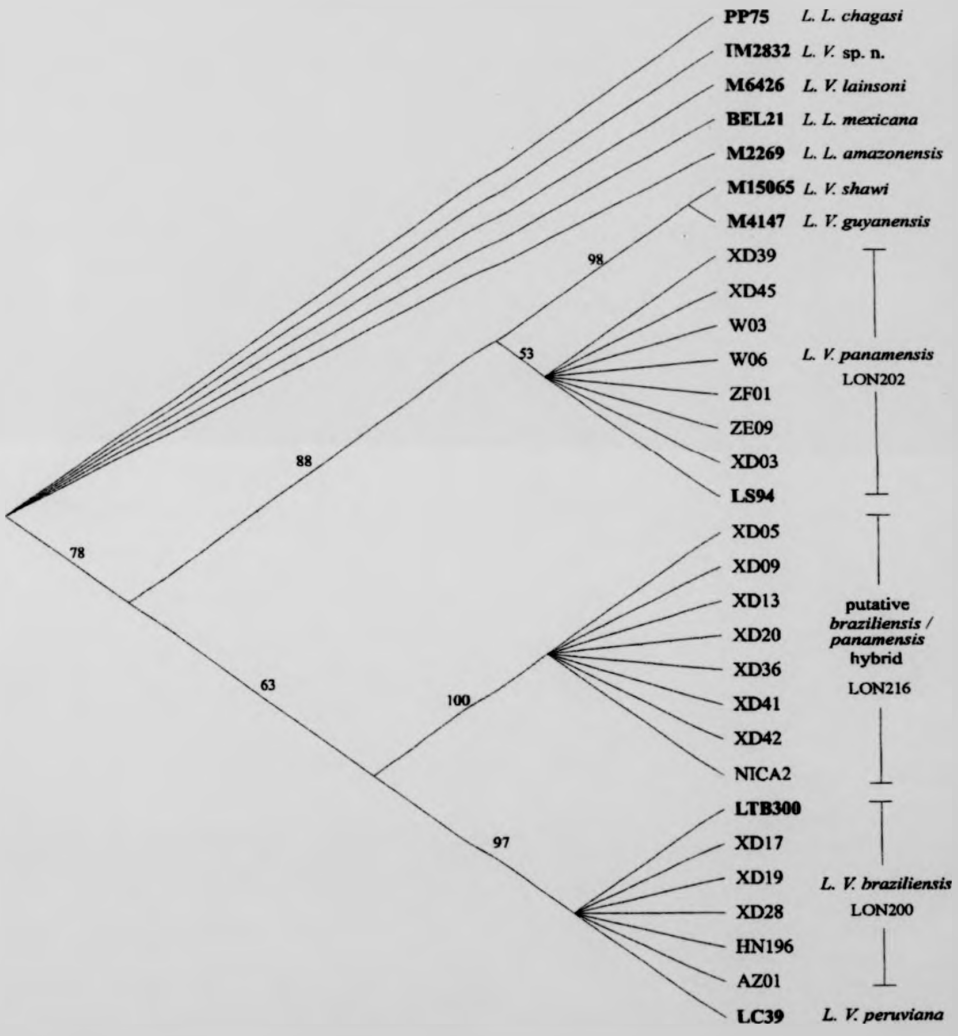


Figure 5.7.1.1: Cladogram showing the results of quartet puzzling for Nicaraguan stocks and reference strains based on zymodemes. Values on branches are "quartet supports", figures which are similar to bootstraps (Strimmer & von Hassler, 1996). These values are the percentage support of each branch, representing the number of quartets supporting each given branching order. Branch lengths are arbitrary. Zymodemes: LON202 - *L. V. panamensis*, LON200 - *L. V. braziliensis*, LON216 - putative *L. V. braziliensis / L. V. panamensis* hybrids.

stocks identified as putative *L. V. braziliensis / L. V. panamensis* hybrids (single zymodeme, LON216). The third clade (97% quartet support) contains reference strains and stocks belonging to the *L. V. braziliensis* complex (2 zymodemes: LON200 - *L. V. braziliensis* and LON201 – *L. V. peruviana*).

The Nicaraguan stocks within each cluster showed a remarkable lack of isoenzyme variation with a single zymodeme observed in each. The putative hybrid stocks clustered slightly closer to *L. V. braziliensis* stocks than to *L. V. panamensis* stocks.

As with the Brazilian stocks, isoenzyme data from the Nicaraguan stocks was also analyzed using a similarity approach. Dendrograms were constructed by UPGMA using the SYN-TAX-pc package (Podani, 1993). *Figure 5.7.1.2* shows the dendrogram constructed for these stocks from a similarity coefficient matrix. Stocks are classified according to similarity (range 0 - 1).

The clustering observed using this method concurs with that from quartet puzzling. The *Viannia* and *Leishmania* subgenera are clearly defined with all reference strains clustering in the correct subgenus (c.f. *figure 4.6.1.2*). As with the cladogram generated by quartet puzzling, the *L. V. braziliensis* and *L. V. guyanensis* species complexes are also clearly separated. Similarly, the putative *L. V. braziliensis / L. V. panamensis* hybrids share greater similarity with members of the *L. V. braziliensis* complex.

5.7.2 Phylogenetic analysis of sequence data from AC01

Molecular phylogenies for sequence data from the AC01 microsatellite locus were constructed as previously described. *Figure 5.7.2.1* shows an unrooted phylogram of the neighbour-joining consensus tree for the Nicaraguan stocks and reference strains based on sequence data from the microsatellite locus AC01. The reliability of tree topologies was assessed by the bootstrap method (n = 1000). Only branches with greater than 50% bootstrap support are indicated. Note the trifurcating layout and the different scale in this figure compared to *figure 4.6.2.1.*

The IEA and AC01 sequence phylogenies generated from data for Nicaraguan stocks are generally concordant, with the notable exceptions of the Nicaraguan hybrid stocks and AZ01, which are discussed in greater detail below.

Figure 5.7.1.2 **UPGMA dendrogram constructed for Nicaraguan stocks and reference strains based on isoenzyme data.**

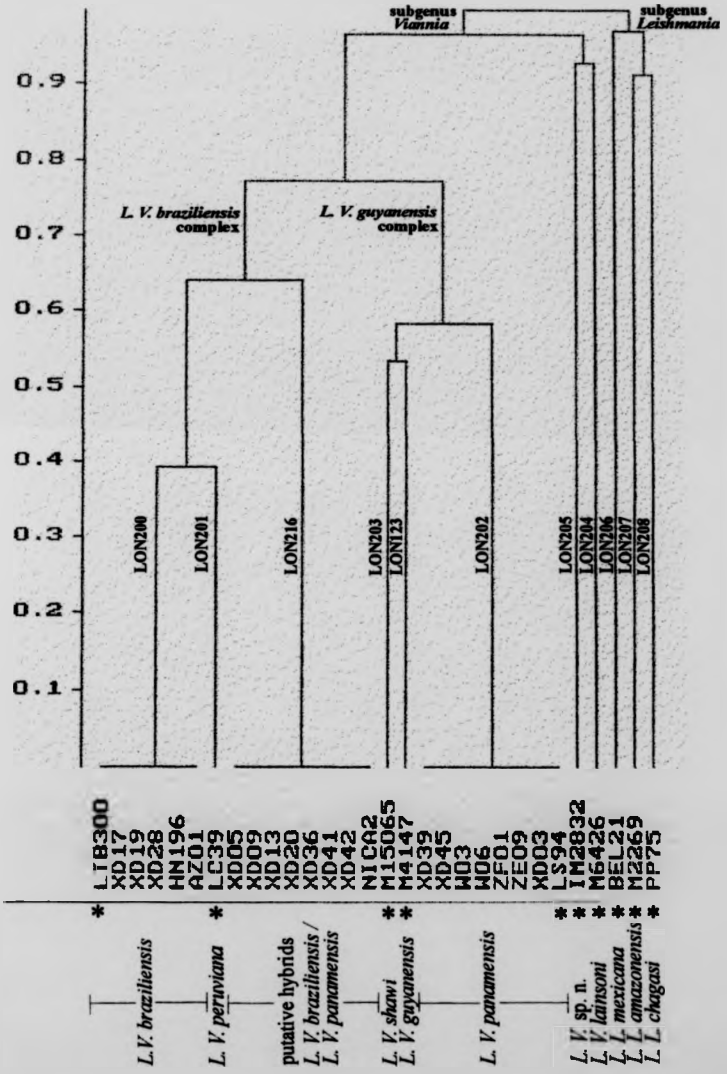


Figure 5.7.1.2: UPGMA dendrogram constructed from the similarity coefficient matrix for Nicaraguan stocks and reference strains based on isoenzyme data. Similarities between zymodemes (y-axis, range 0-1) were calculated, using SYN-TAX-pc (Podani, 1993), by averaging the Jaccard coefficients calculated from data from twelve enzyme loci. Stocks / strains marked with * are reference strains. Zymodemes (LON numbers) are indicated on branches.

***Figure 5.7.2.1*:** **Phylogram based on sequence data from the microsatellite locus AC01 for the Nicaraguan stocks and reference strains.**

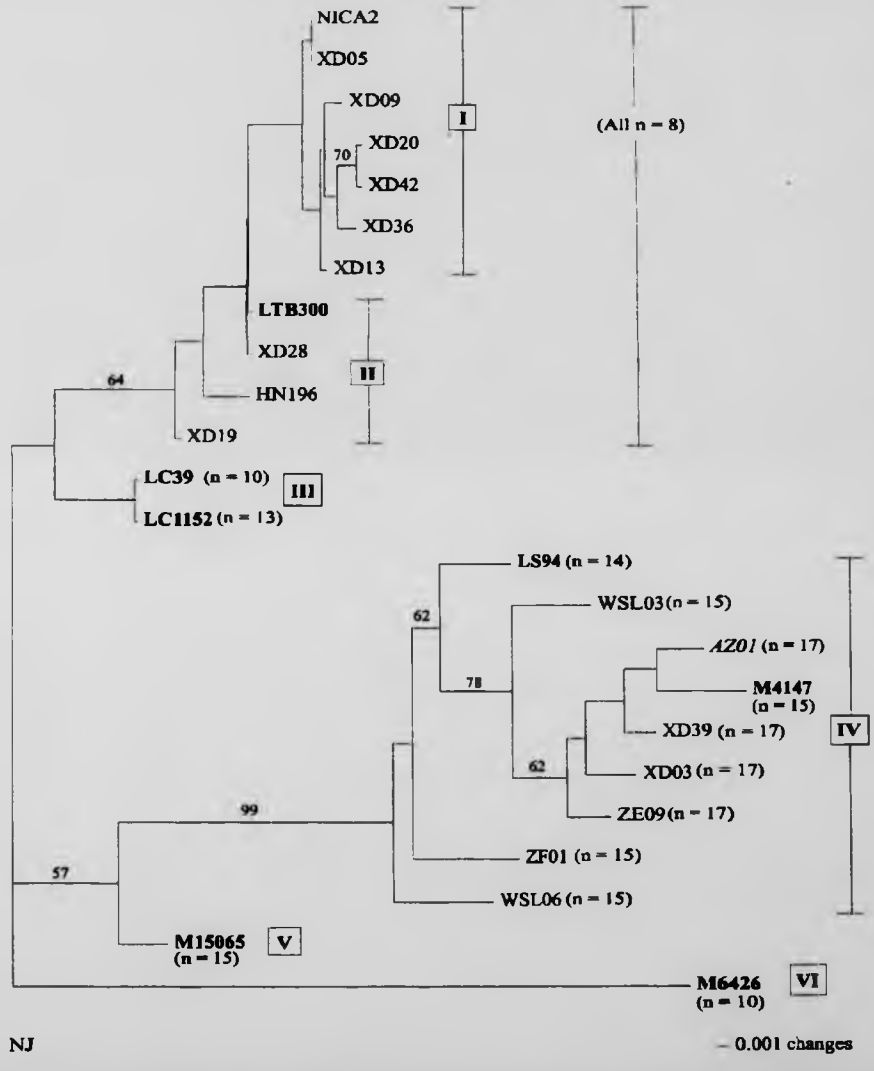


Figure 5.7.2.1: unrooted phylogram of the neighbour-joining consensus tree for the Nicaraguan stocks and reference strains based on sequence data from the microsatellite locus AC01. Numbers indicate the percentage of bootstrap support for each branch. The scale bar represents 0.001 (0.1%) nucleotide changes per sequence position. Numbers in parentheses indicate the number of dinucleotide repeats (n) scored (for *L. V. guyanensis* and *L. V. shawi* stocks the repeats are not perfect). Clusters **I** and **II** stocks / strains are *L. V. braziliensis*, cluster **III** stocks / strains are *L. V. peruviana*, cluster **IV** stocks / strains are, with the exception of M4147 (*L. V. guyanensis* reference strain) *L. V. panamensis*, **V** is *L. V. shawi* and **VI** is *L. V. lainsoni* (both reference strains).

In addition to the *L. V. lainsoni* reference strain M6426 (**VI**), used here as an outgroup, 2 major subdivisions are evident, corresponding to the *L. V. braziliensis* complex (clusters **I** – **III**) and the *L. V. guyanensis* complex (clusters **IV** and **V**).

Among the *L. V. braziliensis* complex, three groups can be seen. **I** contains Nicaraguan stocks identified by IEA as putative *L. V. braziliensis* / *L. V. panamensis* hybrids. **II** includes Nicaraguan stocks identified as *L. V. braziliensis* and the *braziliensis* reference strain LTB300. **III** contains 2 *L. V. peruviana* reference strains: LC39 (n = 10) and LC1152 (n = 13). In the AC01 phylogenetic analysis of Brazilian stocks, these strains were oddly placed in different species complex clusters; it is possible in the previous analysis that a mistake was made during data entry for the phylogenetic analysis which resulted in the misplacing of LC1152.

Cluster **IV** contains Nicaraguan *L. V. panamensis* stocks and the *L. V. panamensis* and *L. V. guyanensis* reference strains (LS94 and M4147, respectively). Nicaraguan *L. V. panamensis* stocks could, by AC01 sequence analysis, be split into 2 groups based on the number of dinucleotide repeats and variation in the downstream flanking region (see *figure 5.6.4.1*). *L. V. panamensis* stocks with n = 17 (ZE09, XD03 and XD39), along with the *L. V. guyanensis* reference strain M4147 and the Nicaraguan stock AZ01, formed a discrete group (62% bootstrap support) within cluster **IV**.

The stock AZ01 was identified as *L. V. braziliensis* using IEA. This stock was, however, found to have 17 dinucleotide repeats using sequence analysis and an allele size of 245 using Genotyper®, both indicative of *L. V. panamensis* status. The most parsimonious explanation for this stock is the occurrence of a recombination event between *braziliensis* and *panamensis* stocks resulting in the exchange of IEA and AC01 markers. A similar event appears to have occurred in the stock NICA2, which was identified as a putative hybrid using IEA, but scored as *braziliensis* by both sequence and Genotyper® analyses (n = 8 and an allele size of 227). This stock therefore appears to be heterozygous at enzyme loci but homozygous for AC01, possibly suggesting that partial recombination, involving the enzyme loci only, has occurred.

All strains / stocks in groups **I** and **II** were shown, using sequence analysis, to have 8 dinucleotide repeats. As discussed in sections 5.6.3.1 and 5.6.4.1, results for the

putative hybrid stocks from sequence and Genotyper® analyses were not in agreement. Genotyper® analysis suggested the presence of 2 alleles (227 and 241) in these hybrid stocks, corresponding to alleles from both *L. V. braziliensis* and *L. V. panamensis*. The phylogenetic position of these stocks should be treated with caution until the discrepancies between sequence and Genotyper® analyses have been resolved. This also applies to the phylogenetic position of the *L. V. panamensis* stock, XD03 (heterozygous 227 / 241 using Genotyper® but 17 dinucleotide repeats by sequence analysis), as previously discussed.

5.8 Discussion

Very little diversity was observed among the Nicaraguan stocks using IEA: just 3 zymodemes were scored, corresponding to *L. V. braziliensis*, *L. V. panamensis* and putative *braziliensis / panamensis* hybrids. In agreement with results from Brazilian stocks, microsatellite analysis revealed greater diversity than IEA. However, the two methods used for microsatellite analysis (sequencing and Genotyper®) were not always in agreement, especially for the putative hybrid stocks. This made phylogenetic analyses difficult to interpret. These discordant results need to be investigated further and resolved. .

Nevertheless, using both IEA and AC01 microsatellite analysis (Genotyper® data), the patterns observed in the putative *L. V. braziliensis / L. V. panamensis* hybrid stocks concur with a juxtaposition of the parental patterns. Although these stocks were not cloned, the IEA results cannot be explained by a mixture of genotypes. These observations, assuming that *Leishmania* are diploid and that these markers are inherited in a Mendelian fashion, suggest that these hybrids may be the equivalent of an F1 generation resulting from a total recombination event between the parental species types. The precise mechanism involved in generating these hybrids remains, in the absence of experimental genetic exchange, unclear. Banuls *et al.* (1999) have recently examined the Nicaraguan hybrids using IEA and RAPD. These authors also raised the possibility that these hybrids may, in fact, represent an ancestral type from which the parental types descended, but suggested that this explanation was less parsimonious than genetic recombination.

CHAPTER 6 - HUÁNUCO, PERU: SYMPATRIC TRANSMISSION OF *L. V. BRAZILIENSIS* AND *L. V. PERUVIANA*

6.0 Leishmaniasis in Peru

Cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) are endemic in Peru. *Leishmania Viannia braziliensis* and *L. V. peruviana* are the most frequently reported species causing CL (Desjeux, 1992b; Lucas *et al.*, 1994, 1998) however, CL due to infection with *L. V. guyanensis*, *L. V. lainsoni* and *L. Leishmania amazonensis* have also been reported (Lucas *et al.*, 1994, 1998; Franke *et al.*, 1990). Mucosal leishmaniasis (ML), with mucosal involvement by contiguity, has been described for all species causing CL in Peru (Lucas *et al.*, 1998). ML is, however, distinct from MCL which involves the metastatic spread of parasites to mucosal sites: MCL is principally associated with *L. V. braziliensis* infection (Lucas *et al.*, 1998). DCL has also been reported in Peru (Lucas *et al.*, 1998) but VL is absent (Desjeux, 1992a).

L. V. braziliensis and *L. V. peruviana* have traditionally been associated with different clinical forms of disease. *L. V. braziliensis* is regarded as the most notorious species since infection may, in addition to CL, result in MCL ("espundia") (Romero *et al.*, 1987). CL resulting from *L. V. peruviana* infection, also known as "uta" or Andean CL (ACL), generally presents as relatively benign, self-limiting, cutaneous lesions. CL caused by *L. V. braziliensis* infection presents a more variable clinical picture ranging from uta-like lesions to multiple aggressive lesions which require therapeutic intervention (Llanos-Cuentas, 1993). However, different grades of lesion severity have been described for both species (Llanos-Cuentas, personal comm.) and clinical examination alone cannot pinpoint accurately the infecting species causing CL in a region where both species occur sympatrically.

Leishmania species have often been incriminated as the cause of CL on the basis of known geographical range. *L. V. peruviana* is found primarily between 800 - 3000m a.s.l. in the western Andes and inter-Andean valleys where it is associated with rural agricultural practices (Guerra, 1988). *L. V. braziliensis* is found predominantly at lower altitudes in Amazonian areas and is associated with activities in forested areas (Guerra, 1988).

There are regions, however, as in the present study area in the unforested Andean Department of Huánuco (Peru), where the Andean and forest regions meet and in which *L. V. peruviana* and *L. V. braziliensis* exist sympatrically.

L. V. braziliensis and *L. V. peruviana* are remarkably similar and the subtlety of discriminatory power available until relatively recently has impeded the study of genetic diversity amongst them. The two species can be differentiated biochemically using isoenzyme analysis (IEA) (Arana *et al.*, 1990), random amplified polymorphic DNA (RAPD) (Dujardin *et al.*, 1995b, Banuls *et al.*, 2000), PCR-RFLP of the *gp63* gene intergenic region (Victoir *et al.*, 1998), molecular karyotyping (Dujardin *et al.*, 1993b; Dujardin *et al.*, 1995b) and microsatellite analysis (Russell *et al.*, 1999).

6.1 Study site

Prior to 1983, sporadic cases of cutaneous leishmaniasis were reported from valleys in the Department of Huánuco, Peru (Llanos-Cuentas, 1993). A concurrent CL and MCL epidemic has occurred in the region since the mid 1980's. *Leishmania* stocks were isolated from humans and dogs as part of a study entitled "The epidemiology of *Leishmania braziliensis* in the unforested eastern Andean highlands of Huánuco Department, Peru" (Davies, C.R. & Llanos-Cuentas, E.A. ISC grant: contract no. C11-CT93-0036). *Figure 6.1* shows a map of the study site, which is situated at an altitude of 2000-3000m. a.s.l., an altitude commonly associated with uta. The study site encompassed rural and peri-urban villages around Huánuco City and from the neighbouring province of Ambo, an area circa 40km in diameter, in three valleys: Higueras, Quera and Chinobamba. Two of the valleys, Quera and Higueras, are relatively dry, with mostly xerophytic vegetation, although some irrigation is *in situ* for cultivation purposes. The third valley, Chinobamba, is more moist: the land here is largely employed in crop cultivation (coffee, potatoes and maize). Most adults of all three valleys are employed in agricultural practices; the extent of visits to forested areas is difficult to ascertain. A full demographic and clinical census of the populations inhabiting villages and peri-urban sites around Huánuco City was carried out as part of this study. Households in these areas routinely keep dogs, donkeys, pigs and chickens. *Leishmania* transmission appears to be domestic (C. Davies, personal comm.). The principal sand fly vector in the area is suspected to be *Lutzomyia (Helcocyrtomyia) tejadai*, an anthropophilic species found both inside and outside houses (C. Davies, personal comm.).

Figure 6.1: **Map of Huanuco study sites, Huanuco Department, Peru.**

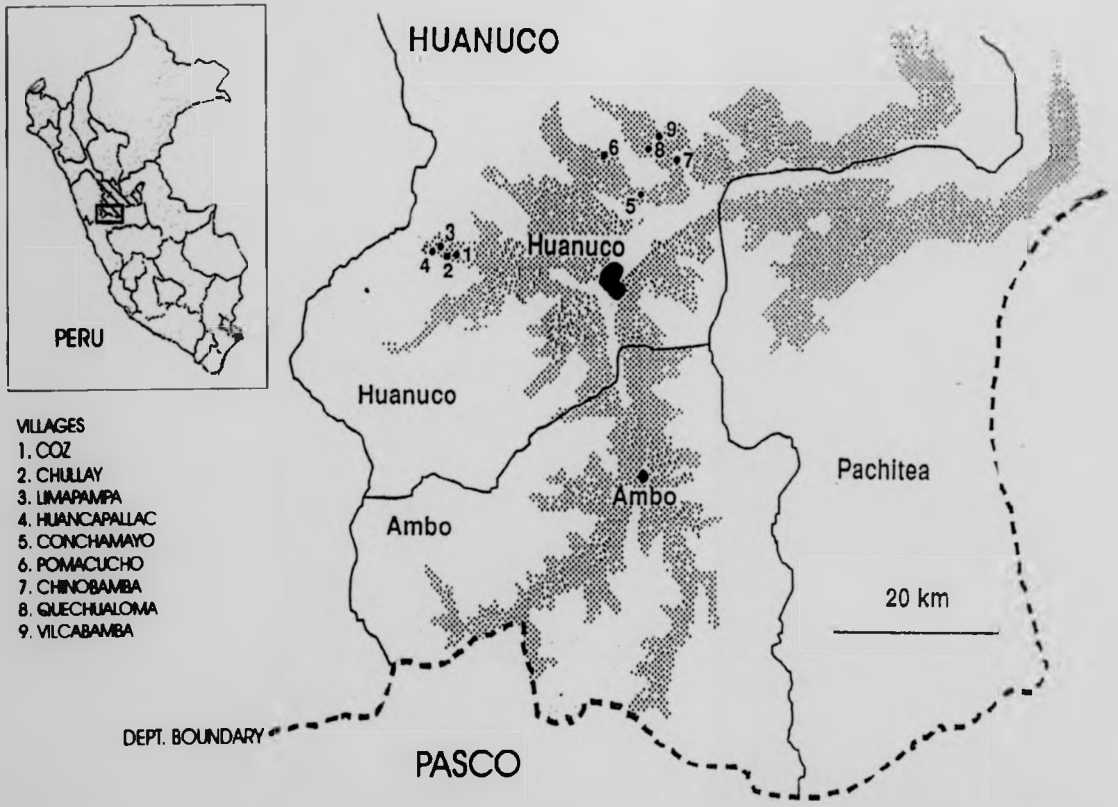


Figure 6.1: Map of Huanuco study site. Shaded areas are situated 2000-3000 above sea-level. Map of Peru (inset) shows location of Huanuco Department. (Courtesy of Clive Davies).

6.2 *Leishmania* reference strains and Huánuco stocks

6.2.1 *Leishmania* reference strains

The reference strains used for comparison include those listed in *table 3.1* (Chapter 3), plus five additional *L. V. peruviana* stocks, two isolated from humans (MHOM/PE/94/LC1152; MHOM/PE/84/LC26) and three isolated from canine lesions (MCAN/PE/92/LRP709; MCAN/PE/92/LRP737; MCAN/PE/92/LRP906). These additional reference strains all originate from the Department of Ancash, Peru.

6.2.2 Huánuco stocks

Fifty-nine *Leishmania* stocks were isolated: 45 from humans and 14 from dogs. These stocks have not been characterized previously, although seven other stocks from the region have been analyzed by IEA, RAPD and molecular karyotyping (Dujardin *et al.*, 1995b). The Huánuco stocks are listed in *table 6.2.2.*

Table 6.2.2 ***Leishmania*** **stocks from Huánuco, Peru.**

STOCK CODE NO.	HOST SPECIES	VALLEY / DISTRICT / VILLAGE	LESION TYPE
MCAN/PE/95/HR78	Dog	Quera / El Valle / Conchumayo	CL
MCAN/PE/95/HR80	Dog	Quera / El Valle / Conchumayo	CL
MCAN/PE/95/HR108	Dog	Quera / El Valle / Conchumayo	CL
MCAN/PE/95/HR110	Dog	Quera / El Valle / Conchumayo	CL
MHOM/PE/94/LC2421	Human	Quera / El Valle / Conchumayo	MCL
MHOM/PE/94/LC2422	Human	Quera / El Valle / Conchumayo	CL
MHOM/PE/94/LC2412	Human	Quera / El Valle / Pomacucho	CL
MHOM/PE/94/LC2452	Human	Quera / El Valle / Huayrajirca	CL
MHOM/PE/94/LC2679	Human	Quera / El Valle / Huaycho	CL
MHOM/PE/95/LC2829	Human	Quera / El Valle / Mirache	MCL
MHOM/PE/95/LC2898	Human	Quera / El Valle / Pachabamba	CL
MHOM/PE/95/LC2900	Human	Quera / El Valle / Naosa	MCL
MHOM/PE/95/LC2875	Human	Chinobamba / Churubamba / Churubamba	CL
MCAN/PE/95/HR529	Dog	Chinobamba / Churubamba / Quechaloma	CL
MHOM/PE/94/LC2434	Human	Chinobamba / Churubamba / Quechaloma	CL
MHOM/PE/94/LC2450	Human	Chinobamba / Churubamba / Quechaloma	CL
MHOM/PE/94/LC2683	Human	Chinobamba / Churubamba / Quechaloma	CL
MCAN/PE/95/HR701	Dog	Chinobamba / Churubamba / Vilcabamba	CL
MHOM/PE/95/LC2901	Human	Chinobamba / Churubamba / Vilcabamba	MCL
MHOM/PE/94/LC2435	Human	Chinobamba / Churubamba / Vilcabamba	CL
MCAN/PE/95/HR399	Dog	Chinobamba / Churubamba / Chinobamba	CL
MCAN/PE/95/HR410	Dog	Chinobamba / Churubamba / Chinobamba	CL
MCAN/PE/95/HR413	Dog	Chinobamba / Churubamba / Chinobamba	CL
MCAN/PE/95/HR419	Dog	Chinobamba / Churubamba / Chinobamba	CL
MCAN/PE/95/HR424	Dog	Chinobamba / Churubamba / Chinobamba	CL
MCAN/PE/95/HR434	Dog	Chinobamba / Churubamba / Chinobamba	CL
MCAN/PE/95/HR797	Dog	Chinobamba / not determined	CL
MCAN/PE/95/HR799	Dog	Chinobamba / not determined	CL
MHOM/PE/94/LC2484	Human	Chinobamba / Churubamba / Chinobamba	MCL
MHOM/PE/94/LC2485	Human	Chinobamba / Churubamba / Chinobamba	MCL
MHOM/PE/94/LC2487	Human	Chinobamba / Churubamba / Chinobamba	CL
MHOM/PE/94/LC2489	Human	Chinobamba / Churubamba / Chinobamba	CL

Table 6.2.2 ***Leishmania*** **stocks from Huánuco, Peru (continued).**

STOCK CODE NO.	HOST SPECIES	VALLEY / DISTRICT / VILLAGE	LESION TYPE
MHOM/PE/94/LC2511	Human	Chinobamba / Churubamba / Chinobamba	CL
MHOM/PE/94/LC2523	Human	Chinobamba / Churubamba / Chinobamba	MCL
MHOM/PE/95/LC3150	Human	Chinobamba / not determined	CL
MHOM/PE/94/LC2491	Human	Chinobamba / Churubamba / Huayucro	CL
MHOM/PE/94/LC2520	Human	Chinobamba / Churubamba / Cascay	CL
MHOM/PE/94/LC2522	Human	Chinobamba / Churubamba / Cascay	CL
MHOM/PE/94/LC2551	Human	Higueras / Kichki / Limapampa	CL
MHOM/PE/94/LC2553	Human	Higueras / Kichki / Limapampa	MCL
MHOM/PE/94/LC2558	Human	Higueras / Kichki / Limapampa	CL
MHOM/PE/94/LC2570	Human	Higueras / Kichki / Huancapallac	CL
MHOM/PE/94/LC2572	Human	Higueras / Yarumayo / Chullay	CL
MHOM/PE/94/LC2576	Human	Higueras / Yarumayo / Chullay	CL
MHOM/PE/94/LC2603	Human	Higueras / Yarumayo / Cozo	MCL
		CITY / BOROUGH / DISTRICT	
MHOM/PE/95/LC2904	Human	Huánuco City /Heroes de Jactay / Las Moras	CL
MHOM/PE/95/LC2905	Human	Huánuco City /Heroes de Jactay / Las Moras	CL
MHOM/PE/95/LC2841	Human	Huánuco City / Amarilis / San Luis	MCL
MHOM/PE/95/LC2842	Human	Huánuco City / Amarilis / San Luis	MCL
MHOM/PE/95/LC2851	Human	Huánuco City / Amarilis / San Luis	CL
MHOM/PE/95/LC2873	Human	Huánuco City / Amarilis / San Luis	CL
MHOM/PE/95/LC2902	Human	Huánuco City / Amarilis / San Luis	CL
MHOM/PE/95/LC2790	Human	Huánuco City / Amarilis / Gonzalez Prada	CL
MHOM/PE/95/LC2843	Human	Huánuco City/Amaralis / Hermilio Valdizan	CL
MHOM/PE/95/LC2876	Human	Huánuco City / Marabamba	CL
MHOM/PE/95/LC2866	Human	Huánuco City / Sariapampa	CL
		PROVINCE / DISTRICT	
MHOM/PE/95/LC2825	Human	Ambo Province / Las Pampas	CL
MHOM/PE/95/LC2844	Human	Ambo Province / Quicacan	CL
MHOM/PE/95/LC2877	Human	Ambo Province / Huacar District	CL

6.3 Isoenzyme analysis

Isoenzyme polymorphism was observed in the 59 Huánuco stocks at all enzyme loci bar NHi2. Four stocks (HR419, HR529, HR701 and LC2679) accounted for virtually all polymorphism at 6 loci: PGM, 6PGD, GPI, G6PD, ASAT and ALAT. Two stocks (LC2435 and LC2877) repeatedly lacked detectable enzyme activity at PGM, 6PGD and ALAT. The remaining 53 Huánuco stocks were polymorphic for MPI, NHi1, NHd, ES and PEPD. Banding patterns observed in the reference strains and Huánuco stocks are shown diagramatically, along with allele designations, for all enzymes in *figure 6.3.1.*

6.3.1 Polymorphic enzymes

6.3.1.1 MPI

MPI is the only enzyme that is described as consistently discriminating between *L. V. braziliensis* and *L. V. peruviana* on TSGE (Arana *et al.*, 1989). The Huánuco stocks were therefore classified initially according to their MPI profile: 6 MPI phenotypes were scored among Huánuco stocks (see *figures 6.3.1* and *6.3.1.1).*

Twenty-five stocks were identified as *L. V. braziliensis* (MPI type 1, inferred genotype 6/6) and 4 as L. V. *peruviana* (MPI type 2, inferred genotype 7/7). A double-banded pattern (MPI type 3, inferred genotype 6/7) suggestive of a *L. V. braziliensis* / *L. V. peruviana* hybrid was observed in 26 stocks. This double-banded phenotype is the expected banding pattern for a heterozygote using a monomeric enzyme such as MPI. Mixed cultures were excluded by both equal band intensities and the occurrence of the same phenotype in biological clones isolated from stocks from the same area (Dujardin *et al.* 1995b). This MPI phenotype was also observed in 2 stocks (including the widely used reference strain M2903) from Brazil: in these stocks however, polymorphism was not observed at any other locus. Three stocks presented another double-banded MPI pattern (MPI type 4, inferred genotype 6/9): the lower band in this pattern corresponded to that of *L. V. braziliensis* but the upper band did not correspond to any of the reference strains used. This MPI pattern was not observed among the 7 Huánuco samples analyzed by Dujardin *et al.* (1995b), however, this phenotype has been previously reported in stocks from Belize (Evans *et al.*, 1984). Three stocks generated banding patterns (MPI type 5, inferred genotype 5/6) identical to the *L. V.* sp. n. reference strain IM2832 and one stock was *L. V. lainsoni* (MPI type 6, inferred genotype 4/4).

***Figure 6.3.1.1*: MPI phenotypes observed in the Huánuco stocks.**

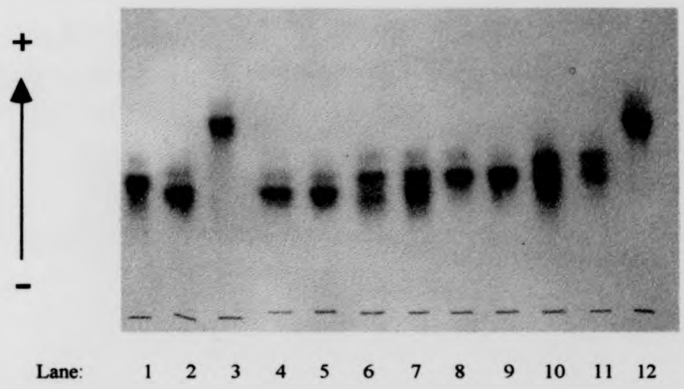


Figure 6.3.1.1: MPI phenotypes observed in the Huánuco stocks. Lanes 1-3: reference strains: lane 1: *L. V. braziliensis* (LTB300); lane 2: *L. V. peruviana* (LC39); lane 3: *L. V. lainsoni* (M6246). Lanes 4-12: Huánuco stocks: lanes 4 & 5: *L. V. peruviana* (HR78 & LC2484); lanes 6, 8 & 9: *L. V. braziliensis* (LC2422, LC2487 & LC2412); lane 7: putative *L. V. braziliensis* / *L. V. peruviana* hybrid (HR399); lanes 10 & 11: *L. V. braziliensis* variant (MPI type 4, LC2603 & LC2842); lane 12: *L. V. lainsoni* (LC2679).

6.3.1.2 NHd

The enzyme NHd was shown to differentiate between the reference strains of *L. V. braziliensis* and *L. V. peruviana* however it did not differentiate these species, as identified by MPI type, in the Huánuco stocks. Nonetheless a triple-banded hybrid *L. V. braziliensis* / *L. V. peruviana* phenotype was observed in all 26 stocks with MPI type 3 (putative *L. V. braziliensis* / *L. V. peruviana* hybrids). A triplet is the expected banding pattern for a heterozygote and a dimeric enzyme such as NHd. The presence of the more intense central band in this triplet adds further evidence to refute the possibility of mixed cultures: the latter would generate a doublet with just 2 bands corresponding to the outer bands of the triplet. Dujardin *et al.* (1995b), also reported finding a triplet pattern with NHd (their NH2) in several stocks from the region. The *L. (V) peruviana* NHd allele was not observed among the Huánuco stocks: this allele was either not sampled from the small number of *L. V. peruviana* stocks identified, or originated from elsewhere. *Figure 6.4.1.2* shows the NHd phenotypes observed among Huánuco stocks.

6.3.1.3 NHi

The enzyme system NHi consists of two loci: NHi1 (faster migration) and NHi2 (slower migration) and does not differentiate between the reference strains of *L. V. braziliensis* and *L. V. peruviana*.

Nhi2 was monomorphic for all of the Huánuco stocks bar LC2679 (*L. V. lainsoni*). Excluding the *L. V. lainsoni* stock LC2679, Huánuco stocks could be divided into 2 groups based on their Nhi1 pattern: 23 stocks generated a single band identical to that observed in *L. V. braziliensis*, *L. V. peruviana* or *L. V.* sp. n. (IM2832) reference strains; the remaining 35 stocks (6 *L. V. braziliensis* stocks and all stocks with MPI types 3 and 4) generated a poorly resolved double-banded pattern which appeared to consist of one band from *L. V. braziliensis* or *L. V. peruviana* ("allele 2") plus another band ("allele 3") similar to that produced by reference stocks from the *L. V. guyanensis* complex (see *figure 6.3.1*). *Figure 6.3.1.3* shows the NHi phenotypes observed among Huánuco stocks.

6.3.1.4 ES and PEPD

Excluding the *L. V. lainsoni* stock LC2679, Huánuco stocks could be divided into 3 groups based on their ES and PEPD profiles: group 1 - stocks identical to *L. V. braziliensis/L. V. peruviana*, group 2 - stocks identical to *L. V.* sp. n. reference strain IM2832 and group 3 - stocks sharing bands from 1 and 2.

At the PEPD locus, group 1 stocks (36) had the inferred PEPD genotype 2/2, group 2 stocks (3) had the inferred PEPD genotype 4/4 and group 3 stocks (19 stocks, i.e. all MPI type 1 stocks bar 3) had the inferred PEPD genotype 2/4.

At the ES locus, group 1 stocks (39) had the inferred PEPD genotype 4/4, group 2 stocks (3) had the inferred PEPD genotype 5/5 and group 3 stocks (16 stocks, i.e. all MPI type 1 stocks bar 3) had the inferred PEPD genotype 4/5.

***Figure 6.3.1.2*: NHd phenotypes observed among Huánuco stocks.**

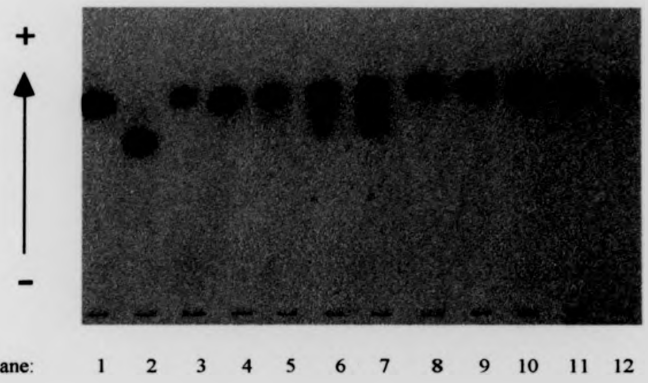


Figure 6.3.1.2: NHd phenotypes observed among Huánuco stocks. Lanes 1-3: reference strains: lane 1: *L. V. braziliensis* (LTB300), lane 2: *L. V. peruviana* (LC39); lane 3: *L. V. lainsoni* (M6246). Lanes 4-12: Huánuco stocks: lanes 4 & 5: *L. V. peruviana* (HR78 & LC2484); lanes 6, 8 & 9: *L. V. braziliensis* (LC2422, LC2487 & LC2412); lane 7: putative *L. V. braziliensis* / *L. V. peruviana* hybrid (HR399); lanes 10 & 11: *L. V. braziliensis* variant (MPI type 4, LC2603 & LC2842); lane 12: *L. V. lainsoni* (LC2679). Note different mobilities of reference strain and Huánuco stocks of *L. V. peruviana*.

***Figure 6.3.1.3*: NHi 1 / 2 phenotypes observed among Huánuco stocks.**

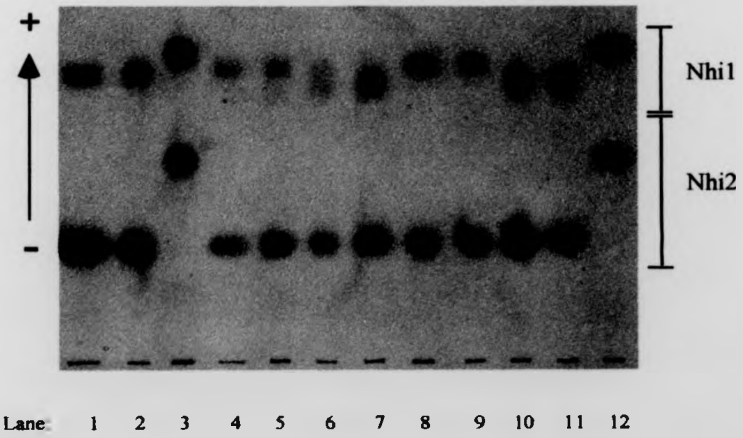


Figure 6.3.1.3: NHi phenotypes observed among Huánuco stocks. Lanes 1-3: reference strains: lane 1: *L. V. braziliensis* (LTB300); lane 2: *L. V. peruviana* (LC39); lane 3: *L. V. lainsoni* (M6246). Lanes 4-12: Huánuco stocks: lanes 4 & 5: *L. V. peruviana* (HR78 & LC2484); lanes 6, 8 & 9: *L. V. braziliensis* (LC2422, LC2487 & LC2412); lane 7: putative *L. V. braziliensis* / *L. V. peruviana* hybrid (HR399); lanes 10 & 11: *L. V. braziliensis* variant (MPI type 4, LC2603 & LC2842); lane 12: *L. V. lainsoni* (LC2679).

6.3.2 Zymodemes

The 59 Huánuco stocks showed a remarkable degree of isoenzyme diversity considering that they originated from such a small area. Three species *L. V. peruviana*, *L. V. braziliensis* and *L. V. lainsoni* and a new, as yet unnamed parasite, *L. V.* sp. n. (type strain IM2832) were found to occur sympatrically in the region. *L. V. guyanensis* and *L. L. amazonensis*, previously reported from Peru (Lucas *et al.*, 1994; 1998) were not identified among the Huánuco stocks.

Twelve zymodemes were identified using eleven enzyme systems (12 loci). Alleles and zymodeme designations for the Huánuco stocks are summarized in *table 6.3.2.1*: allele designations are the same as those given in *figure 6.3.1*. Stocks characterized as *L. V. lainsoni* and *L. V.* sp. n. (type strain IM2832) accounted for two of the zymodemes scored. LON227 included 3 dog stocks which were characterized as *L. V.* sp. n.: these stocks differed from the *L. V.* sp. n. reference strain IM2832 (LON5) at 3 loci. LON228 contained a single human stock, LC2679, which differed from the *L. V. lainsoni* reference strain M6426 (LON204) at two loci (NHi1 and GPI).

Ten zymodemes encompassed the remaining 55 stocks which could be split into 3 groups: *L. V. braziliensis* (LON222 - LON226), *L. V. peruviana* (LON217) and putative *L. V. braziliensis / L. V. peruviana* hybrids (LON218 - 221). *L. V. braziliensis* stocks differed from the reference strain LTB300 (LON200) at 1 or 2 loci; *L. V. peruviana* stocks differed from the reference strain LC39 (LON201) at a single locus. In contrast to the putative *L. V. braziliensis / L. V. panamensis* hybrids from Nicaragua, the genotypes of these hybrids were not a simple combination of "parental" genotypes. All 4 *L. V. peruviana* stocks were zymodeme LON217. Most *L. V. braziliensis* stocks were LON225 (15/25): 4 other zymodemes were scored, 3 containing 3 stocks each and one containing a single stock. Most hybrid stocks were LON218 (23/26): 3 other zymodemes were scored, each containing a single stock.

Figure 6.3.1: **Diagrammatic representation of banding patterns observed in reference strains and Huánuco stocks.**

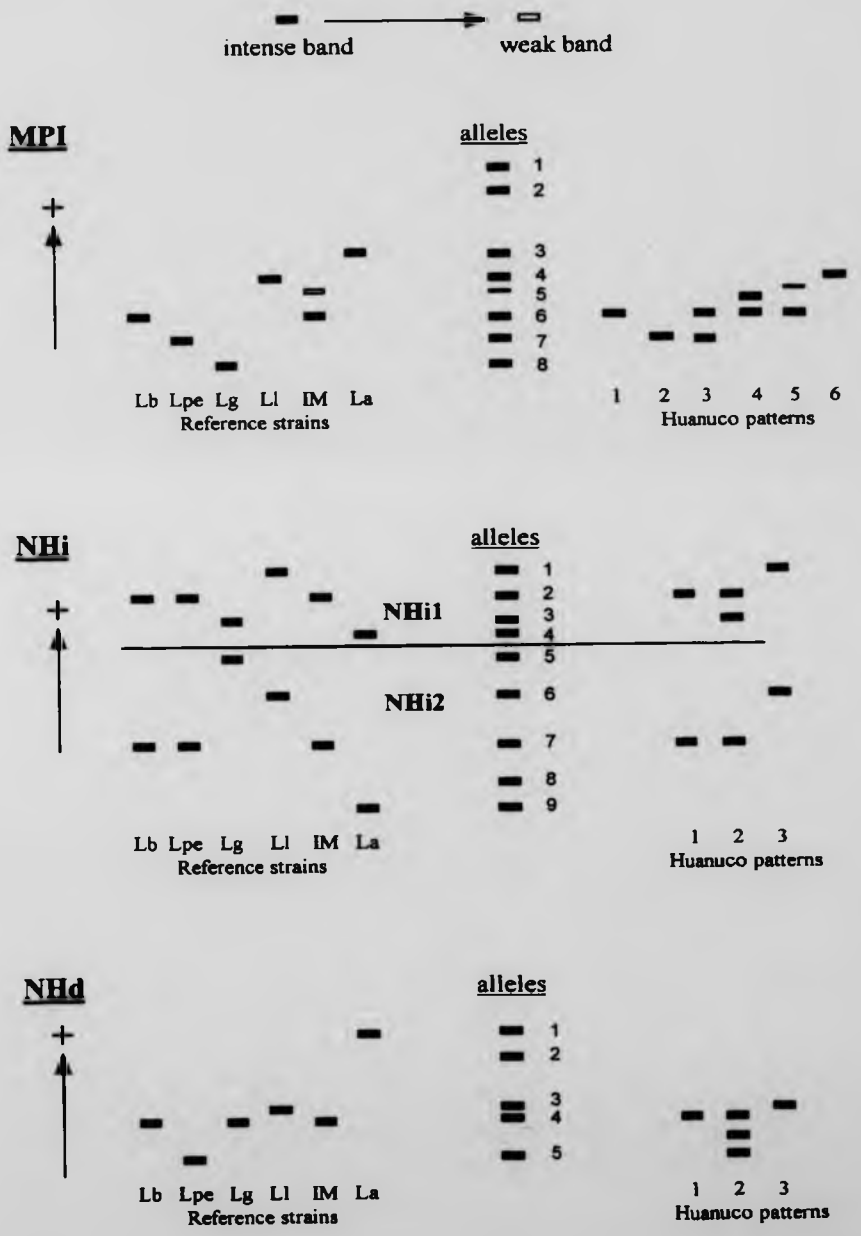


***Figure 6.3.1*:** **Diagrammatic representation of banding patterns observed in reference strains and Huánuco stocks (continued).**

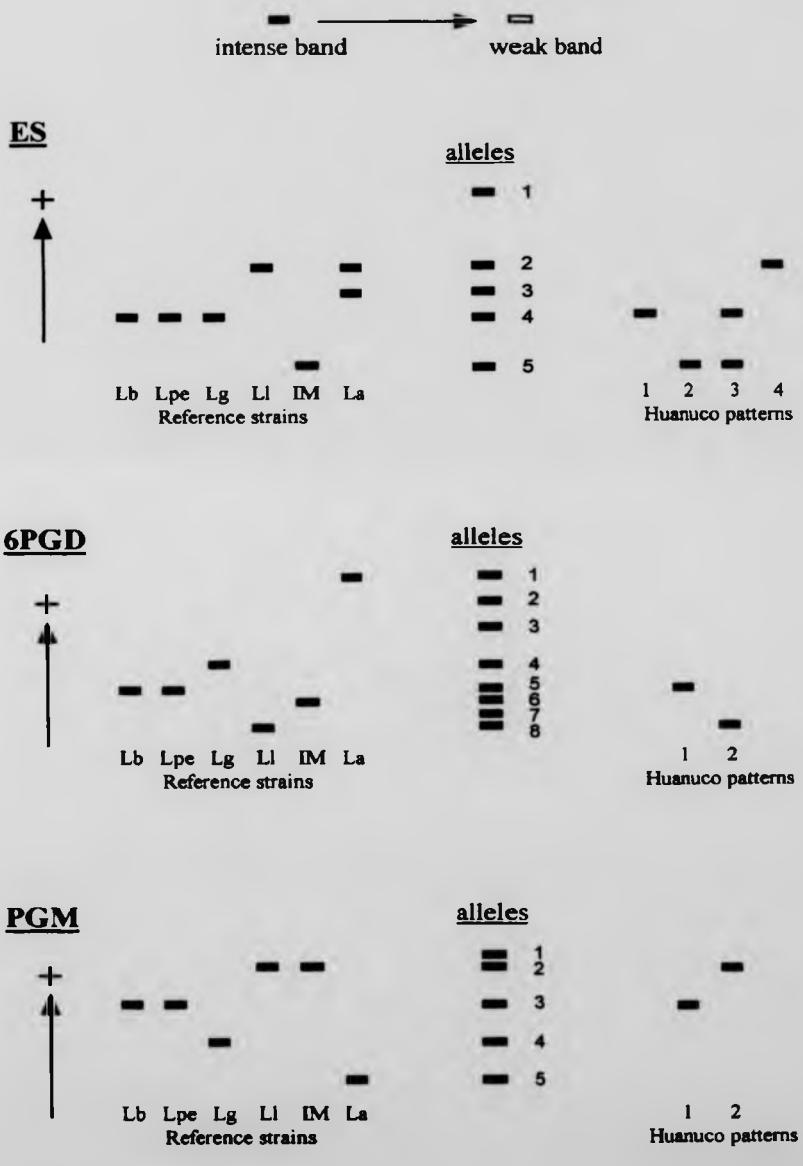


Figure 6.3.1: **Diagrammatic representation of banding patterns observed in reference strains and Huánuco stocks (continued).**

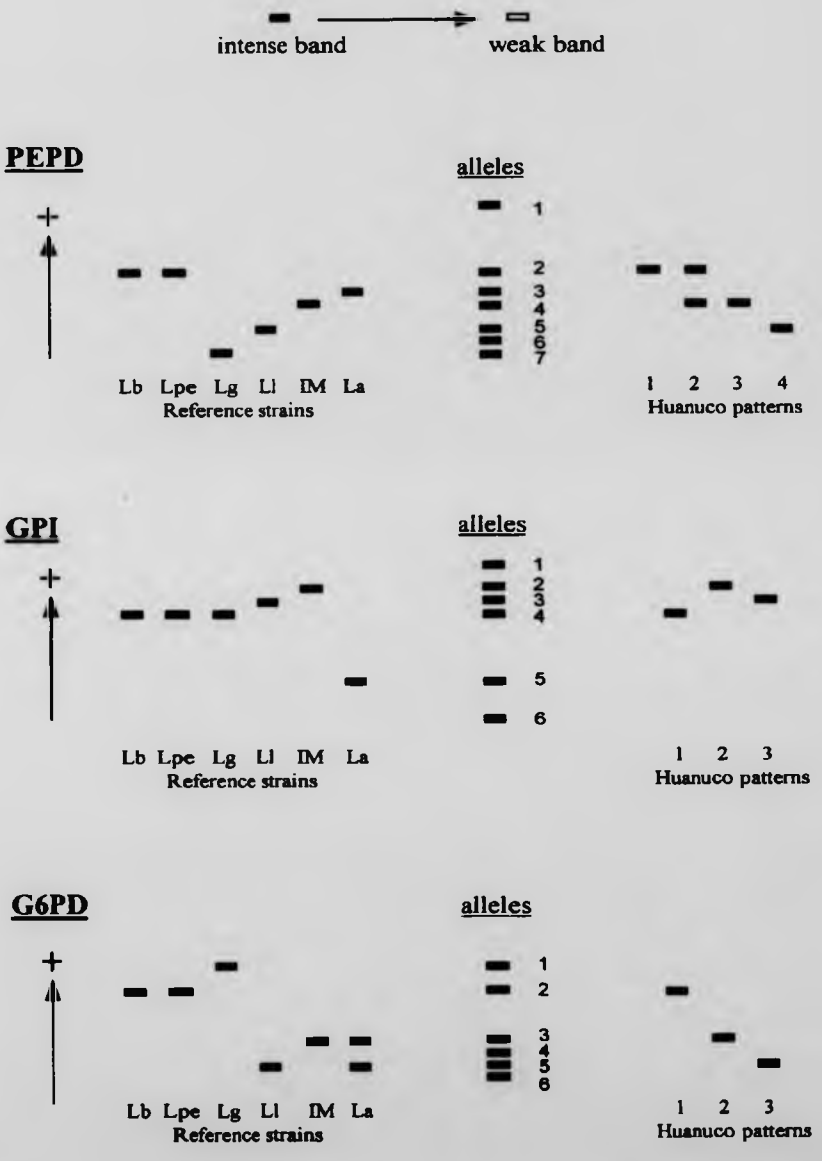
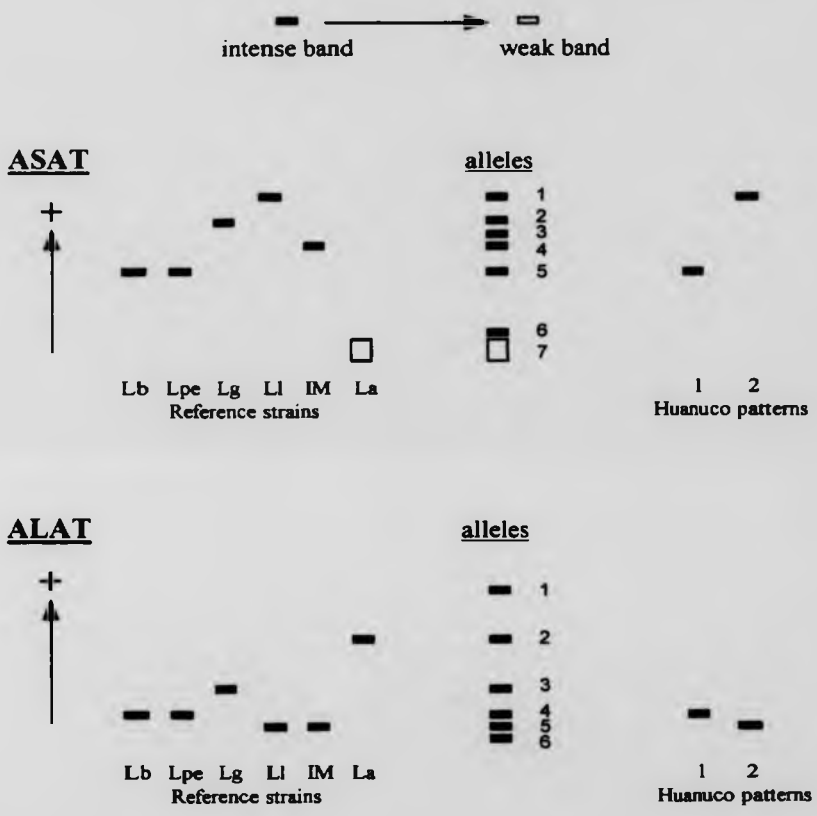


Figure 6.3.1: **Diagrammatic representation of banding patterns observed in reference strains and Huánuco stocks (continued).**



Notes to *table 6.3.2.1* (next page): allele and zymodeme designations for Huánuco stocks. Code names given in **bold** are reference strains. Z = zymodeme; * LON numbers above 200 have been reassigned by the author as previous records have been lost (zymodemes are assigned based on the IEA conditions used, i.e. for *Leishmania Viannia* species). For full enzyme names, see chapter 2. See *figure 6.3.1* for allele scoring scheme. Data given assumes diploidy, see introduction.

Table 6.3.2.1: **Allele and zymodeme designations for Huánuco stocks.**

	ENZYME												
STOCK CODE NOs.	**MPI**	**NHi 1**	**NHi 2**	**NHd**	**ES**	**PEPD**	**PGM**	**6PGD**	**GPI**	**G6PD**	**ASAT**	**ALAT**	**Z***
HR78, LC2434, LC2484, LC2851	7/7	2/2	7/7	4/4	4/4	2/2	3/3	5/5	4/4	2/2	5/5	4/4	**LON217**
HR80, HR108, HR110, HR399, HR413, HR424, HR434, HR797, HR799, LC2422, LC2485, LC2511, LC2553, LC2570, LC2576, LC2790, LC2825, LC2841, LC2844, LC2901, LC2902, LC2904, LC3150	6/7	2/3	7/7	4/5	4/4	2/2	3/3	5/5	4/4	2/2	5/5	4/4	**LON218**
HR410	6/7	2/3	7/7	4/5	4/5	2/2	3/3	5/5	4/4	2/2	5/5	4/4	**LON219**
LC2435	6/7	2/3	7/7	4/5	4/4	2/2	-	5/5	4/4	2/2	5/5	-	**LON220**
LC2877	6/7	2/3	7/7	4/5	4/4	2/2	-	-	4/4	2/2	5/5	4/4	**LON221**
LC2551, LC2603, LC2842	6/9	2/3	7/7	4/4	4/4	2/2	3/3	5/5	4/4	2/2	5/5	4/4	**LON222**
LC2450, LC2452, LC2523,	6/6	2/3	7/7	4/4	4/4	2/4	3/3	5/5	4/4	2/2	5/5	4/4	**LON223**
LC2520, LC2876, LC2900,	6/6	2/3	7/7	4/4	4/4	2/2	3/3	5/5	4/4	2/2	5/5	4/4	**LON224**
LC2412, LC2421, LC2487, LC2489, LC2491, LC2522, LC2558, LC2572, LC2683, LC2829, LC2843, LC2866, LC2875, LC2898, LC2905,	6/6	2/2	7/7	4/4	4/5	2/4	3/3	5/5	4/4	2/2	5/5	4/4	**LON225**
LC2873	6/6	2/2	7/7	4/4	4/4	2/4	3/3	5/5	4/4	2/2	5/5	4/4	**LON226**
HR419, HR529, HR701	5/6	2/2	7/7	4/4	5/5	4/4	2/2	5/5	2/2	3/3	5/5	4/4	**LON227**
LC2679	4/4	1/1	7/7	3/3	2/2	5/5	2/2	8/8	5/5	5/5	1/1	5/5	**LON228**

6.3.2.1 Zymodemes: associations with host species, clinical presentation and geographical origin

Huánuco stock zymodemes are summarized by host species, clinical presentation and by geographical origin in *table 6.3.2.2*. Of these 59 stocks, 45 (76.3%) were of human origin and 14 (23.7%) were isolated from dogs.

Among the 45 stocks isolated from humans, 3 (6.7%) were identified as *L. V. peruviana*, 25 (55.6%) as *L. V. braziliensis*, 16 (35.6%) as putative *L. V. braziliensis / L. V. peruviana* hybrids and a single stock (2.2%) as *L. V. lainsoni*, a species only recently reported from Peru (Lucas *et al.*, 1994). Eleven of the 45 (24.4%) human stocks originated from patients with MCL. Six MCL stocks were identified as *L. V. braziliensis* (6/25 stocks from 4/5 *L. V. braziliensis* zymodemes), underlining the association of this species with MCL. A single MCL stock was identified as *L. V. peruviana*. Lucas *et al.* (1998) attributed a single case of ML in Peru to *L. V. peruviana* but made no comment on this observation in the text. No distinction was made between ML and MCL in their study however, so it is possible that the lesion from which this stock originated was ML, as defined previously, not MCL. Nevertheless, the isolation here of *L. V. peruviana* from an MCL lesion is an important finding linking yet another *Viannia* species with this clinical presentation. The putative *L. V. braziliensis/L. V. peruviana* hybrid was also associated with MCL (4/26 stocks, 1/4 hybrid zymodemes). The implication of these findings is that all patients from Huánuco infected with *L. V. braziliensis*, *L. V. peruviana* and putative hybrids thereof, must be considered at risk of developing MCL.

Fourteen stocks were isolated from dogs (from cutaneous lesions on ears or nose): these dogs came from 2 of the regions in the study (Quera and Chinobamba). Four zymodemes were scored from these dogs: LON217 (*L. V. peruviana*, 1 stock); LON 218 and LON219 (putative hybrid, 9 and 1 stocks respectively) and LON227 (*L. V.* sp. n., 3 stocks). The 3 dog stocks accounting for LON227 (HR419, HR529 and HR701) were almost identical to the *L. V.* sp. n. reference strain IM2832, which has been proposed to be a new species in the subgenus *Viannia* (Grimaldi *et al.*, 1991): as far as I am aware, this is the first time that this *Viannia* phenotype has been identified in Peru or in dogs (IM2832 is a sand fly isolate from Amazonas State, Brazil). Interestingly, this phenotype was not sampled among the human stocks. *L. V. braziliensis* was not found among the dog stocks examined.

Table 6.3.2.2: **Summary of Huánuco stock zymodemes by host species, clinical presentation and by geographical region.**

		HUMAN STOCKS													DOG STOCKS						
		CL[a]						MCL[b]						Human stocks						Dog stocks	All stocks
		Region§					T[1]	Region§					T[2]		Region§						
Species†	Z*	A	C	H	Q	HC	All	A	C	H	Q	HC	All	Total	A	C	H	Q	HC	Total	Total
pe	217	-	1	-	-	1	2	-	1	-	-	-	1	3	-	-	-	1	-	1	4
b/pe	218	2	3	2	1	3	11	-	2	1	-	1	4	15	-	6		3	-	9	24
	219	-	-	-	-	-	0	-	-	-	-	-	0	0	-	1	-	-	-	1	1
	220	-	1	-	-	-	1	-	-	-	-	-	0	1	-	-	-	-	-	0	1
	221	1	-	-	-	-	1	-	-	-	-	-	0	1	-	-	-	-	-	0	1
b	222	-	-	1	-	-	1	-	-	1	-	1	2	3	-	-	-	-	-	0	3
	223	-	1	-	1	-	2	-	1	-	-	-	1	3	-	-	-	-	-	0	3
	224	-	1	-	-	1	2	-	-	-	1	-	1	3	-	-	-	-	-	0	3
	225	-	5	2	2	3	12	-	-	-	2	-	2	14	-	-	-	-	-	0	14
	226	-	-	-	-	1	1	-	-	-	-	-	0	1	-	-	-	-	-	0	1
*	227	-	-	-	-	-	0	-	-	-	-	-	0	0	-	3	-	-	-	3	3
l	228	-	-	-	1	-	1	-	-	-	-	-	0	1	-	-	-	-	-	0	1
							34						11	45						14	59

Table 6.3.2.2: summary of Huánuco stock zymodemes by host species, clinical presentation and by geographical region. Z = zymodeme; * LON numbers above 200 have been reassigned by the author as previous records have been lost (zymodemes are assigned based on the IEA conditions used, i.e. for *Leishmania Viannia* species). † Species codes: pe - *peruviana*; b - *braziliensis*; b/pe - putative *braziliensis / peruviana* hybrid; * *L. V.* sp. n. (IM2832, Grimaldi *et al.*, 1991); l - *lainsoni*. T^1 - total number of human CL stocks examined. T^2 - total number of MCL stocks examined. §Region codes: A - Ambo; C - Chinobamba; H - Higueras, Q - Quera, HC - Huánuco City. [a]CL - cutaneous leishmaniasis; [b]MCL - mucocutaneous leishmaniasis.

The finding here of *L. V. peruviana* and *L. V. braziliensis* / *L. V. peruviana* hybrid phenotypes in dogs and humans from the same area indicates that both are exposed to the same infective sand fly populations. Dogs are reservoirs of visceral disease and the isolation of apparently identical parasites from dogs and humans has led to the belief that they are also reservoir hosts of CL/MCL. The role of dogs in the epidemiology of CL has recently been extensively reviewed by Reithinger & Davies (1999).

It is difficult to pinpoint any association between geographical origin and zymodeme for these stocks because the area from which they originated was so small and it is not therefore possible to say how much movement occurred between the different regions and villages therein. Nevertheless, it is interesting to note that the 3 dog stocks of zymodeme LON227 all came from the same region (Chinobamba): this zymodeme (species) was not found in human stocks from this region or in dog or human stocks from other regions. It is tempting to speculate that these dogs may have had contact with a sylvatic cycle. Zymodeme LON218 (putative hybrid) was sampled among stocks from all regions and from both human and dog stocks. The zymodeme LON225 (*L. V. braziliensis*), although sampled from 4/5 regions, was only scored from human stocks: in fact, *L. V. braziliensis* was not scored from dogs at all. Too few stocks from each zymodeme were identified to allow any further comment.

6.4 Microsatellite analysis

6.4.1 PCR of microsatellite loci

6.4.1.1 Null alleles

As observed among the Brazilian and Nicaraguan stocks, several Huánuco stocks had null alleles at one or more of the microsatellite loci. Two *L. V. peruviana* stocks (LC2484 and LC2851) had null alleles at all three microsatellite loci. Two hybrid dog stocks (HR399 and HR410) failed to amplify AC52.

6.4.1.2 Agarose gel electrophoresis

PCR products generated from Huánuco stocks for all loci were within the expected size range. Size variation was evident, especially using AC16 and AC52 (data not shown), however the resolution was insufficient for characterization.

6.4.2 Non-denaturing PAGE analysis

6.4.2.1 AC01

Figure 6.4.2.1 shows AC01 products from Huánuco stocks analyzed using non-denaturing PAGE and silver staining.

Figure 6.4.2.1 **AC01 products from Huánuco stocks (PAGE analysis)**

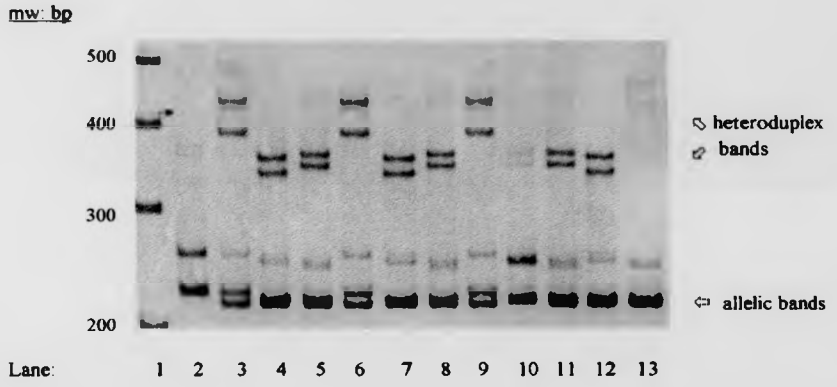


Figure 6.4.2.1: AC01 products from Huánuco stocks analyzed using non-denaturing 6% PAGE and silver staining. Lane 1 - 100 bp molecular weight marker (Gibco BRL, UK); lane 2 - LC2873; lane 3 - LC2842; lane 4 - LC2841; lane 5 - LC2905; lane 6 - LC2603; lane 7 - LC2570; lane 8 - LC2558; lane 9 - LC2551; lane 10 - LC2523; lane 11 - LC2487; lane 12 - LC2485; lane 13 - LC2484. PCR products from *L. V. braziliensis* stocks (identified using IEA) are in lanes 2, 3, 5 6, 8 -11; an *L. V. peruviana* stock is in lane 13 hybrids; putative hybrid stocks are in lanes 4, 7 & 12. Note the prominent heteroduplex bands in hybrid and some *L. V. braziliensis* lanes.

Many pattern types could be scored among the Huánuco stocks at AC01 using PAGE: the majority of stocks appeared to be heterozygous with 2 allelic bands. All stocks presented the locus-specific additional band, described previously: heteroduplexes were particularly evident for apparently heterozygous stocks. For example, in *figure 6.4.2.1*, stocks in lanes 3, 6 and 9 have 2 clearly-resolved allelic bands and 2 heteroduplex bands which are widely spaced. Stocks in lanes 4, 7 and 12 appear to have 2 allelic bands (poorly resolved): they also have 2 heteroduplex bands but these are lower and more closely spaced. Stocks in lanes 5, 8 and 11 also appear to have 2

allelic bands but their heterozygous bands are more closely spaced than in the 2 previous patterns. Stocks in lanes 2, 10 and 13 have very weak heteroduplex bands; the band in lane 2 is clearly larger than the bands in lanes 10 and 13.

Stocks identified as *L. V. braziliensis* / *L. V. peruviana* hybrids using IEA presented several different PAGE patterns, all with heteroduplex bands. AC01 PAGE pattern type correlated almost exactly with zymodeme. For example, the PAGE pattern generated by all stocks of zymodeme LON222 were identical (lanes 3, 6 and 9); this pattern was not found in other stocks. All stocks of zymodeme LON218 generated the pattern seen, for example, in lane 4.

6.4.2.2 AC16

Figure 6.4.2.2 shows the AC16 products from Huánuco stocks. As with AC01, AC16 products from different stocks could be grouped according to pattern type. The interpretation of these PAGE patterns was complicated owing to the presence of small differences in the size of the allelic band, heterozygous allelic bands, additional locus-specific bands and heteroduplex molecules. One PAGE pattern, however, could be correlated with a zymodeme: all stocks of zymodeme LON223 generated the pattern visible in lane 1 of *figure 6.4.2.2*.

6.4.2.3 AC52

Figure 6.4.2.3 shows AC52 products from Huánuco stocks. Stocks could be grouped for AC52, as for other loci, by comparison of pattern type. The interpretation of AC52 PAGE patterns was complicated owing to the multiplicity of apparently allelic bands (see lanes 3, 8, 9 & 11), additional bands and heteroduplex bands. Clear differences in the sizes of the allelic bands were evident (see lanes 2, 6, 10 & 13). AC52 PAGE patterns could usually be correlated with species type and/or zymodemes: for example, 3 bands (possibly 2 allelic, 1 additional) were generated by hybrid stocks (lanes 8, 9 & 11); all *L. V. peruviana* stocks and one hybrid stock (LC2422) generated large products; all stocks from zymodeme LON223 generated the pattern observed in lane 6.

Figure 6.4.2.2 **AC16 products from Huánuco stocks (PAGE analysis)**

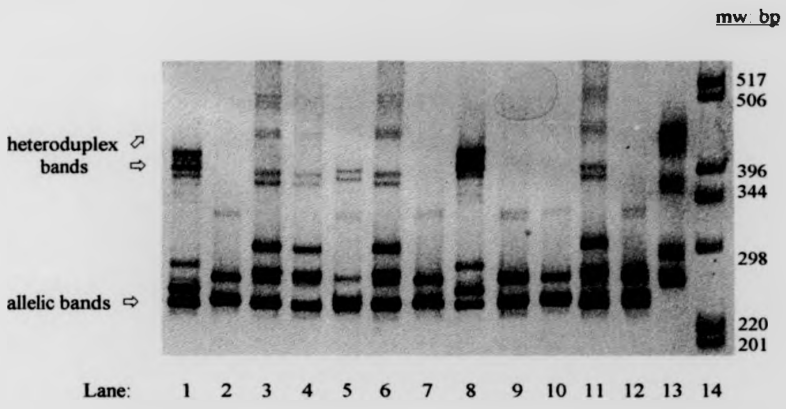


Figure 6.4.2.2: AC16 products from Huánuco stocks analyzed using non-denaturing 6% PAGE and silver staining. Lane 1 - LC2450; lane 2 - LC2683; lane 3 - LC2901; lane 4 - LC2435; lane 5 - LC2484; lane 6 - LC2485; lane 7 - LC2487; lane 8 - LC2523; lane 9 - LC2551; lane 10 - LC2558; lane 11 - LC2570; lane 12 - LC2603; lane 13 - additional *L. V. peruviana* reference strain, LC1152; lane 14 - 1kbp molecular weight marker (Gibco BRL, UK). *L. V. braziliensis* stocks (identified using IEA) lanes 1, 2, 7-10 & 12, *L. V. peruviana* stock lane 5; hybrid stocks lanes 3, 4, 6 & 11.

Figure 6.4.2.3 **AC52 products from Huánuco stocks (PAGE analysis)**

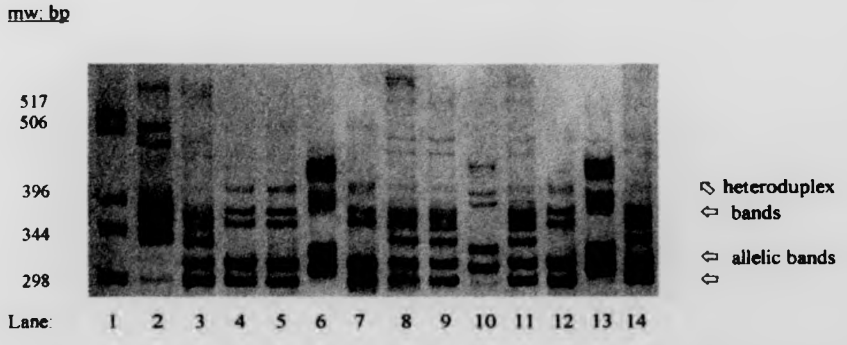


Figure 6.4.2.3: AC52 products from Huánuco stocks analyzed using non-denaturing 6% PAGE and silver staining. Lane 1 - 1kbp molecular weight marker (Gibco BRL, UK); lane 2 - LC2422; lane 3 - LC2679; lane 4 - LC2898; lane 5 - LC2875; lane 6 - LC2450; lane 7 - LC2683; lane 8 - LC2901; lane 9 - LC2435; lane 10 - LC2484; lane 11 - LC2485; lane 12 - LC2487; lane 13 - LC2523; lane 14 – LC2844. *L. V. braziliensis* stocks - lanes 4, 5 - 7, 12 & 13; *L. V peruviana* stock - lane 10; hybrid stocks - lanes 2, 8, 9, 11 & 14; *L. V. lainsoni* stock - lane 3.

6.4.3 Genescan® and Genotyper® analyses

6.4.3.1 AC01

Eight distinct AC01 alleles were scored amongst the Huánuco stocks giving rise to 9 genotype combinations: *table 6.4.3.1a* summarizes the AC01 genotypes observed in Huánuco stocks using Genotyper®. *Figure 6.4.3.2* illustrates the AC01 alleles observed in Huánuco stocks using Genotyper®. AC01 Genotyper® results for these stocks can generally be explained by variation of the number of dinucleotide repeats (see AC01 sequencing, section 6.4.4).

Five Peruvian *L. V. peruviana* reference strains were included for comparison in this study: alleles from these strains, and from the *L. V. peruviana* reference strain LC39, were more variable in size, but generally larger, than those associated with *L. V. braziliensis* (allele sizes: 231, 235, 235 and 237). Only 2 of the 4 *L. V. peruviana* stocks from Huánuco generated an AC01 product. These stocks generated single peaks of sizes 235 (HR78) and 241 (LC2484) and were assumed to be homozygous, genotypes 235/235 and 241/241 respectively. The genotype 235/235 was also found in 1 of the *L. V. peruviana* reference strains; the allele 241 and inferred genotype 241/241 is associated with Nicaraguan *L. V. panamensis* stocks (see chapter 5).

From previously examined populations (chapters 4 and 5), the AC01 allele 227 was associated with *L. V. braziliensis* stocks and was usually scored as a single peak with the inferred homozygous genotype 227 / 227. In contrast to *L. V. braziliensis* stocks from Brazil and Nicaragua, however, Huánuco stocks identified as *L. V. braziliensis* using IEA did not present the homozygous 227/227 genotype. Indeed, all *L. V. braziliensis* stocks bar one presented a heterozygous pattern (i.e. 2 peaks were scored). Most *L. V. braziliensis* stocks clearly presented the heterozygous genotype 227/231. This genotype was also scored in 3 *L. V. braziliensis* stocks from Brazil. The variation among Huánuco *L. V. braziliensis* stocks also included 3 other heterozygous patterns: 227/233 (1 stock), 227/237 (3 stocks) and 229/233 (3 stocks). A single *L. V. braziliensis* stock, LC2873, presented a single peak, inferred homozygous genotype 237/237. Two *L. V. braziliensis* AC01 genotypes correlated with zymodemes: all 3 stocks with genotype 227/237 were LON222 and all 3 stocks with the genotype 229/233 were LON223.

***Table 6.4.3.1a*: Comparison of AC01 genotypes observed in Huánuco stocks using Genotyper®, by species**

Observed genotype	Stocks: species status assigned using IEA data†				
	b	**h**	**pe**	*****	**l**
225 / 225	–	–	–	HR701	–
227 / 227	**LTB300**	–	–	–	–
227 / 231	LC2412, *LC2421* LC2487, LC2489 LC2491, LC2522 LC2558, LC2572 LC2683, *LC2829* LC2866, LC2875 LC2898, LC2905 LC2520, *LC2900* LC2843	HR108, HR110 HR399, HR410 HR413, HR424 HR434, HR797 HR799, LC2435 *LC2485*, LC2570 LC2576, LC2790 LC2825, *LC2841* LC2844, LC2877 *LC2901*, LC2902 LC2904, LC3150	–	–	–
227 / 233	LC2876	–	–	–	–
227 / 237	LC2551, *LC2603* *LC2842* (all LON222)	–	–	–	–
227 / 245	–	–	–	**IM2832**	–
229 / 231	–	HR80	–	–	LC2679
229 / 233	LC2450, LC2452, *LC2523* (all LON223)	–	–	–	–
231 / 231	–	–	**LC26, LC39, LRP906**	–	**M6426**
231 / 235	–	–	**LRP709**	–	–
235 / 235	–	–	**LRP737**, HR78	HR419	–
237 / 237	LC2873	LC2422	**LC1152**	–	–
241 / 241	–	–	*LC2484*	–	–
F	–	–	–	HR529	–
X	–	–	LC2434, LC2851	–	–
ND	–	LC2511, *LC2553*	–	–	–

Table 6.4.3.1a: Comparison of AC01 genotypes observed in Huánuco stocks using Genotyper®, by species. Species abbreviations: b - *L. V. braziliensis*; h - putative *L. V. braziliensis / L. V. peruviana* hybrid; pe - *L. V. peruviana*; * - *L. V.* sp. n.; l - *L. V. lainsoni*. Status determined from isoenzyme analysis. X - did not generate a PCR product (null allele(s)). F - failed to generate scorable Genotyper® peak. ND - not done. Code names in **bold** indicate reference strains, not from Huánuco. Stocks which are in *italics* are from MCL lesions.

Figure 6.4.3.2 **Genotyper® output from the AC01 locus for selected Huánuco stocks, illustrating the peaks observed in putative parental and hybrid profiles.**

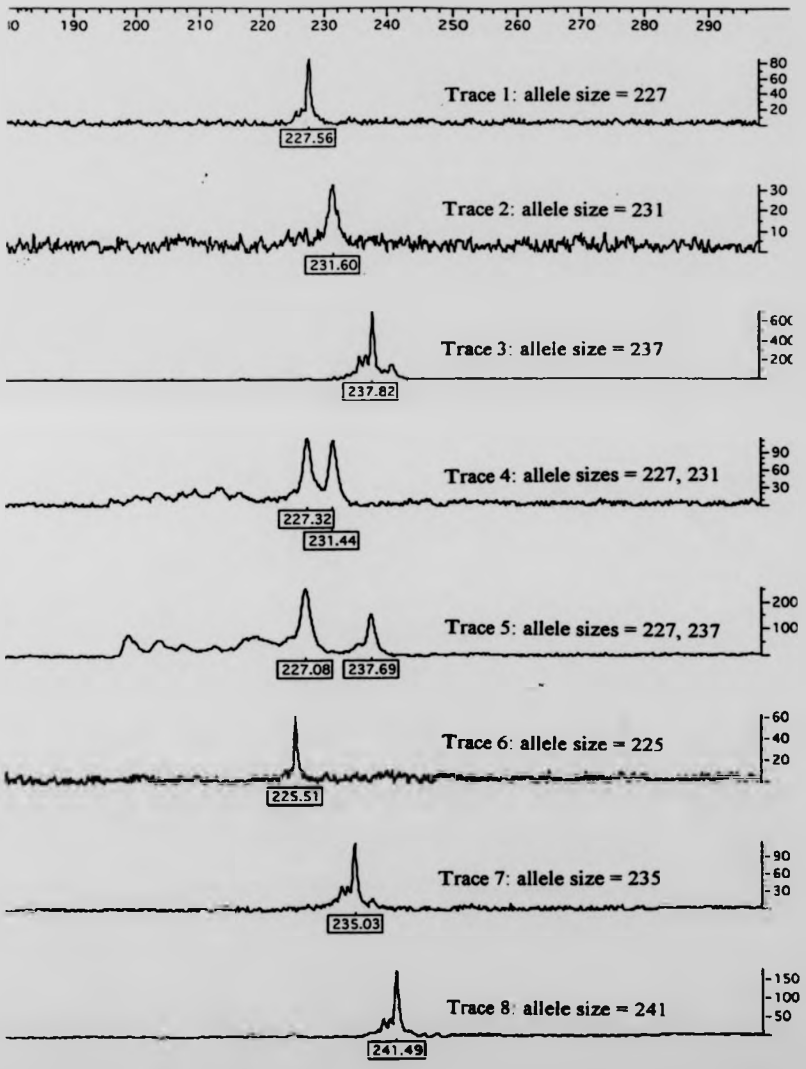


Figure 6.4.3.2: Genotyper® output from the AC01 locus for selected Huánuco stocks, illustrating the peaks observed in putative parental (single peaks, traces 1 – 3 & 6 – 8) and hybrid (double peaks, traces 4 & 5) profiles. Numbers across the top of the figure represent peak size, in bases. The exact sizes of individual peaks were scored automatically by the software. The scale to the right of each trace indicates the peak intensity. See chapter 3 for discussion of allele scoring. Traces 1 – 3 & 6 – 8: peaks with alleles sizes corresponding to 8, 10, 13. 7, 12 and 15 dinucleotide repeats, respectively; traces 4 & 5: two peaks with alleles sizes corresponding to 8 & 10 and 8 & 13 dinucleotide repeats, respectively.

All bar 2 of the putative hybrid stocks generated peaks corresponding to the genotype 227/231 which might be expected from a heterozygous locus having one 227 allele (i.e. from *L. V. braziliensis*) and one 231 allele (i.e. from *L. V. peruviana*). The 231 allele was not sampled among *L. V. peruviana* stocks from Huánuco, but was observed in 4 of the *L. V. peruviana* reference strains. One putative hybrid stock (LC2422) generated a single peak of size 237 (inferred genotype 237/237): this genotype was also observed in one *L. V. braziliensis* stock (LC2873) and one of the additional *L. V. peruviana* reference strains, LC1152.

Only 2/3 IM2832-type stocks generated scorable AC01 data: both generated single peaks of sizes 225 and 235 which were assumed to indicate homozygous genotypes 225/225 and 235/235 respectively. Both of these alleles were distinct from those scored in the IM2832 reference strain (227 / 245).

Most of the Huánuco stocks identified using IEA as *L. V. braziliensis* and putative *L. V. braziliensis* / *L. V. peruviana* hybrids were heterozygous at the AC01 locus. The most common AC01 genotype was 227/231, which accounted for 39/54 (72.2%) of Huánuco stocks. The allele 231, in addition to its presence in the genotype 227/231, was found in the homozygous state in 3 *L. V. peruviana* stocks (all additional *L. V. peruviana* reference strains) and in the *L. V. lainsoni* reference strain, M6426. This allele was also scored from the inferred genotype 229/231 in 2 stocks (1 putative hybrid and the Huánuco *L. V. lainsoni* stock, LC2679). These observations, in agreement with Russell *et al.* (1999), indicate that the alleles scored at this locus alone are not always correct in discriminating between *Viannia* species, as identified by IEA. The fact that most Huánuco *L. V. braziliensis* stocks were heterozygous suggests that the AC01 locus is not linked to enzyme loci: there is, in fact, no reason why different sets of markers should be simultaneously homozygous or heterozygous.

There appears to be considerably greater diversity in AC01 allele size amongst Huánuco stocks than was observed among Brazilian and Nicaraguan stocks. Huánuco stocks were isolated from a small geographical area in which, it is proposed that, the natural transmission cycle had recently been interrupted by the introduction of a new species. This interruption resulted in the current epidemic and provided the ideal setting for the occurrence of genetic recombination events. *Table 6.4.3.2* compares the allele sizes scored in *L. V. peruviana* and *L. V. braziliensis* stocks / strains from this study with data from Peruvian strains reported by Russell *et al.* (1999).

***Table 6.4.3.1b*:** **AC01 alleles scored in Peruvian stocks and strains**

	L. V. peruviana	*L. V. braziliensis*
This thesis	null 231 235 237 241	227 229 231 233 237
No. stocks examined	10	25
Russell *et al.* (1999)†	227 (8*) 229 (9) 231 (10) 233 (11*) 237 (13) 239 (14)	221 (5) 231 (10)
No. stocks examined	27	3

† sizes calculated, assuming no insertions or deletions in flanking regions, from the number of dinucleotide repeats reported (given in brackets - scored from sequence data). See *table 3.8.5.1* (page 136, chapter 3) for calculation. * alleles scored once each from a heterozygous stock (inferred equivalent genotype 227/233).

There was no apparent correlation between AC01 allele/genotype and clinical presentation. Ten out of eleven stocks isolated from MCL lesions generated AC01 products and were assigned to 4 genotypes: 227/231 (3/17 *L. V. braziliensis* and 3/22 hybrid stocks); 227/237 (1/3 stocks - *L. V. braziliensis)*; 229/233 (2/3 stocks - *L. V. braziliensis*) and 241/241 (1/1 stock - *L. V. peruviana*). The inferred genotype 241/241 was previously found associated with *L. V. panamensis* stocks from Nicaragua, where it was not associated with MCL. There was no apparent association between geographical origin and AC01 genotype.

6.4.3.2 AC16

Ten distinct AC16 alleles were scored amongst the Huánuco stocks giving rise to 13 genotype combinations: *table 6.4.3.2* summarizes the AC16 genotypes observed in Huánuco stocks using Genotyper®.

Of the six *L. V. peruviana* reference strains used, 3 (including LC39) failed to generate scorable Genotyper® data, 2 generated a single peak of 266 (inferred homozygous genotype 266 / 266) and 1 generated 2 peaks of 244 and 274 (inferred heterozygous genotype 244 / 274). Two Huánuco *L. V. peruviana* stocks failed to amplify this locus; the two remaining stocks presented different profiles.

Table 6.4.3.2: **Comparison of AC16 genotypes observed in Huánuco stocks using Genotyper®, by species**

Observed genotype	Stocks: species status assigned using IEA data				
	b	h	pe	*	l
240 / 240	--	HR399, HR410, HR799	--	--	--
240 / 244	--	LC2570, LC2435	--	--	--
240 / 270	--	--	--	--	**M6426**
244 / 244	**LTB300**, LC2520, LC2843 LC2873	HR108, HR110, HR413, HR424, HR434, *LC2485*, LC2576, LC2790 *LC2841*, LC2844 *LC2901*, LC2904 LC3150, LC2877	--	--	--
244 / 246	LC2551, *LC2603* *LC2842* (all LON222)	--	--	--	--
244 / 248	--	HR797	--	--	--
244 / 250	*LC2900*	LC2825	--	--	--
244 / 266	--	--	**HR78**	--	--
244 / 274	LC2876	--	**LC26**	--	--
248 / 248	LC2412, *LC2421* LC2487, LC2489 LC2491, LC2522 LC2558, LC2572 LC2683, LC2866 LC2875, LC2898 LC2905	--	--	--	--
248 / 262	LC2450, LC2452 *LC2523* (all LON223)	--	--	--	--
250 / 250	--	--	*LC2484*	--	--
250 / 252	--	LC2422, HR80	--	HR529, HR701	LC2679
250 / 254	--	--	--	HR419	--
266 / 266	--	--	**LRP737, LC1152**	--	--
F	*LC2829*	LC2902	**LC39, LRP906 LRP709**	--	--
X	--	--	LC2434, LC2851	**IM2832**	--
ND	--	LC2511, *LC2553*	--	--	--

Table 6.4.3.2: Comparison of AC16 genotypes observed in Huánuco stocks using Genotyper®, by species. Species abbreviations: b - *L. V. braziliensis*; h - putative *L. V. braziliensis* / *L. V. peruviana* hybrid; pe - *L. V. peruviana*; * - *L. V.* sp. n.; l - *L. V. lainsoni*. Status determined from isoenzyme analysis. X - did not generate a PCR product (null allele(s)). F - failed to generate scorable Genotyper® peak. ND - not done. Code names in **bold** indicate reference strains, not from Huánuco. Stocks which are in *italics* are from MCL lesions.

HR78 generated 2 peaks (inferred heterozygous genotype 244 / 266, the 266 allele was also identified in 2 *L. V. peruviana* reference strains), whilst LC2484 generated a single peak of 250 (inferred homozygous genotype 250 / 250).

Three Huánuco *L. V. braziliensis* stocks generated a single peak, inferred genotype 244 / 244, identical to the *L. V. braziliensis* reference strain LTB300. The majority of Huánuco *L. V. braziliensis* stocks (13/25), however, presented a single peak of 248 (inferred homozygous, genotype 248 / 248). Five stocks generated double peaks: three stocks (all stocks from zymodeme LON222) generated peaks of 244 and 246 (inferred genotype 244 / 246), one stock generated peaks of 244 and 250 (inferred genotype 244 / 250) and another stock generated peaks of 244 and 274 (inferred genotype 244 / 274, the 274 allele was also scored from one of the *L. V. peruviana* reference strains).

In the absence of sequence data for AC16 it is difficult to make comparisons between these results and those of Russell *et al.* (1999), however, in their study, allele sizes of *L. V. peruviana* (inferred from the number of dinucleotide repeats scored, n, range 7-12) were, with a single exception (n = 7), considerably smaller than those identified from *L. V. braziliensis* stocks (n, range 15-56). .

The majority of putative *L. V. braziliensis / L. V. peruviana* hybrid stocks (14/26) generated a single peak, size 244, for the AC16 locus (inferred genotype, homozygous 244 / 244). Three stocks generated single products of 240 (inferred genotype 240 / 240) and 6 stocks generated double peaks with inferred genotypes of 240 / 244 (2 stocks), 244 / 248 (1 stock), 244 / 250 (1 stock) and 250 / 252 (2 stocks).

All 3 IM2832-type Huánuco stocks generated 2 AC16 products: two were heterozygous 250 / 252 and the third 250 / 254.

Six AC16 genotypes were observed among stocks isolated from MCL lesions: 244 / 244 (also associated with MCL in Brazilian *L. V. braziliensis* stocks and a single Nicaraguan *L. V. panamensis* stock), 244 / 246, 244 / 250, 248 / 248, 248 / 262 and 250 / 250 (also associated with MCL from a single Brazilian *L. V. braziliensis* stock).

6.4.3.3 AC52

Many Huánuco stocks failed to generate readable Genotyper® data from the AC52 locus. Of those stocks which could be scored (34/59), 5 distinct AC52 alleles were scored in 4 genotype combinations: *table 6.4.3.3* summarizes the AC52 genotypes observed among Huánuco stocks using Genotyper®.

***Table 6.4.3.3*: Comparison of AC52 genotypes observed in Huánuco stocks using Genotyper®, by species**

Observed genotype	Stocks: species status assigned using IEA data				
	b	h	pe	*	l
265 / 295	LC2520, LC2843 LC2876, LC2551 *LC2603*, *LC2421* LC2487, LC2489 LC2491, LC2522 LC2558, LC2572 LC2683, LC2866 LC2875, LC2898 LC2905, *LC2829*	LC2570, LC2435 HR108, HR110, HR413, HR424, HR434, *LC2485* LC2576, LC2790 *LC2841*, LC2844 LC2877, LC2904 LC3150, LC2825	–	–	–
281 / 283	–	–	–	–	**M6426**
283 / 283	**LTB300**	–	–	–	–
287 / 289	–	–	–	HR701	–
295 / 295	–	–	**LC26**	–	LC2679
291 / 291	–	–	–	HR419	–
F	*LC2900*, *LC2842* LC2412, LC2450 LC2452, *LC2523* LC2873	HR799, *LC2901* HR797, HR80, LC2422, LC2902	HR78, *LC2484* **LRP906**, **LRP709**, **LRP737**, **LC1152, LC39**	HR529	–
X	–	HR399, HR410	LC2434, LC2851	**IM2832**	–
ND	–	LC2511, *LC2553*	–	–	–

Table 6.4.3.3: Comparison of AC52 genotypes observed in Huánuco stocks using Genotyper®, by species. Species abbreviations: b - *L. V. braziliensis*; h - putative *L. V. braziliensis* / *L. V. peruviana* hybrid; pe - *L. V. peruviana*; * - *L. V.* sp. n.; l - *L. V. lainsoni*. Status determined from isoenzyme analysis. X - did not generate a PCR product (null allele(s)). F - failed to generate scorable Genotyper® peak. ND - not done. Code names in **bold** indicate reference strains, not from Huánuco. Stocks which are in *italics* are from MCL lesions.

Only 1 *L. V. peruviana* strain (LC26) generated a scorable Genotyper® peak, size 295, inferred genotype 295 / 295. All other reference strains, and the 2 Huánuco stocks which produced a PCR amplification product for AC52, failed to produce peaks.

All *L. V. braziliensis* and putative *L. V. braziliensis / L. V. peruviana* stocks from Huánuco which were scorable generated 2 peaks of sizes 265 and 295, with an inferred heterozygous genotype of 265 / 295. The 265 allele has not been described from other populations or reference strains. The 295 allele was scored from an inferred homozygous genotype 295 / 295 in 2 Huánuco stocks: the *L. V. lainsoni* stock LC2679 and from one of the L. V. peruviana reference strains, see above. This genotype was also scored from a single Nicaraguan *L. V. panamensis* stock. The 2 other inferred AC52 genotypes were accounted for by 2 IM2832-type stocks, inferred genotypes 287 / 289 and 291 / 291: the latter has also been scored from the Nicaraguan population, mostly from *L. V. panamensis* and putative *L. V. braziliensis / L. V. panamensis* hybrid stocks.

It is not clear why so many of the Huánuco stocks failed to generate readable AC52 Genotyper® data: the AC52 products were analyzed at the same time in the same lanes of the same gel as AC01 and AC16 products which were easily scored. The run was repeated with the same results; AC52 peaks were very short and diffuse, and could not be reliably scored.

Comparing the AC52 non-denaturing PAGE results for these stocks (*figure 6.4.2.3*) with stocks from other populations (*figure 4.5.2.4*, Brazil and *figure 5.6.2.3*, Nicaragua), it is evident that, overall, the AC52 patterns from Huánuco stocks are more complex, with many additional bands staining as intensely as the presumed allelic bands. These many bands may account for the multiple diffuse peaks of low intensity seen in the Huánuco stocks using Genotyper®. A possible explanation for these results could be slippage during PCR amplification resulting in various product sizes, however, slippage presumably occurs randomly and it would therefore be unlikely that consistent pattern types would have been scored from PAGE analysis.

Russell *et al.* (1999), using sequence analysis, also found the AC52 locus to be complex, notably in *L. V. peruviana* isolates, with a long dinucleotide repeat sequence

interspersed with (TA) inserts. They found fewer (TA) inserts and smaller numbers of repeats in isolates of other species.

Genotyper® results at other microsatellite loci for the Huánuco stocks indicated that this population was extremely heterogeneous. AC52 Genotyper® scoring was impossible for some stocks in all groups (i.e. identified as species, hybrids and the IM type). To address this problem, several approaches could be taken: (i) re-examining the PCR conditions used for this locus; (ii) using an AC52 probe (derived from a single AC52 PCR product) under high- stringency conditions on Southern blots of genomic DNA-RFLPs to see if this locus has been duplicated in these stocks.

6.4.3.4 Multilocus analysis of microsatellite data

Missing data complicated assigning microdemes in the Huánuco stocks: stocks with data at all 3 loci were assigned to microdemes first, followed by stocks with data at both AC01 and AC16. Microdeme numbers were assigned (solely on Genotyper® data) with increasing AC01, then AC16, allele size, where possible. Stocks with data from AC52 plus one other locus were assigned to given microdemes based on similar PAGE patterns at the locus for which Genotyper® data was missing. Stocks with data from only 1 locus were not assigned to a microdeme (scored "?") but grouped with other similar stocks based on the data available. Microdemes observed in stocks from Huánuco are shown in *table 6.4.3.4*.

Twenty microdemes, all new, were scored from this data: 3 from the additional *L. V. peruviana* reference strains and 17 from the Huánuco stocks. Of those scored from Huánuco stocks, 10 contained a single stock and 4 contained more than 2 stocks. Of the 7 microdemes which contained more than one stock, 3 (LMS46, LMS48 and LMS52) contained stocks identified using IEA as different species / types. Microdemes LMS46 (identified by IEA as putative *L. V. braziliensis* / *L. V. peruviana* hybrid stocks plus 3 stocks *L. V. braziliensis*) and LMS51 (all *L. V. braziliensis*) contained the majority of stocks (17 and 13 respectively). Two microdemes correlated exactly with 2 *L. V. braziliensis* zymodemes: all 3 stocks identified as LON222 were assigned to microdeme LMS50 and all 3 stocks identified as LON223 were microdeme LMS53. Stocks isolated from MCL lesions were assigned to 6 microdemes.

Table 6.4.3.4 **Summary of microdemes† observed in stocks from Huánuco and additional *L. V. peruviana* reference strains.**

Microdeme †	Microsatellite locus genotype						Stock(s)	*Viannia* species§
	AC01		AC16		AC52			
LMS1	227	227	244	244	283	283	*LTB300*	b
LMS2	231	231	F	F	F	F	LC39	pe
LMS6	231	231	240	270	281	283	M6426	l
LMS7	227	245	X	X	X	X	IM2832	*
LMS42	225	225	250	252	287	289	HR701	*
	F	F					HR529	*
LMS43	227	231	240	240	X	X	HR399, HR410	b/pe
LMS44	227	231	240	240	F	F	HR799	b/pe
LMS45	227	231	240	244	265	295	LC2570, LC2435	b/pe
LMS46	227	231	244	244	265	295	HR108, HR110, HR413, HR424, HR434, *LC2485*, LC2576, LC2790, *LC2841*, LC2844, LC2877, LC2904, LC3150	b/pe
							LC2520, LC2843	b
			F	F			*LC2829*	b
					F	F	*LC2901*	b/pe
LMS47	227	231	244	248	F	F	HR797	b/pe
LMS48	227	231	244	250	265	295	LC2825	b/pe
					F	F	*LC2900*	b
LMS49	227	233	244	274	265	295	LC2876	b
LMS50	227	237	244	246	265	295	LC2551, *LC2603*	b
					F	F	*LC2842*	
LMS51	227	231	248	248	265	295	*LC2421*, LC2487, LC2489, LC2491, LC2522, LC2558, LC2572, LC2683, LC2866, LC2875, LC2898, LC2905	b
					F	F	LC2412	
LMS52	229	231	250	252	295	295	LC2679	l
					F	F	HR80	b/pe
LMS53	229	233	248	262	F	F	LC2450, LC2452, *LC2523*	b
LMS54	231	231	244	274	295	295	LC26	pe
(? LMS2)	231	231	F	F	F	F	LRP906	pe
?	231	235	F	F	F	F	LRP709	pe
LMS55	235	235	244	266	F	F	HR78	pe
LMS56	235	235	250	254	291	291	HR419	*
LMS57	235	235	266	266	F	F	LRP737	pe
LMS58	237	237	244	244	F	F	LC2873	b
LMS59	237	237	250	252	F	F	LC2422	b/pe
LMS60	237	237	266	266	F	F	LC1152	pe
LMS61	241	241	250	250	F	F	*LC2484*	pe
?	227	231	F	F	F	F	LC2902	b/pe
±NA	X	X	X	X	X	X	LC2434, LC2851	pe
ND							LC2511, *LC2553*	b/pe

Table 6.4.3.4 (previous page): summary of microdemes† observed in stocks from Huánuco and additional *L. V. peruviana* reference strains. † microdeme is the term coined here to describe *Leishmania* sharing the same microsatellite multilocus genotype (i.e. combined genotype data from the 3 microsatellite loci: AC01, AC16 and AC52). "LMS" numbers have been used to identify different microdemes (i.e. LMS1, 2, 3 etc.). "?" - not assigned owing to missing data. Code names in **bold** type indicate reference strains which, although not from Huánuco, are included for comparison. ±NA - stocks which failed to generate PCR products (X) at any of the three loci were not assigned a microdeme number because the genetic basis of failure to amplify could not be determined. F - failed to generate scorable Genotyper® peak. ND - not done. §*Viannia* species codes: b - *braziliensis*; pe - *peruviana*; l - *lainsoni*; * *L. V.* sp. n. (reference strain IM2832, Grimaldi *et al.*, 1991); b/pe - putative *L. V. braziliensis* / *L. V. peruviana* hybrid. Stocks in *italics* are from MCL lesions.

6.4.4 AC01 sequencing

Readable AC01 sequence data was obtained from all 55 Huánuco stocks which generated a PCR product for the AC01 locus. *Figure 6.4.4.1* shows the alignment of sequences from the AC01 microsatellite locus for Huánuco stocks and *Viannia* reference strains.

6.4.4.1 Interpretation of sequence data from Huánuco stocks

The large numbers of Huánuco stocks which were shown, using Genotyper®, to have 2 distinct AC01 alleles complicated sequence analysis. Direct cycle sequencing of these stocks generated products from both alleles which overlapped when electrophoresed. For most stocks, differences in allele size could be interpreted in terms of different numbers of dinucleotide repeats (see below). Since the sequence upstream of the repeat was conserved, downstream sequences could be aligned and compared. Despite the overlapping downstream sequence in stocks heterozygous for this locus, sequence data was usually unambiguous to manual interpretation. All samples were coded for sequencing and subsequent analysis to prevent bias from manual scoring. Scoring was carried out using the standard coding system format for nucleotides and nucleotide combinations (*Appendix IV*).

Figure 6.4.4.1 **Sequence alignment of the AC01 locus from Huánuco stocks and *Viannia* reference strains: bases 1 - 80.**

Species §	Strain/stock* code no	10	20	30	40	50	60	70	80
b	**LTB300**	ATGTGCCTCT	CCCACCCTTA	GTGCTTGTCT	TCTTCCTGCT	TTGCCTCTCT	GTC----TGT	GTGTGTGTGT	GTG-------
pe	LC2484	....	....	....	....	....	...----...	....	...-------
*	HR419 group	....	....	.......C..	....	....	...----...	....	.---------
l	**M6426**	....	....	..A....C..	C	......T.GC	C..----...	....	...TGTG---
l	LC2679	....	....	..A....C..	C	......T.GC	C..----...	.......---	----------
s	**M15065**	....	....	..... .C..	....	....	...TGTC...	....	...TGTGCGT
pa	**LS94**	....	....	....C..C..	....	....	...----...	....	...TGTGTGT
g	**M4147**	....	....	.......C..	....	C	...----...	....	...TGYGYGT
pe	**LC39 group†**	....	....	....	....	....	...----...	....	...TGTG---
pe	LC1152†	....	....	....	....	....	...----...	....	...TGTGTGT
pe	LRP737†	....	....	....	....	....	...----...	....	...TGTGTGT
pe	HR78	....	....	....	....	....	...----...	....	...TGTGTGT
h / b	LC2422 group	....	....	....	....	....	...----...	....	...TGTGTGT
b	LC2452 group	....	....	....	....	....	...----...	....	...TG-----
b	consensus 1	....	....	....	....	....	...----...	....	...TSYG---
h / b	consensus 2	....	....	....	....	....	R..----...	..S.......	...TSYG---
b	LC2551 group	....	....	....	....	....	R..----...	..S.......	...TSYGYKT

Notes to *figure 6.4.4.1*: sequence alignment of the AC01 locus from Huánuco stocks and *Viannia* reference strains. §Species abbreviation: b - *braziliensis*; l - *lainsoni*, s - *shawi*, pa - *panamensis*; g - *guyanensis;* pe - *peruviana*, h - putative *braziliensis* / *peruviana* hybrid. Species / type designation determined using IEA. † indicates additional *L. V. peruviana* reference strain. Reference strains are indicated in **bold** type. * Stock groups: HR419 group (HR419, HR529 and HR701); LC39 group (LC39, †LC26, †LRP709 and †LRP906); LC2422 group (LC2422 and LC2873); LC2452 group (LC2452, LC2450 and LC2523); LC2551 group (LC2551, LC2603 and LC2842). Consensus groups 1 and 2 are explained in the text. Numbers above sequence data indicate the base position used for alignment purposes. Bases in coloured type are explained in the text. Only these 165 bases of the total AC01 product were aligned and used for phylogenetic analysis (see chapter 3). See *Appendix IV* for base coding system.

Figure 6.4.4.1 **Sequence alignment of the AC01 locus from Huánuco stocks and *Viannia* reference strains (c'td): bases 81 - 165.**

Species §	Strain/stock* code no.	90	100	110	120	130	140	150	160	165
b	**LTB300**	----------	-TCCGCTTCA	GTGGGCCGAT	CCGTTTCACT	TTTTGCCGGT	GACGTTTGTG	TGCTGACGTG	TCTGGTGGCC	TCWCA
pe	LC2484	----------	-.....	.....	.....	.....	A....K....	...W.....	.....	.....
*	HR419 group	----------	-.....	.....	.....	.....	.....	.....	.....	.....
l	**M6426**	----------	-.....	.....T.	..A.....	...C.....	.....	...W.....	.....	..A..
l	LC2679	----------	-.....	......T...	..A.....	...C.....	.....	...N.....	.....	W.T..
s	**M15065**	GCG-------	-.....	.....	.....	..K.....	....CK....	...W.....	.....	..A..
pa	**LS94**	GTGTG-----	-.....YW..	......M...	....Y.....	..K..SS..W	....CG....	...A..Y...	..K..K....	..A..
g	**M4147**	GTGYGYS---	-.S...CA..	.K...SA..Y	S...CW....	..GY.SS..A	S...CG....	..SA..Y...	.SG..K....	W.A..
pe	**LC39 group†**	----------	-.....	.....	.....	.....	.....K....	...W.....	..K.....	W.A..
pe	LC1152†	GTG-------	-.....YW..	......M...	....Y.....	..K...S..W	.....K....	...W.....	..K.....	W.A..
pe	LRP737†	GTG-------	-.....YW..	......M...	....Y.....	..K...S..W	.....G....	...W.....	..K.....	W.A..
pe	HR78	GTG-------	-.....CW..	......MR..	....C.....	..G..M....	.....R....	...A.....	..G..K..M.	A.A..
h / b	LC2422 group	GTG-------	-.....Y...	......M...	....Y.....	..K.....W	.....K....	...W.....	..K.....	W....
b	LC2452 group	----------	-.SYS.KYYW	SW.K.SVSMK	MYSYK..W.W	Y....YSSSK	.WSAYG....	..YKS.SAYK	.S.SKK.KSV	H..M.
b	consensus 1	----------	-YYYSMK.SR	.YV.RY.SR.	YYSWC.YWY.	GYYG.YSRSD	K.Y..G..Y.	KA.DKRYV..	KYG.SY.VHH	A.A..
h / b	consensus 2	----------	-YYYSMKTSR	.YS.RYMSR.	YYSWC.YWY.	GYYG.YSASD	K.Y..G..YK	KA.DKRYV..	KYG.SY.SH.	A.A..
b	LC2551 group	SWG-------	-.SS..CKMW	SY.KKY.AM.	YYK.GY.RS.	KWCGKYY.KD	KR.KKAY...	.SYD.WS.YS	..DVRK...M	WM...

Notes to *figure 6.4.4.1*: sequence alignment of the AC01 locus from Huánuco stocks and *Viannia* reference strains. §Species abbreviation: b - *braziliensis*; l - *lainsoni*; s - *shawi*; pa - *panamensis*; g - *guyanensis*; pe - *peruviana*; h - putative *braziliensis* / *peruviana* hybrid. Species / type designation determined using IEA. † indicates additional *L. V. peruviana* reference strain. Reference strains are indicated in **bold** type. * Stock groups: HR419 group (HR419, HR529 and HR701); LC39 group (LC39, †LC26, †LRP709 and †LRP906); LC2422 group (LC2422 and LC2873); LC2452 group (LC2452, LC2450 and LC2523); LC2551 group (LC2551, LC2603 and LC2842). Consensus groups 1 and 2 are explained in the text. Numbers above sequence data indicate the base position used for alignment purposes. Bases in coloured type are explained in the text. Only these 165 bases of the total AC01 product were aligned and used for phylogenetic analysis (see chapter 3). See *Appendix IV* for base coding system.

6.4.4.2 AC01 sequence variation among Huánuco stocks

Based on overall AC01 sequence, Huánuco stocks could be divided into 9 groups, 6 of which contained more than 1 stock. All stocks within each of 7 groups had identical sequences. Stocks in the remaining 2 groups (consensus groups 1 and 2) showed a high degree of similarity with other members in their group but varied in downstream sequence. Consensus group 2 stocks could be differentiated from consensus group 1 stocks based on sequence variation at positions 51 (in the upstream region, see i, below) and 53 (at the beginning of the microsatellite repeat, see ii, below). Owing to the high level of conservation in these regions, this variation was assumed to be more significant than downstream variation in separating the stocks within consensus groups 1 and 2. Consensus group 1 contained all *L. V. braziliensis* stocks (identified using IEA) bar 3 with inferred AC01 genotype 227 / 231 (from Genotyper® data). Consensus group 2 contained all putative *L. V. braziliensis / peruviana* hybrid stocks with the genotype 227 / 231 plus the 3 *L. V. braziliensis* stocks. Sequence variation will be considered in 3 sections: i) sequence upstream of the dinucleotide (microsatellite) repeat (bases 1-57 of the alignment shown in figure 6.4.4.1); ii) the microsatellite repeat unit (bases 58-91) and iii) sequence downstream of the dinucleotide repeat (bases 92-165).

i) sequence upstream of the dinucleotide (microsatellite) repeat (bases 1-57)

In agreement with AC01 sequencing results from stocks in other populations (chapters 4 and 5), there was little sequence variation upstream of the dinucleotide repeat region (bases 1-57) in Huánuco stocks: 4 different upstream sequences could be identified. One stock (LC2679), identified using IEA as *L. V. lainsoni*, had an identical sequence in this region to that of the *L. V. lainsoni* reference strain M6426. All 3 IM2832-type stocks (*HR419, HR529 and HR701) had a "C" at position 28, a feature identified in all non-*L. V. braziliensis* complex isolates. Many stocks (21/59: LC2484, HR78, LC2422 group [3], LC2452 group [3] and consensus [14]) had upstream sequence identical to that of the *L. V. braziliensis* reference strain LTB300. The remaining stocks (30/59: LC2551 group [3] and consensus 2 [27]) had a double peak at position 51 with "A" and "G" scored (R). Strains used as additional *L. V. peruviana* references were, like the *L. V. peruviana* reference strain LC39, identical in upstream sequence to that of the *L. V. braziliensis* reference strain LTB300.

ii) the microsatellite repeat unit (bases 58-91)

Among the Huánuco stocks, the number of "TG" repeats scored ranged from 5 – 13 (in blue type). The *L. V. lainsoni* stock LC2679 had 5 dinucleotide repeats, half the number observed in the *L. V. lainsoni* reference strain M6426. All 3 IM2832-type stocks (*HR419, HR529 and HR701) had 7 repeats. Both of these repeat numbers (5 and 7) are lower than any described by Russell *et al.* (1999) for this locus. Of the additional *L. V. peruviana* reference strains examined, 3 (LC26, LRP709 and LRP906) had 10 repeats, the same number as the *L. V. peruviana* reference strain LC39; 2 others however, (LC1152 and LRP737) had 13. Both 10 and 13 repeats were commonly scored in *L. V. peruviana* isolates by Russell *et al.* (1999). Among the Huánuco stocks identified as *L. V. peruviana*, only 1 stock (HR78) had 13 repeats; none had 10. A single *L. V. peruviana* stock (LC2484) had 8 repeats: this number of repeats is associated with *L. V. braziliensis* (chapters 4 and 5 here, and Russell *et al.*, 1999). Similarly, a single stock identified as *L. V. braziliensis* (LC2873) and a single hybrid stock (LC2422) had 13 repeats. These stocks will be discussed further in section 6.4.4.3, below. Of the remaining stocks, all had 2 overlapping repeat sequences which could generally be interpreted in terms of 8 and 9 repeats, 8 and 10 repeats or 8 and 13 repeats (see *figure 6.4.4.2*, below).

iii) sequence downstream of the dinucleotide repeat (bases 92-165)

For stocks homozygous for AC01, the dinucleotide repeats were followed by a highly conserved motif of "TCCGC (red type)." This motif was used to denote the start of the downstream flanking region. All stocks in the HR419 group (IM2832 type) were identical to the *L. V. braziliensis* reference strain, LTB300, in the downstream region. A single stock, LC2484, assigned as *L. V. peruviana* using IEA, differed from the *L. V. braziliensis* reference strain, LTB300, at only 3 positions. The *L. V. lainsoni* stock, LC2679, was similar, but not identical, to the *L. V. lainsoni* reference strain M6426. *L. V. peruviana* stocks in the LC39 group (n = 10) were all identical and differed from the *L. V. braziliensis* reference strain, LTB300, in 5 downstream bases. Stocks in the LC2422 group, LC1152 and LRP709 (all n = 13, all identified as *L. V. peruviana*, bar LC2873 in the LC2422 group) were remarkably similar in the downstream region, with the same heterozygous peaks usually scored at the same nucleotide positions. Another *L. V. peruviana* stock with n = 13, HR78, shared less overall similarity with other n = 13 stocks, however the same nucleotide positions were often variable.

***Figure 6.4.4.2*: Dinucleotide repeat region illustrating the overlapping sequence reads in stocks with 2 distinct alleles.**

i) Stocks with 2 alleles containing 8 and 10 dinucleotide repeats

§Sp. (n)	allele		sequence
b (8)	LTB300 group	1	GTC----TGT GTGTGTGTGT GTGTCCGCTT CAGTGGGCCG
pe (10)	LC39 group	2	GTC----TGT GTGTGTGTGT GTGTGTGTCC GCTTCAGTGG
h (8/10)	**EXPECTED**	3	GTC----TGT GTGTGTGTGT GTGTSYGYYY SMKTSRGYSG
	consensus group 1		...----... ....V.
	consensus group 2		R..----... ..S..... ....

ii) LC2551 group stocks (alleles containing 8 and 13 dinucleotide repeats)

§Sp. (n)	allele		sequence
b (8)	LTB300 group	1	GTC----TGT GTGTGTGTGT GTGTCCGCTT CAGTGGGCCG
pe (13)	LC1152 group	2	GTC----TGT GTGTGTGTGT GTGTGTGTGT GTGTCCGCYW
h (8/13)	**EXPECTED**	3	GTC----TGT GTGTGTGTGT GTGTSYGYKT SWGTSSGCYD
	LC2551 group		R..----... ..S..... ....CK

iii) LC2452 group stocks (alleles containing 9 and 10 dinucleotide repeats)

§Sp. (n)	allele		sequence
? (9)	?	1	GTC----TGT GTGTGTGTGT GTGTGTCCGC TTCAGTGGGC
pe (10)	LC39 group	2	GTC----TGT GTGTGTGTGT GTGTGTGTCC GCTTCAGTGG
? (9/10)	**EXPECTED**	3	GTC----TGT GTGTGTGTGT GTGTGTSYSC KYYWSWGKGS
	LC2452 group		...----... ....

Notes to *figure 6.4.4.2*: dinucleotide repeat region, illustrating the overlapping sequence reads in stocks with 2 distinct alleles. The **EXPECTED** sequence of heterozygous stocks (i.e. having 1 copy each of the alleles in rows 1 & 2) is given in row 3 of each example i) – iii) (see *Appendix IV* for base coding system). The sequences observed in heterozygous stocks are compared to this expected sequence. Dots indicate that an identical base was scored to that expected. §Sp.: species abbreviation: b - *braziliensis*, pe - *peruviana*, h - putative *braziliensis* / *peruviana* hybrid, ? - unknown. Species / type designation determined using IEA. Consensus groups 1 and 2 are explained in the text. (n): number of dinucleotide repeats. Bases in blue type indicate the dinucleotide repeats, bases in red indicate the TCCGC motif used to identify the end of the dinucleotide repeat region, bases in green indicate bases which are invariant despite the overlapping sequence reads.

In 4 Huánuco groups (consensus group 1, consensus group 2, LC2551 group and LC2452 group), significant variation was scored in the downstream flanking region owing to the superimposition of sequence generated from 2 distinct alleles. The number of dinucleotide repeats (blue type) contained in each allele was determined by identifying the TCCGC end motif in each (red type, see *figure 6.4.4.2*). It was possible to align the mixed sequences based on sites which were invariant (in green type), despite the overlap.

Figures 6.4.4.3 and *6.4.4.4* illustrate the expected sequence that would be presented by stocks with alleles encoding AC01 products with 8 / 10 and 8 / 13 dinucleotide repeats, respectively. This expected sequence was then compared with observed sequence(s). For stocks in consensus groups 1 and 2, the overlapping sequences could be explained by the presence of 2 alleles, one with 8 dinucleotide repeats and the other with 10 (see *figure 6.4.4.3*). For stocks in the LC2551 group, the overlapping sequences could be explained by the presence of 2 alleles, one with 8 dinucleotide repeats and the other with 13 (see *figure 6.4.4.4*). For these 3 groups, the 2 alleles had been previously scored from Peruvian stocks and reference strains. Differences between expected and observed sequences of heterozygous stocks may be explained by the fact that the 2 sequences used as "parental" 8 and 10 dinucleotide repeat-containing alleles were from strains not originating from Huánuco.

In one group of stocks (LC2452 group), significant downstream variation was observed which, like the 3 previous groups, suggested overlapping sequence from alleles containing different numbers of repeats. For the LC2452 group stocks, however, 9 dinucleotide repeats were scored unequivocally: this number of repeats had not been scored among any of the stocks examined previously, although Russell *et al.* (1999) recorded this allele in *Viannia* stocks (scored once each from *L. V. braziliensis*, *L. V. peruviana* and *L. V. panamensis* isolates from Bolivia, Peru and Colombia, respectively). By identifying the TCCGC end motif, the other allele in LC2452 group stocks was found to contain 10 repeats (see *figure 6.4.4.2*).

Figure 6.4.4.3 **Expected sequences of stocks heterozygous at the AC01 locus for alleles encoding 8 and 10 dinucleotide repeats.**

Species	Strain / stock	10	20	30	40	50	60	70	80
b (8)	LTB300 group	ATGTGCCTCT	CCCACCCTTA	GTGCTTGTCT	TCTTCCTGCT	TTGCCTCTCT	GTC----TGT	GTGTGTGTGT	GTGTCCGCTT
pe (10)	LC39 group	ATGTGCCTCT	CCCACCCTTA	GTGCTTGTCT	TCTTCCTGCT	TTGCCTCTCT	GTC----TGT	GTGTGTGTGT	GTGTGTGTCC
h (8/10)	**EXPECTED**	ATGTGCCTCT	CCCACCCTTA	GTGCTTGTCT	TCTTCCTGCT	TTGCCTCTCT	GTC----TGT	GTGTGTGTGT	GTGTSYGYYY
	consensus group 1	.....	.....	.....	.....	.....	...----...	.....	.....
	consensus group 2	.....	.....	.....	.....	.....	R..----...	..S.....	.....

Species	Strain / stock	90	100	110	120	130	140	150
b (8)	LTB300 group	CAGTGGGCCG	ATCCGTTTCA	CTTTTTGCCG	GTGACGTTTG	TGTGCTGACG	TGTCTGGTGG	CCTCWCA
pe (10)	LC39 group	GCTTCAGTGG	GCCGATCCGT	TTCACTTTTT	GCCGGTGACG	TKTGTGTGCW	GACGTGTCKG	GTGGCCWCACA
h (8/10)	**EXPECTED**	SMKTSRGYSG.	RYCSRTYYSW	YTYWYTKYYK	GYSRSKKWYG	TKTGYKKRCD	KRYSTGKYKG	SYKSHCWCACA
	consensus group 1	.....V.	.....	C.....G..G	.....D.T..	.G...T.A..	...V....G.	..TV.HA....
	consensus group 2	.....	..M.....	C.....G..G	...A.D.T..	.G.....A..	...V....G.	..T..CA....

Notes to *figure 6.4.4.3*: Expected sequences of stocks heterozygous at the AC01 locus for alleles encoding 8 and 10 dinucleotide repeats. §Species abbreviation: b - *braziliensis*, pe - *peruviana*, h - putative *braziliensis* / *peruviana* hybrid. Species / type designation determined using IEA. Consensus groups 1 and 2 are explained in the text. Numbers above sequence data indicate the base position used for alignment purposes. Bases in blue type indicate the dinucleotide repeats; bases in red type indicate the TCCGC motif used to identify the end of the dinucleotide repeat region; bases in green type indicate bases which are invariant despite the overlapping sequence reads. See *Appendix IV* for base coding system.

Figure 6.4.4.4 **Expected sequences of stocks heterozygous at the AC01 locus for alleles encoding 8 and 13 dinucleotide repeats.**

Species	Strain / stock	10	20	30	40	50	60	70	80
b (8)	LTB300 group	ATGTGCCTCT	CCCACCCTTA	GTGCTTGTCT	TCTTCCTGCT	TTGCCTCTCT	GTC----TGT	GTGTGTGTGT	GTGTCCGCTT
pe (13)	LC1152 group	ATGTGCCTCT	CCCACCCTTA	GTGCTTGTCT	TCTTCCTGCT	TTGCCTCTCT	GTC----TGT	GTGTGTGTGT	GTGTGTGTGT
h (8/13)	**EXPECTED**	ATGTGCCTCT	CCCACCCTTA	GTGCTTGTCT	TCTTCCTGCT	TTGCCTCTCT	GTC----TGT	GTGTGTGTGT	GTGTSYGYKT
	LC2551 group	.....	.....	.....	.....	.....	R..----...	..S.....	.....

Species	Strain / stock	90	100	110	120	130	140	150	160
b (8)	LTB300 group	CAGTGGGCCG	ATCCGTTTCA	CTTTTTGCCG	GTGACGTTTG	TGTGCTGACG	TGTCTGGTGG	CCTCWCA	
pe (13)	LC1152†	GTGTCCGCYW	CAGTGGGCMG	ATCCGTYTCA	CTTTKTGCSG	GWGACGTKTG	TGTGCWGACG	TGTCKGGTGG	CCWCACA
h (8/13)	**EXPECTED**	SWGTSSGCYD	MWSYGKKYMR	MTYYKTBYCR	STKWBKKYBG	KDKRCKKDYG	TGTSYDGWSG	YSTCDSRTGG	CCWCACA
	LC2551 group	........CK	........CA	......G...	....CG..Y.	.......A..	.....	.....V.K..	.M.MW..

Notes to *figure 6.4.4.4*: Expected sequences of stocks heterozygous at the AC01 locus for alleles encoding 8 and 13 dinucleotide repeats. §Species abbreviation: b - *braziliensis*, pe - *peruviana*, h - putative *braziliensis* / *peruviana* hybrid. Species / type designation determined using IEA. Numbers above sequence data indicate the base position used for alignment purposes. Bases in blue type indicate the dinucleotide repeats, bases in red type indicate the TCCGC motif used to identify the end of the dinucleotide repeat region; bases in green type indicate bases which are invariant despite the overlapping sequence reads. See *Appendix IV* for base coding system.

6.4.4.3 Comparison of AC01 data from sequencing and Genotyper®

Table 6.4.4.3 compares the number of dinucleotide repeats scored from sequence data with Genotyper® results. For the majority of Huánuco stocks and additional *L. V. peruviana* reference strains, the number of dinucleotide repeats scored from sequence analysis correlated exactly with allele size scored from Genotyper® analysis (assuming an allele size of 227 equates to 8 dinucleotide repeats, see chapter 3). This correlation was all the more impressive since the majority of stocks were scored as heterozygous using Genotyper®. For example, all *L. V. braziliensis* stocks scored using Genotyper® as 227 / 231 were found to have overlapping sequence reads which could be interpreted as 2 alleles differing by 2 repeats (i.e. 8 and 10), giving allele sizes which varied by 4 base pairs.

For several stocks, small discrepancies were noted. As discussed previously (chapters 3 - 5), these could have arisen from insertions or deletions ('indels") in the extreme 5' sequence (not used in the alignments), thus giving rise to Genotyper® sizes larger or smaller, respectively, than expected by extrapolation from the number of dinucleotide repeats. This explanation could possibly account for the observed disparity between methods for the *L. V. peruviana* stocks LRP737 and HR78 where 13 repeats were scored from sequence data (expected allele size 237) but where Genotyper® results indicated an allele size of 235 (? 2 base deletion). Similarly, in stocks scored using Genotyper® as heterozygous for AC01, a small indel in one of the 2 alleles could account for the discrepancy between the 2 methods. For example, in stocks of the LC2542 group, Genotyper® analysis scored the stocks as 229 / 233 (suggesting alleles containing 9 and 11 repeats) whereas sequence data indicated that 9 and 10 repeats were in fact, present - equivalent to a 2 base insertion in the 233 allele.

In a few stocks, however, there were significant differences between the 2 methods in which large indels would be required to explain Genotyper® data or where Genotyper® results indicated a stock heterozygous for AC01 but where sequence data suggested a single allele. For example, a 14 base pair insertion would be required to account for the disparity between these methods for the *L. V. peruviana* stock LC2484 (Genotyper® peak of 241, expected number of repeats = 15: 8 repeats were scored by sequence analysis). For the Huánuco *L. V. lainsoni* stock LC2679, 5 repeats were repeatedly scored by sequence analysis suggesting a single allele (Genotyper® data of 229 / 231 suggesting 2 distinct alleles, inferring 9 and 10 dinucleotide repeats). The

significant discrepancies are difficult to explain. These may be the result of labeling errors: all stocks concerned should be re-examined by both techniques to confirm this.

***Table 6.4.4.3*: Comparison of the number of dinucleotide repeats scored from sequence data with Genotyper® results.**

Strain, stock or group from sequencing data†	No. stocks	Species designation using IEA§	No. of dinucleotide repeats scored	AC01 Genotyper® data±
M6426†	-	l	10	231 / 231
LC2679	1	l	5	229 / 231
HR419 group	3	IM2832 type	7	225 / 225 (1) 235 / 235 (1) failed (1)
LTB300†	-	b	8	227 / 227
consensus group 1	14	b	8 / 10	227 / 231
LC2452 group	3	b	9 / 10	229 / 233
LC2551 group	3	b	8 / 13	227 / 237
LC2873	1	b	13	237 / 237
consensus group 2	27	b / pe	8 / 10	227 / 231 227 / 233 (1) 229 / 231 (1)
LC2422	1	b / pe	13	237 / 237
LC2484	1	pe	8	241 / 241
LC39 group†	4	pe	10	231 / 231 (3) 231 / 235 (1)
LC1152†	1	pe	13	237 / 237
LRP737†	1	pe	13	235 / 235
HR78	1	pe	13	235 / 235

† indicates a reference strain. Consensus groups 1 and 2 are explained in the text. §Species abbreviations: b - *braziliensis*; pe - *peruviana*, b / pe - putative *braziliensis / peruviana* hybrid; l - *lainsoni*; IM2832-type - potential new *Viannia* species (Grimaldi *et al*, 1991). ± numbers in parentheses indicate the number of stocks / strains with the given Genotyper® data.

6.4.4.4 Comparison of microsatellite and isoenzyme data

Two Huánuco stocks had conflicting results by IEA and AC01 microsatellite locus analysis which need to be readdressed to eliminate the possibility of an error. The stock LC2873, identified as *L. V. braziliensis* using IEA, was found to be highly similar to *L. V. peruviana* stocks and strains both in the number of dinucleotide repeats

scored (13), DNA sequence and in Genotyper® allele size (237). This number of repeats was not found to be associated with *L. V. braziliensis* by Russell *et al.* (1999), although they examined relatively few isolates.

Another stock, LC2484, was identified using IEA as *L. V. peruviana* but found to have just 8 dinucleotide repeats from sequence analysis, a repeat number associated with *L. V. braziliensis* (here, Russell *et al.*, 1999). However, this stock generated a Genotyper® peak of 241 suggestive of 15 repeats; *L. V. peruviana* stocks generally have greater numbers of repeats at the AC01 locus compared to *L. V. braziliensis* stocks (here, Russell *et al.*, 1999), however 15 repeats have only been described in *L. V. panamensis* stocks (again, here, Russell *et al.*, 1999).

6.5 Phylogenetic analysis of Huánuco stocks

Phylogenetic analysis of isoenzyme data (multilocus data) included data from all *Leishmania* reference strains. Phylogenetic analysis of sequence data from the microsatellite locus AC01 included data from all reference strains of the *Viannia* subgenus, with the exception of *L. V.* sp. n. reference strain IM2832 which failed to produce readable sequence data. The AC01 microsatellite locus is specific to the *Viannia* subgenus hence it was not possible to include reference strains from the subgenus *Leishmania* in this phylogenetic analysis.

6.5.1 Phylogenetic analysis of isoenzyme data

Isoenzyme phylogenies were constructed, as described in chapter 2, using a maximum-likelihood approach, quartet puzzling (Strimmer & von Hassler, 1996). *Figure 6.5.1.1* shows the consensus cladogram constructed after quartet puzzling analysis of isoenzyme data from *Leishmania* reference strains and from Huánuco stocks. Stocks from Huánuco spanned 3 *Viannia* species (*L. V. braziliensis*, *L. V. peruviana* and *L. V. lainsoni*) plus a potentially new *Viannia* species (*L. V.* sp. n.: Grimaldi *et al.*, 1991), and included putative *L. V. braziliensis / L. V. peruviana* hybrids.

Using this approach, the Huánuco *L. V. lainsoni* stock LC2679 clustered with the reference strain for this species (M6426), although the former had a distinct zymodeme (LON228 and LON206, respectively). The status of the potential new *Viannia* species (type and reference strain IM2832, LON205) as distinct from other members of this subgenus is not supported by this analysis. IM2832 differed from the

L. V. braziliensis reference strain LTB300 at 9/12 loci: the 3 Huánuco stocks (HR419, HR529 and HR701, LON227), which were found to be identical to IM2832 at 6 of these 9 loci, did, however, cluster separately from other stocks (73% support). It seems likely that the data for IM2832 was incorrectly entered for this analysis (compare the position of IM2832 in this analysis with its position in *figure 6.5.1.2*).

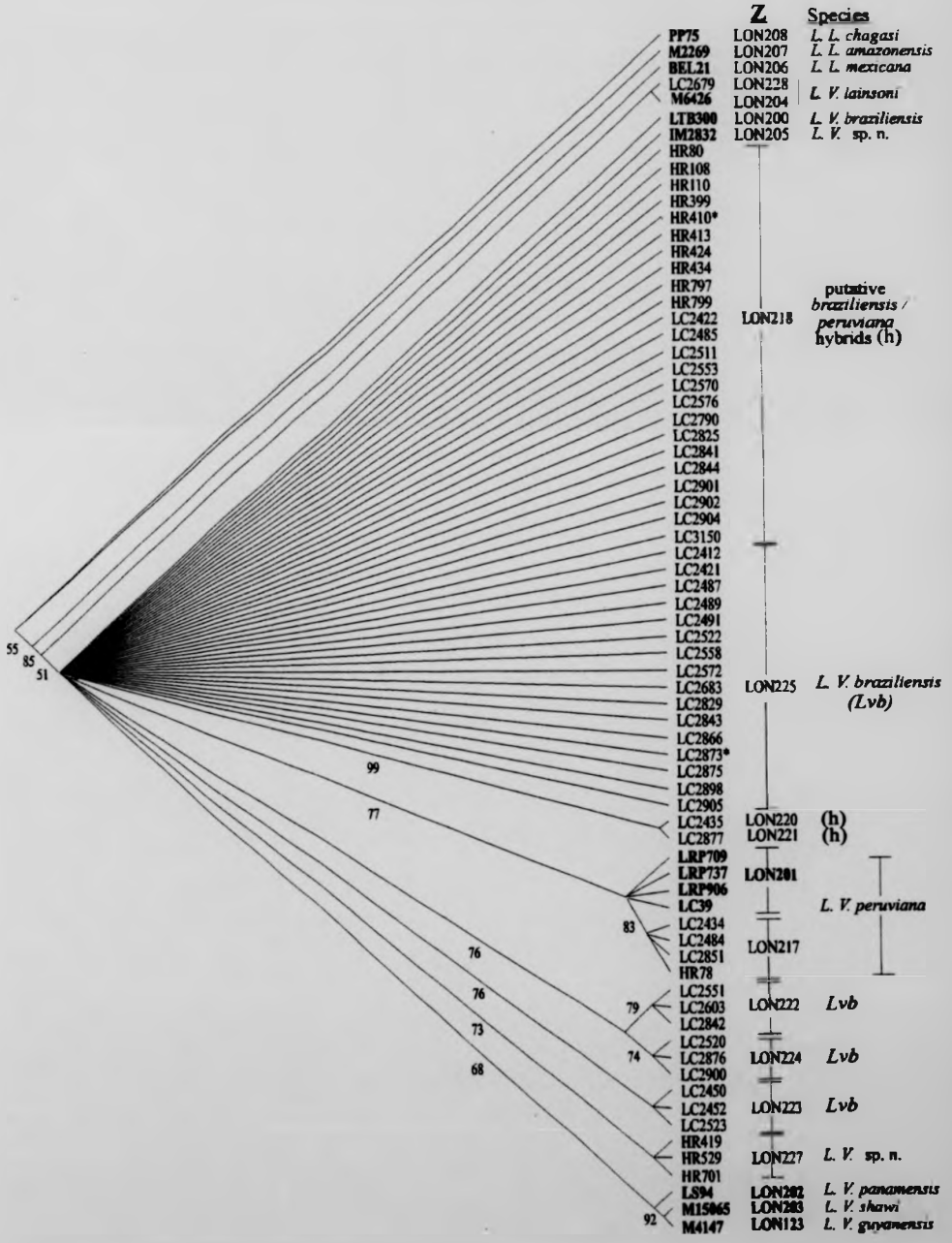
Reference strains of the *L. V. guyanensis* complex (*L. V. guyanensis*, M4147; *L. V. shawi*, M15065 and *L. V. panamensis*, LS94) formed a clade (68% support): the close relationship reported between *L. V. shawi* and *L. V. guyanensis* (Shaw *et al.*, 1991) is supported here, by 92% of quartets.

All strains and stocks identified as *L.V. peruviana* were identified in a discrete clade (77% quartet support), with the *L. V. peruviana* stocks from Huánuco (LON217) separate from the reference strains (LON201) of this species (83% support).

The *L. V. braziliensis* (LON225) and putative hybrid (LON218) zymodemes were supported by less than 50% of quartets: stocks belonging to each of these zymodemes thus have separate (collapsed) branches and do not appear as a discrete cluster. Several clusters can, however, be seen for other Huánuco *L. V. braziliensis* and putative hybrid stock zymodemes. Stocks from *L. V. braziliensis* zymodeme LON223 forms a discrete cluster (76% quartet support). Two *L. V. braziliensis* zymodemes, LON222 and LON224, form a clade (76% support) with stocks from each zymodeme clustering as a group (79% and 74% support, respectively). Two putative hybrid stocks (LC2435 - LON220 and LC2877 - LON221) also formed a clade (99% support). The separate standing of these stocks is likely due to the absence of bands at 2 enzyme loci (see section 6.3).

Figure 6.5.1.1 (next page): Cladogram showing the results of quartet puzzling for Huánuco stocks and reference strains based on isoenzyme data. Values on branches are "quartet supports", figures which are similar to bootstraps (Strimmer & von Haeseler, 1996). These values are the percentage support of each branch, representing the number of quartets (n = 1000) supporting each given branching order. Branches with less than 50% support have been collapsed. Branch lengths are arbitrary. Zymodemes and species designations are indicated. "h" indicates stocks which were putative *braziliensis / peruviana* hybrids. "*" indicates LON219 (HR410) and LON226 (LC2873).

***Figure 6.5.1.1*:** **Cladogram derived from quartet puzzling of isoenzyme data for Huánuco stocks and reference strains.**



As with stocks from previous chapters, isoenzyme data from the Huánuco stocks was also analyzed using a similarity approach. Dendrograms were constructed by UPGMA using the SYN-TAX-pc package (Podani, 1993). *Figure 6.5.1.2* shows the dendrogram constructed for these stocks from a similarity coefficient matrix. Stocks are classified according to similarity (range 0 - 1).

Greater resolution is obtained using this analysis with each *Viannia* species-complex being identified. Similarly to its position in *figure 6.5.1.1*, the *L. V. lainsoni* stock LC2679 clusters with the reference strain M6426: using this analysis, however, these stocks show more similarity to, and cluster with, reference strains from the *Leishmania* subgenus. Reference strains of the *L. V. guyanensis* complex form a discrete cluster, again with *L. V. shawi* and *L. V. guyanensis* sharing more similarity than with *L. V. panamensis*.

In contrast to its position in *figure 6.5.1.1*, the reference strain and potential new *Viannia* species IM2832 (*L. V.* sp. n., Grimaldi *et al.*, 1991) clearly forms a distinct group, together with the Huánuco dog stocks HR419, HR529 and HR701. This group clusters between the *L. V. braziliensis* and *L. V. guyanensis* species complexes.

The majority of Huánuco stocks were identified as belonging to the *L. V. braziliensis* complex, i.e. were *L. V. braziliensis*, *L. V. peruviana* or putative hybrids thereof. Using the UPGMA approach, based on IEA data, all stocks from each zymodeme were shown to cluster together. Four major clades are evident: clades 1 and 4 contain *L. V. braziliensis* stocks (comprising stocks from 4 and 2 zymodemes, respectively), clade 3 contains *L. V. peruviana* stocks (from 2 zymodemes) and clade 3 contains all putative *braziliensis / peruviana* hybrid stocks (from 4 zymodemes). The *L. V. braziliensis* clade 4 (containing stocks from zymodemes LON225 and LON226) falls outside the clades containing the reference strains of both *L. V. braziliensis* (LON200) and *L. V. peruviana* (LON201).

Figure 6.5.1.2 (next page): UPGMA dendrogram constructed from the similarity coefficient matrix for Huánuco stocks and reference strains based on isoenzyme data. Similarities between zymodemes (y-axis, range 0-1) were calculated, using SYN-TAX-pc (Podani, 1993), by averaging the Jaccard coefficients calculated from data from twelve enzyme loci. Stocks / strains marked with * are reference strains. Zymodemes (LON numbers) are indicated on branches. "h" indicates putative *braziliensis / peruviana* hybrid stocks.

Banuls *et al.* (1999), based on data from IEA and RAPD analysis, argue that the species status of *L. V. peruviana* is valid. In their analyses, all *L. V. peruviana* stocks and strains clustered into a discrete typing unit (DTU) which showed "a clear structuring between the 2 species". In this analysis, based on IEA data, *L. V. peruviana* strains and stocks, whilst forming a discrete clade (clade 3), did so nestled amongst the *L. V. braziliensis* clades (1 and 4). This observation does not, therefore, support their suggestion that *L. V. peruviana* is an indisputable species in its own right.

6.5.2 Phylogenetic analysis of sequence data from AC01

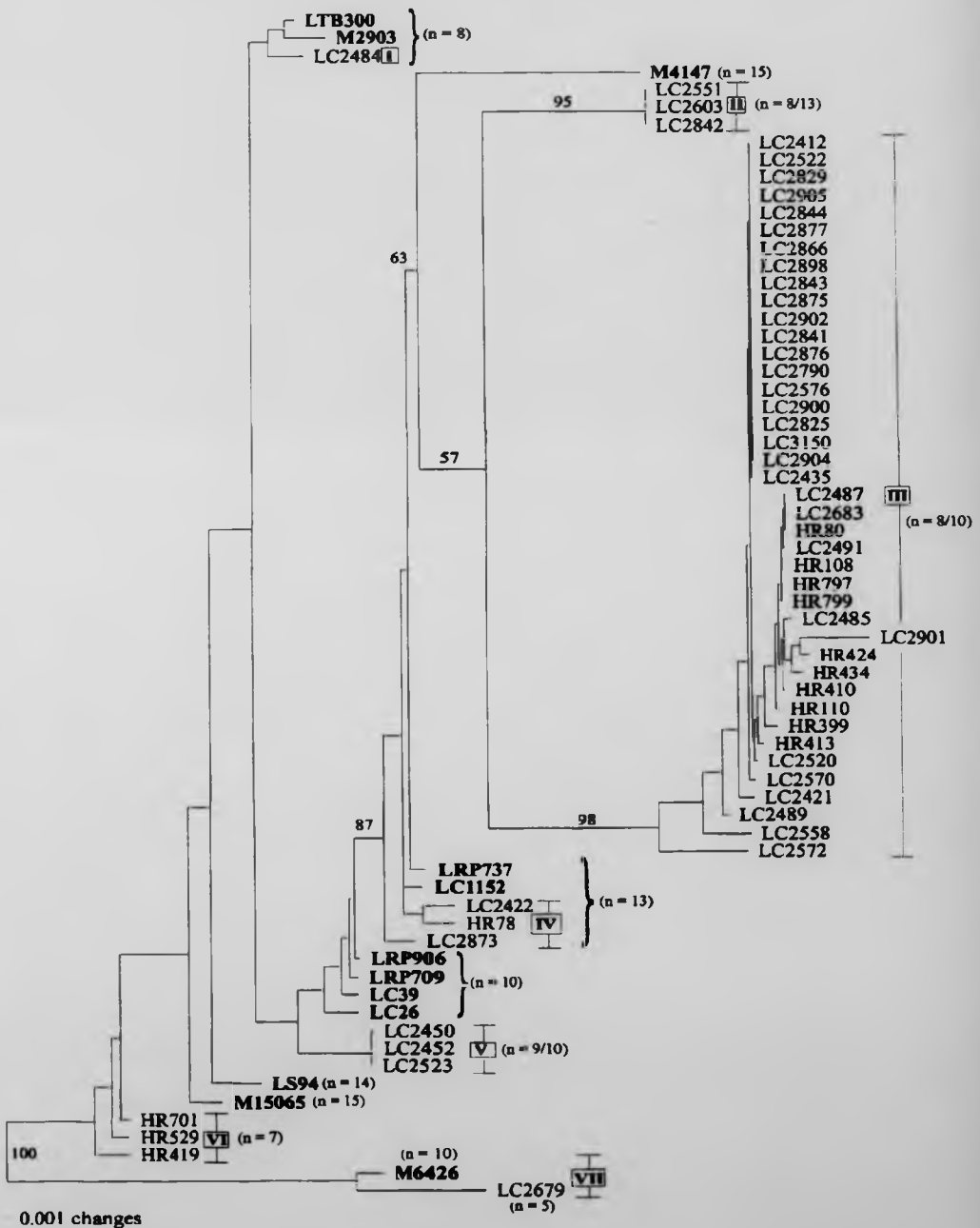
Molecular phylogenies for sequence data from the AC01 microsatellite locus were constructed as previously described. *Figure 6.5.2* shows an unrooted phylogram of the neighbour-joining consensus tree for the Huánuco stocks and reference strains based on sequence data from the microsatellite locus AC01. The reliability of tree topologies was assessed by the bootstrap method (n = 1000).

The interpretation of this phylogram in meaningful terms is difficult bearing in mind discussions from previous chapters regarding the applicability of current software for analyzing sequence data from microsatellite loci. Nevertheless, several clusters are evident which will be discussed briefly.

The phylogram was drawn using the *L. V. lainsoni* reference strain M6426 as an outgroup. *L. V. lainsoni* was used as the most distinct species in the *Viannia* subgenus (the PCR primers used to amplify this microsatellite locus do not amplify subgenus *Leishmania* stocks or parasites from other genera (Russell *et al.*, 1999). The Huánuco *L. V. lainsoni* stock, LC2679, grouped closely with the reference strain in cluster **VII**.

Figure 6.5.2 (next page): unrooted phylogram of the neighbour-joining consensus tree for the Huánuco stocks and reference strains based on sequence data from the microsatellite locus AC01. Numbers indicate the percentage of bootstrap support for each branch. The scale bar represents 0.001 (0.1%) nucleotide changes per sequence position. Code names in **bold** type indicate reference strains. Numbers in parentheses indicate the number of dinucleotide repeats (n) scored (for the reference strains of *L. V. guyanensis*, M4147, and *L. V. shawi*, M15065, the repeats are not perfect). The species designations for, and explanation of, clusters are given in the text.

***Figure 6.5.2*:** **Phylogram based on sequence data from the microsatellite locus AC01 for Huánuco stocks and reference strains.**



The three dog stocks, HR419, HR529 and HR701, shown using IEA to be closely related to the *L. V.* sp. n. reference strain IM2832, cluster together (cluster **VI**) and, like *L. V. lainsoni* stocks, are distinct from strains and stocks of the two main *Viannia* subgenus.

Using this analysis, two of the reference strains from the *L. V. guyanensis* complex (*panamensis* - LS94 and *shawi* - M15065) are distinct from members of the *L. V. braziliensis* complex, however, the *guyanensis* reference strain (M4147) nestles between *L. V. peruviana* stocks and the majority of Huánuco stocks. From looking at the AC01 sequence alignment (*figure 6.4.4.1*), this is likely due to ambiguity in the downstream sequence of all of these stocks.

Cluster **I** contains 2 *L. V. braziliensis* reference strains (LTB300 and M2903) which, like the vast majority of *L. V. braziliensis* stocks, had alleles with n = 8. As discussed previously, the only Huánuco stock with n = 8 was LC2484, which was identified using IEA as *L. V. peruviana* and not *L. V. braziliensis*. The vast majority of Huánuco stocks grouped together in cluster **III**. This cluster contained all stocks which were heterozygous for alleles containing 8 and 10 dinucleotide repeats. As discussed previously, the stocks in this cluster were identified using IEA as *L. V. braziliensis* (18) and putative *L. V. braziliensis* / *L. V. peruviana* hybrids (23). Cluster **II** contains all 3 LON222 stocks which were heterozygous for AC01 with alleles containing 8 and 13 dinucleotide repeats. The remaining Huánuco stocks and all *L. V. peruviana* reference strains formed a distinct group outside of clusters **II** and **III**. All Huánuco stocks with an AC01 allele where n = 13 were grouped together in cluster **IV** and were most closely related to the 2 *L. V. peruviana* reference strains (LC1152 and LRP737) which also had n = 13. As discussed previously, only one of these 3 stocks had been identified as *L. V. peruviana* using IEA. The final group of Huánuco stocks formed cluster **V** which comprised all stocks belonging to LON223 (*L. V. braziliensis*) which each had 2 AC01 alleles containing 9 and 10 dinucleotide repeats.

Thus, the relationships between Huánuco stocks from the phylogenetic analysis of AC01 microsatellite sequence data shows that these stocks comprise a complex population. Several groupings (i.e. clusters **II**, **V** and **VI**) however, correlate with those identified using IEA data and hence are likely "true" groups of stocks. Some stocks did not, however, cluster with the same stocks as had been observed from the

phylogenetic analysis of IEA data. These observations may be explained by several points. Firstly, as reported by Russell *et al.* (1999), these microsatellite markers do not always correlate with *Leishmania* species status as determined using IEA. Secondly, there is no reason why microsatellite alleles should be homozygous in an individual homozygous for enzyme loci, or indeed vice versa. Lastly, as discussed previously, the phylogeny constructed from the analysis of microsatellite data is likely to be inherently inaccurate owing to the lack of an appropriate and applicable model for such loci.

Other molecular markers have been used to construct phylogenies of *Viannia* stocks in which *L. V. peruviana* formed a discrete DTU [karyotyping, Dujardin *et al.*, 1993b, 1995b; *gp63* gene analysis, Victoir *et al.*, 1998; RAPD analysis, Dujardin *et al.*, 1995b and Banuls *et al.*, 1999; 2000]. However, the Huánuco stocks have only recently become available and hence could not have been included in previous studies. It would be interesting to see the phylogenetic positions of these stocks, particularly those of zymodemes LON225 and LON226, using data from the molecular markers and techniques employed by the aforementioned authors.

6.6 Population genetic analyses

It has been proposed that *Leishmania* have a clonal population structure, with sexual reproduction rare or absent in natural populations (Tibayrenc *et al.*, 1990). To test this hypothesis, data from the Huánuco stocks were examined for evidence of segregation and recombination, basic consequences of sexual reproduction, using standard tests. The validity of using these approaches for the analysis of data from these organisms is discussed, in context with the results, in section 6.6.3.

6.6.1 Test for Hardy-Weinberg equilibrium

Observations of deviations from Hardy-Weinberg (HW) equilibrium, fixed heterozygosity and "missing" genotypes indicate the absence of possible segregation and therefore suggest a clonal population structure. Here, Hardy-Weinberg (HW) exact tests were used to provide evidence for segregation at 10 loci: HW analysis was not possible at 4 enzyme loci as they were monomorphic for all stocks, HW analysis was not carried out on data from the microsatellite locus AC52 because data were missing from many stocks. Exact P-values describing departure from HW equilibrium (panmixia) were calculated as described in chapter 2, by comparing observed and expected genotype frequencies.

Observed and expected allele and genotype frequencies for the Huánuco stocks based on IEA and microsatellite data, respectively, are given in *tables V-1* and *V-2 (Appendix V)*. The Huánuco stock identified as *L. V. lainsoni*, LC2679, was excluded from all analyses as it was distinct from the other stocks at almost every enzyme locus. The three stocks identified as IM2832-type (HR419, HR529 and HR701), proposed to be a new *Viannia* species (Grimaldi *et al.*, 1991), were included in the analysis because the alleles scored for these stocks were identical to those of the *L. V. braziliensis/L. V. peruviana* stocks at 2 polymorphic loci (NHi1 and NHd) and 4 monomorphic loci (NHi2, 6PGD, ASAT and ALAT). The polymorphism observed at 3 loci (PGM, GPI and G6PD) was due entirely to these stocks, which were homozygous for a different allele to that of the *L. V. braziliensis / L. V. peruviana* stocks. At 2 loci, ES and PEPD, the IM2832-type stocks were homozygous for a different allele to that observed in *L. V. braziliensis / L. V. peruviana* stocks homozygous at these loci. However, many *L. V. braziliensis / L. V. peruviana* stocks were heterozygous for these loci, with one allele apparently from the IM2832-type stocks. *Table 6.6.1* summarizes the results of

HW exact tests, by locus, for IEA and microsatellite data from these stocks. For each locus, an exact P-value is given to describe the probability of departure from HW equilibrium having occurred by chance. In addition, the observed and expected numbers of heterozygotes are shown along with a calculation of the fixation index (Fis, Weir & Cockerham, 1984).

Table 6.6.1. **Summary of Hardy-Weinberg exact test results†, by locus, for IEA and microsatellite data from Huánuco stocks±**

Locus	N	A	Deviation from HW†	exact P-value	Heterozygotes		Fis* (W & C)
					Observed	Expected	
MPI	58	5	No	0.5212	32	28.287	-0.133
NHi1	58	2	Yes	0.0010	35	24.652	-0.425
NHd	58	2	No	0.0508	26	20.348	-0.281
ES	58	2	No	0.4035	16	17.983	+0.111
PEPD	58	2	No	0.7115	19	19.783	+0.040
PGM	58	2	Yes	0.0000	0	5.740	+1.000
GPI	58	2	Yes	0.0000	0	5.740	+1.000
G6PD	58	2	Yes	0.0000	0	5.740	+1.000
AC01^{M}	53	8	Yes	0.0000	47	36.581	-0.288
AC16^{M}	52	10	Yes	0.0000	18	37.854	+0.527
NHi2	58	1	only one allele, analysis not possible				
6PGD	58	1					
ASAT	58	1					
ALAT	58	1					

± analysis excludes *L. V. lainsoni* stock LC2679.

N Number of stocks analyzed for locus

A Number of alleles scored at locus from all stocks

† HW exact tests - results were computed using Genepop (Raymond & Roussett, 1995), see Chapter 2 for details. Deviation from HW equilibrium assumes a 5% confidence interval (i.e. $P < 0.05$). P-values: a complete enumeration was determined for loci with up to 4 alleles and an estimation of exact P-value was determined by the Markov chain method for loci with more than 4 alleles.

* Fis: Within-population fixation index: $= \frac{He - Ho}{He}$

where He is the expected number of heterozygotes and Ho is the observed number of heterozygotes. Range of Fis = $-1 > 0 < +1$. +1 indicates that all stocks were homozygous at that locus. A negative value indicates an excess of heterozygotes. Fis computed, using Genepop, as in Weir & Cockerham (W & C, 1984).

Clonal reproduction, by definition, generates individuals identical to the original clone (assuming no mutation). Observations of: 1. deviation from Hardy-Weinberg (HW) equilibrium, 2. fixed heterozygosity and 3. the absence of possible segregation types are all evidence of clonal expansion. Results from these tests are discussed by the above points:

1. Deviation from Hardy-Weinberg (HW) equilibrium:

Analysis of the Huánuco stocks (10 loci, were amenable to statistical testing, see *table 6.6.1*) has shown that 3 enzyme loci (MPI, ES and PEPD) showed no deviation from Hardy-Weinberg (HW) equilibrium ($P > 0.05$) suggesting segregation of alleles at these loci. 1 enzyme locus (NHd) showed marginal deviation at the 5% level. The remaining 6 loci, however, (4 enzyme and both microsatellite loci) showed considerable deviation ($P < 0.001$) from HW equilibrium indicating that alleles at these loci were not segregating, an observation suggestive of clonal propagation.

2. Fixed heterozygosity:

Heterozygosity appears not to be totally fixed. Although several multilocus genotypes were sampled repeatedly (suggestive of clonal expansion), many other combinations were also identified, albeit less frequently. In addition, not all heterozygotes were identical. An unusual finding in this population is the observation of excess heterozygotes (denoted by a negative Fis value). Excess heterozygosity is a rare phenomenon which suggests the increased fitness of the heterozygote form. This finding will be discussed further in section 6.6.3.

3. The absence or lack of possible segregation genotypes.

The absence or lack of possible segregation genotypes (see *table V-1, Appendix V*) was evident but particularly at loci where more than 2 alleles were scored (and therefore particularly at microsatellite loci). As a result of large numbers of alleles being scored, the expected numbers of many genotypes at several loci were very small. Given that some alleles (enzyme and microsatellite) were rare and that a relatively small number of stocks were analyzed, it is not surprising that some genotypes were not sampled. Thus, the lack of all possible segregation genotypes here is not necessarily the result of a lack of segregation, but possibly due to the small sample size.

6.6.2 Tests of linkage disequilibrium

Tests of linkage disequilibrium (LD, non-random association of alleles) between alleles at different loci can be used to provide evidence for recombination. As with segregation, evidence of a lack of recombination suggests that the population is not mating randomly and hence may have a clonal population structure. Here, the occurrence of recombination between loci was examined using three statistical tests for detecting LD: the Index of Association (I_A; Maynard-Smith *et al.*, 1993); the D' index (Lewontin, 1964) and the r^2 (square of the correlation coefficient) index (Hill and Robertson, 1968). The tests differ in that the I_A index examines the data for evidence of LD in the population over all loci, whereas the D' and r^2 indices examine pairwise combinations of loci and hence may be able to pinpoint which loci are involved in generating any LD.

6.6.2.1 Index of Association (I_A)

The Index of Association (I_A) has been proposed as a method for characterizing the population structures of microorganisms (Maynard-Smith *et al.*, 1993). The index is a relatively simple measurement of the degree of association between loci in a given population: I_A is expected to be zero in a large panmictic population. Values of I_A which differ significantly from zero may represent departure due to linkage disequilibrium (LD).

In addition to the I_A value obtained for the population as a whole, Maynard-Smith *et al.* (1993) state that this test can also be used to elucidate underlying population structures. LD may, they argue, be due in part to the presence of sub-groups (which may consist of, for example, discrete taxonomic units or geographically-separated populations) in the population. Linkage disequilibrium observed in the population as a whole may be resolved by calculating the I_A values of these sub-groups. It may be shown from the I_A values of the latter, that one or more subgroups is/are responsible for the LD in an otherwise clonal population. In addition, these authors suggest that the bias due to the presence of over-represented clones can be removed by comparing the I_A value obtained for all individuals in a population with the I_A values obtained from analyzing only the electrophoretic / microsatellite types (ETs or MSTs) observed.

So, according to Maynard-Smith *et al.* (1993), the structure of a given population may be categorized by comparing values of I_A calculated for the whole population, sub-groups and electrophoretic types thereof. Different types of population structure are described including "true" clonality, "epidemic" clonality, and panmixia. Organisms exhibiting true clonality generate clones which are stable in space and time, organisms exhibiting epidemic clonality undergo occasional bouts of clonal propagation although the species is basically sexual. Using I_A values, parasitic protozoa have been categorized (see Maynard-Smith *et al.*, 1993) as clonal (*Trypanosoma cruzi*), epidemic (*T. brucei*) and epidemic/panmictic (*Plasmodium falciparum*).

The equations required to calculate I_A values (taken from Maynard-Smith *et al.*, 1993) are given in *Appendix V*, with allele frequencies of whole Huánuco population and of subgroups therein by locus (*table V-3*). I_A values were calculated for all individuals and for 4 sub-groups: sub-groups were based on taxonomic groups identified using IEA (subgroup 1 [SG1] - stocks identified as *L. V. braziliensis*; SG2 - stocks identified as *L. V. peruviana*; SG3 - stocks identified as putative *L. V. braziliensis / L. V. peruviana* hybrids; SG4 - stocks identified as IM2832-type). I_A results are given in detail in *table V-4 (Appendix V)* and summarized in *table 6.6.2.1*, along with I_A values from previous studies involving trypanosomatids.

***Table 6.6.2.1*:** **I_A values for Huánuco *Leishmania* stocks and for other trypanosomatids.**

Parasite	No. of isolates	I_A	Reference
Leishmania Viannia	58	**0.97** (all loci) **0.59** (enzyme loci) **2.77** (microsat. loci)	present work
T. cruzi	524	2.63 +/- 0.06	Zhang *et al.* (1988)
T. brucei	321	0.45 +/- 0.007	Mihok *et al.* (1989)
	73	0.53 +/- 0.003	Stevens & Tibayrenc (1996)
	67	1.14 +/- 0.002	Stevens & Tibayrenc (1996)
	44	0.80 +/- 0.009	Stevens & Tibayrenc (1996)
	142	0.30 +/- 0.00	Stevens & Tibayrenc (1996)

The I_A values for the Huánuco *Leishmania* stocks obtained for enzyme loci and for all loci combined are relatively small and comparable to values obtained for *Trypanosoma brucei* (Mihok *et al.*, 1989; Stevens & Tibayrenc, 1996). *T. brucei* is considered to exhibit epidemic clonality, i.e. undergoing occasional bouts of clonal propagation although the species is basically sexual. The I_A value for microsatellite loci (2) is more comparable to that of *T. cruzi* (Zhang *et al.*, 1988) which is considered to exhibit true clonality, i.e. generating clones which are stable in space and time. The I_A value calculated from microsatellite data for the Huánuco *Leishmania* stocks is, however, higher owing to the absence of possible genotypes. It is not possible to determine whether these genotypes are actually missing or have not been sampled since the sample size was too small to score all the possible, rare genotypes from these hypervariable loci.

It is noteworthy that different levels of taxonomic speciation are employed by the various researchers of these genera. Strains of *T. cruzi*, for example, are known to be as different genetically as some species of *Leishmania* (Miles *et al.*, 1983). Researchers studying *Leishmania* have tended to liberally impose species status based on distinct clinical presentation and geographical location, despite the fact that the parasites involved are genetically very similar. This discordant level of taxonomic speciation complicates comparison of I_A values between different genera.

In an attempt to pinpoint any groups of stocks responsible for the reduced I_A values, the population was divided into 4 taxonomic subgroups (see *table V-4, Appendix V*). The validity of this approach for these stocks is questionable since the sample sizes of subgroups 2 and 4 were very small and contained only 1 electrophoretic type, making further analysis impossible. The overall I_A values for those subgroups which could be analyzed increased from the value calculated for all stocks together, possibly indicating that stocks in each subgroup had a clonal structure but that some exchange had occurred between groups. One of the subgroups (SG3), for example, appeared to be products of a clonal expansion following a recombination event between 2 supposed species (*L. V. braziliensis* x *L. V. peruviana*).

According to Maynard-Smith *et al.* (1993), it is possible to distinguish "true" and "epidemic" clonality by comparing the I_A values from the whole population with those

of the electrophoretic and microsatellite types, thereby eliminating the bias from over-represented clones. If the I_A value is reduced in the latter it is taken that the organism exhibits epidemic clonality.

Accordingly, I_A values of ETs and MSTs were calculated using enzyme and microsatellite data for the whole population and for each subgroup (see *table-V-4, Appendix V*). I_A was found to increase in all but one instance. According to Maynard-Smith *et al.* (1993), in the cases where I_A was found to increase, this would be interpreted as indicative of clonal structure masked in the whole population analysis by a superabundance of clones. In a single case the I_A value decreased when comparing the I_A value of all stocks with that of just the ETs (IEA data, SG1); this suggests that the stocks within this SG are not necessarily clonal but, perhaps the products of genetic exchange event(s).

These results reinforce the note of caution sounded by Stevens and Tibayrenc (1996), regarding the danger of inadvertently combining inappropriate data from distantly related (but apparently similar) groups of stocks. If this analysis is correct (and which is by no means clear to the author), the results indicate that the Huánuco "population" in fact contained several different populations of stocks which might more properly be considered separately. Furthermore, the structure of each subgroup that was amenable to analysis was found, bar one, to be clonal (again, assuming that this analysis is correct), even after the removal of apparently over-represented clones.

6.6.2.2 D' and r^2 indices

The D' index (Lewontin, 1964) and the r^2 (square of the correlation coefficient) index (Hill and Robertson, 1968) provide a more conventional examination for evidence of LD because they compare observed and expected data from pairwise combinations of loci. As a result, both tests can "pinpoint" where linkage, if any, occurs. For a comprehensive explanation and description of D' and r^2 indices, see Hedrick (1985).

The D' index is a measure of the disequilibrium (D) between the most common allele at each of two loci compared to its theoretical maximum (Dmax). D' has a potential range between +/- 1.0. Zero indicates no linkage; non-zero values signify incomplete linkage and 1 denotes complete linkage (i.e. the loci are always inherited together).

The + or - value reflects which alleles at the loci were compared. Since more than 2 alleles may be scored at a locus, a negative value may arise if the allele frequency of the most common allele is less than the sum of the remaining allele frequencies.

The regression correlation coefficient, r^2, provides a more conservative analysis (i.e. approaches 0 or 1 slowly) than D' because r^2 analysis is not skewed by a missing gametic type. The r^2 index takes into account the level of significance (P value) and has a range of 0 to 1.0 (0 indicating no linkage and 1 indicating complete linkage).

Calculations of D' and r^2 values require the identification of all gametic types (as opposed to allelic types for segregation tests at a single locus). It is assumed for these analyses that *Leishmania* are diploid at all loci examined and that all alleles, at each locus, are co-dominant (see introduction). The gametes which make up each of the observed phenotypes can be identified except for the two types present in the double heterozygote which, because the alleles are co-dominant, are indistinguishable (see notes in *Appendix V*). Hence D' and r^2 calculations are based on maximum-likelihood estimates of gametic frequencies. The equations required to calculate D' and r^2 values are given in *Appendix V*.

D' and r^2 values were calculated for pairwise combinations of loci. Only those polymorphic loci where the frequency of the most common allele was less than 0.90 were, used for analyses of LD because these loci were highly informative statistically. A test of significance (Q) was calculated for each D score from which probability (P) values were assigned. Results were scored as not significant at a P value of > 0.05 (i.e. more than 5% probability that the result could have occurred by chance). D' and r^2 results are summarized in *table 6.6.2.2* overleaf.

The majority of pairwise combinations (see *table 6.6.2.2*) generated high D' values indicating linkage and suggesting that the inheritance of those locus combinations resulted from clonal reproduction. Seven pairwise combinations, however, showed a deviation ($D' < 0.7$) from complete linkage (D' approximately = 1), of which only 3 (MPI x NHi1, AC16 x NHi1 and AC16 x NHd) gave values which were statistically-significant. These D' values were, however, still large compared to values reported for LD in *Plasmodium falciparum* (Conway *et al.*, 1999).

Table 6.6.2.2 **Summary of linkage disequilibrium results†, by pairwise locus combination, for IEA and microsatellite data from Huánuco stocks±**

2-locus combination†	N	D'	P value	r^2
MPI^{6} x NHi1^{2}	**58**	**0.687**	**Sig. P < 0.005**	**0.383**
MPI^{6} x NHd^{4}	58	1.000	Sig. P < 0.005	0.548
MPI^{6} x ES^{4}	58	- 0.579	Not Sig.	0.041
MPI^{6} x PEPD^{2}	58	- 0.644	Not Sig.	0.060
NHi1^{2} x NHd^{4}	58	1.000	Sig. P < 0.005	0.664
NHi1^{2} x ES^{4}	58	- 0.976	Sig. P < 0.025	0.097
NHi1^{2} x PEPD^{2}	58	- 0.981	Sig. P < 0.01	0.115
NHd^{4} x ES^{4}	58	- 1.000	Sig. P < 0.05	0.068
NHd^{4} x PEPD^{2}	58	- 1.000	Sig. P < 0.05	0.080
ES^{4} x PEPD^{2}	58	0.946	Sig. P < 0.005	0.763
AC01^{227} x MPI^{6}	53	1.000	Sig. P < 0.005	0.313
AC01^{227} x NHi1^{2}	53	1.000	Sig. P < 0.005	0.313
AC01^{227} x NHd^{4}	53	1.000	Sig. P < 0.005	0.241
AC01^{227} x ES^{4}	53	1.000	Sig. P < 0.005	0.185
AC01^{227} x PEPD^{2}	53	1.000	Sig. P < 0.005	0.194
AC16^{244} x MPI^{6}	52	- 0.333	Not Sig.	0.070
AC16^{244} x NHi1^{2}	**52**	**- 0.489**	**Sig. P < 0.01**	**0.146**
AC16^{244} x NHd^{4}	**52**	**- 0.576**	**Sig. P < 0.01**	**0.129**
AC16^{244} x ES^{4}	52	0.882	Sig. P < 0.01	0.145
AC16^{244} x PEPD^{2}	52	0.805	Sig. P < 0.01	0.143
AC01^{227} x AC16^{244}	51	0.191	Not Sig.	0.032

† number in superscript after each enzyme locus indicates the most common allele (A_1) used for all calculations (see *Appendix V*).

Complete linkage is represented by an r^2 index of 1; the r^2 values for the 3 pairs of loci which generated a statistically-significant, non-1 value for D' were all less than 0.400. These low values suggest that incomplete linkage was present between these pairs of loci.

The calculated r^2 values were generally low compared to D' values. The D' and r^2 indices are not directly comparable, however, because the latter is calculated using the value of D (not D', which is effectively the maximum D possible from the Dmax). The low r^2 values obtained in this analysis illustrate the lack of statistical power due to the relatively small sample size. The small sample size is of particular relevance to the pairwise combinations involving microsatellite loci since many alleles were present at these loci.

6.6.3 Summary of population genetic analyses

The results herein provide evidence for a degree of genetic exchange having occurred amongst Huánuco stocks. This evidence stems from 3 observations:

1. segregation: a lack of deviation from HW equilibrium was observed at 3 enzyme loci (MPI, ES and PEPD);
2. linkage disequilibrium: relatively low I_A values were obtained for enzyme and combined loci;
3. linkage disequilibrium: D' values of significantly less than 1 were obtained for 3 pairwise locus combinations (MPI x NHi1; AC16 x NHi1 and AC16 x NHd, shown in **bold** type in *table 6.6.2.2.*)

The finding of evidence for genetic exchange is, perhaps, the result of the short time period and the small geographical area over which the samples were isolated (evidence of exchange between samples collected from wide geographical areas is less likely).

Population genetic analyses were carried out here primarily to provide statistical support for the proposed occurrence of genetic exchange among these stocks, however, these tests also give an insight into the prevalent mode of reproduction occurring in the population. The combined findings suggest the population structure is predominantly clonal, despite at least one recombinational event. Observations of stocks with different zymodemes sharing microsatellite markers, and vice versa,

emphasizes the fact that these genetic markers were independent. The expansive clonal propagation of one of the putative hybrid types suggests an increased fitness relative to the parental strains present.

The results presented indicate that genetic exchange as a method for generating genetic diversity cannot be rejected. There are, however, several doubts as to the applicability of classical population genetic analyses to *Leishmania* and to this population specifically. *Leishmania* reproduce in a predominantly clonal manner whereas classical analyses assume a starting point of random mating. Segregation tests are suitable for examining data from organisms of known, fixed, ploidy. Whilst *Leishmania* are thought to be diploid at most housekeeping (i.e. enzyme) loci, there have been reports using karyotype analysis which suggest a multiclonal structure / aneuploidy (Pages *et al.*, 1989; Bastien *et al.*, 1990) and a recent report has described multiclonal strains, identified by the analysis of microsatellite loci, in the related trypanosomatid, *Trypanosoma cruzi* (Oliveira *et al.*, 1998). Population genetic tests have traditionally been carried out on data from a single species; the *Leishmania* stocks analyzed here comprised at least 2 notional (very closely-related) species and putative hybrids thereof. It would not have been possible to analyze each group individually in a manner which was statistically meaningful.

6.7 Discussion of the Huánuco population

By comparison with the Brazilian and Nicaraguan populations, a tremendous level of diversity was identified among the Huánuco stocks. The results and analyses described indicate that this may be due, in part at least, to genetic exchange. [As discussed previously, there is accumulating evidence for the occurrence of genetic exchange in *Leishmania*. Possible reasons why genetic exchange does not occur (or is not identified) more frequently are discussed in greater detail in chapter 7.]

The Huánuco stocks showed a remarkable degree of diversity: 12 zymodemes were identified from 12 enzyme loci and 17 microdemes identified from 3 microsatellite loci. In addition, 9 AC01 sequence types were scored. Several of the inferred genotypes were sampled only once whereas others were repeatedly sampled from both geographically-separate locations and different host species.

Using IEA, 3 species and a new, as yet unnamed, parasite, *L. V.* sp. n. (type strain IM2832, Grimaldi *et al.*, 1991) were found to occur sympatrically in the region. This is a remarkable finding in itself considering the small geographical area (circa 40km^2) concerned. Stocks were identified as: *L. V. peruviana* (4 stocks, 6.8%), *L. V. braziliensis* (25 stocks, 42.4%), *L. V. lainsoni* (1 stock, 1.7%) and *L. V.* sp. n. (3 stocks, 5.1%). In addition, 26 stocks (44.1%) were identified as putative *L. V. braziliensis* / *L. V. peruviana* hybrids.

A single human stock was identified as *L. V. lainsoni* : this is the second report of this species occurring in Peru (Lucas *et al.*, 1994). At the present time it cannot be excluded that this patient may have acquired *L. V. lainsoni* infection elsewhere.

Three dog stocks were almost identical to the type strain, IM2832, of a potential new species of the *Viannia* subgenus (*L. V.* sp. n., Grimaldi *et al.*, 1991). As far as I am aware, this is the first report of this *Viannia* phenotype occurring in Peru or in dogs. These stocks will be discussed in greater detail later.

L. V. peruviana was recorded in the Department of Huánuco prior to the current epidemic, however CL was seldom encountered and no cases of MCL were recorded (Llanos-Cuentas 1993). The recent introduction of *L. V. braziliensis* (Dujardin *et al.*, 1995b) has, without doubt, resulted in the current epidemic since it is this species, and hybrids consisting of this species and *L. V. peruviana*, which have been isolated from more than 85% of recent cases (this thesis; Dujardin *et al.*, 1995b). *L. V. braziliensis* may have been introduced by human immigration from another region or by human or canine intrusion into an unidentified sylvatic transmission cycle. It is recognized that *L. V. braziliensis* can be transmitted by a wider range of sand fly species than other New World *Leishmania* species (Killick-Kendrick, 1999). It seems likely therefore, that *L. V. braziliensis* rapidly adapted to the local vector species after introduction. Presumably, one or more local vector species and/or reservoir hosts (including humans) had a prior *L. V. peruviana* infection such that dual infection facilitated the occurrence of recombination. It is not known where (i.e. in host or vector) any such exchange would occur (see chapter 7 for discussion), however mixed *Leishmania* infections have been reported from both sand flies and humans (Barrios *et al.*, 1994; and Silveira *et al.*, 1984; Mehbratu *et al.*, 1991; Ibrahim *et al.*, 1994; Hernandes-Montes *et al.*, 1998, respectively).

Seventeen microdemes were scored from 54 Huánuco stocks: 3 microdemes were shared between different species / hybrid groups and 4 contained more than 1 stock. Microdemes did not always correlate with species status as identified using IEA. Stocks isolated from MCL lesions were assigned to 6 microdemes. Nine AC01 sequence types were scored from 55 Huánuco stocks: 1 sequence type was shared by several stocks identified as *L. V. braziliensis* and *L. V. braziliensis / L. V. peruviana* hybrids and 6 sequence types contained more than one stock. The numbers of zymodemes, microdemes and AC01 sequence types scored among the Huánuco stocks are summarized, by species type, in *table 6.7.1*.

Table 6.7.1 **Summary of zymodemes, microdemes and AC01 sequence types scored from Huánuco stocks, by species group.**

Species	No. stocks	No. zymodemes (59 stocks[a])	No. microdemes (54 stocks[b])	No. AC01 sequence types (55 stocks[c])
L. V. braziliensis	25	5	7[d]	4[f]
L. V. peruviana	4	1	2	2
L. b / L. pe hybrids	26	4	8[d,e]	2[f]
L. V. lainsoni	1	1	1[e]	1
L. V. sp. n.	3	1	2	1
Totals	**59**	**12**	**17[d,e]**	**9[f]**

Notes to *table 6.7.1*: [a] 59 stocks were isolated and examined; [b] 54 stocks could be assigned to a microdeme (see section 6.4.3.4); [c] AC01 sequence type was determined for all 55 stocks which amplified the AC01 locus; [d] 2 microdemes were shared by several stocks identified as *L. V. braziliensis* or *L. braziliensis / L. peruviana* (L. b / L. pe) hybrids; [e] 1 stock identified as a *L. braziliensis / L. peruviana* hybrid shared a microdeme with the Huánuco *L. V. lainsoni* stock; [f] several stocks identified as *L. V. braziliensis* and *L. braziliensis / L. peruviana* hybrids had an identical AC01 sequence type.

Of the 59 Huánuco stocks, 45 (76.3%) were of human origin and 14 (23.7%) were isolated from dogs. Eleven of the human stocks (24.4%) were isolated from mucocutaneous lesions. Six MCL stocks were identified as *L. V. braziliensis*, underlining the association of this species with MCL. A single stock isolated from an MCL lesion was identified as *L. V. peruviana*: this is an important finding linking yet another *Viannia* species with this clinical presentation. *L. V. braziliensis / L. V. peruviana* hybrids were also associated with MCL. The implication of these findings is that all patients from Huánuco infected with *L. V. braziliensis*, *L. V. peruviana* and

putative hybrids thereof, must be considered at risk of developing MCL. *Table 6.7.2* summarizes the host and clinical origin of the Huánuco stocks, by species group.

Table 6.7.2 **Summary of the host and clinical origin of the Huánuco stocks, by species group.**

Species	Humans			Dogs (CL)	No. stocks
	CL	MCL	Total		
L. V. braziliensis	19	6	25	0	25
L. V. peruviana	2	1	3	1	4
L. b / L. pe hybrids	12	4	16	10	26
L. V. lainsoni	1	0	1	0	1
L. V. sp. n.	-	-	0	3	3
Totals	**34**	**11**	**45**	**14**	**59**

Fourteen stocks were isolated from dogs: one was identified as *L. V. peruviana*, 10 as putative *L. V. braziliensis / L. V. peruviana* hybrids and 3 as *L. V.* sp. n. ('IM2832-type').

The finding here of identical *L. V. peruviana* and *L. V. braziliensis / L. V. peruviana* hybrid phenotypes in dogs and humans from the same area would seem to support the widely held belief that dogs can act as reservoir hosts of CL and MCL (a subject reviewed extensively by Reithinger & Davies, 1999). Of course, this finding may simply reflect the fact that dogs and humans are exposed to the same infective sand fly populations. However, several species types were found only in humans or dogs; for example, *L. V. braziliensis* was not isolated from dogs and the 'IM2832-type' *L. V.* sp. n. was not isolated from humans. The latter finding may suggest that dogs and humans are not, in fact, exposed to the same sand fly populations or that dogs and humans differ in their susceptibility to infection with the various parasite types present. The role of dogs as reservoirs of human infection cannot be proven conclusively without further ecoepidemiological investigation of the dynamics of the transmission cycle. Entomological studies are needed to investigate biting rates, host preferences, etc., further parasitological studies are needed to compare the genetic diversity of stocks from an extended vector and host range (to include dogs, humans, other domestic and sylvatic reservoirs and especially local sand fly fauna), and xenodiagnosis studies are

needed to investigate whether local sand flies can become infected from identified animal reservoirs.

The origin of the stocks identified as 'IM2832-type' is unclear. IM2832 itself is a sand fly isolate from Amazonas State, Brazil (Grimaldi *et al.*, 1991): to my knowledge, this strain has not been reported since its original discovery, from Brazil or from any other location. One can speculate that these 3 dogs, all from the same village, contracted this parasite strain after interrupting a previously undisturbed sylvatic cycle, perhaps on a foray into the forest. This phenotype was not sampled among the human stocks however so, if this speculation were true and assuming that dogs may be reservoirs of human infection, it would appear that *Lu. tejadai*, the sand fly species responsible for the peri-domestic transmission of the other species and hybrids in the study site, may not be capable of transmitting the IM2832-type strain. The 'IM2832-type' *L. V.* sp. n. dog stocks and the *L. V. lainsoni* stock LC2679 were shown, using phylogenetic analysis of IEA data, to be genetically distinct from Huánuco stocks of the two main *Viannia* subgenus complexes.

The geographical separation and distinct clinical presentations of CL and MCL in Peru have supported the concept that these diseases are caused by discrete *Leishmania* species (Shaw, 1994). *L. V. braziliensis* and *L. V. peruviana* stocks can now be differentiated using a wide range of phenotypic and genotypic approaches, (Arana *et al.*, 1990; Dujardin *et al.*, 1993b; 1995b; Victoir *et al.*, 1998; Banuls *et al.*, 1999; Russell *et al.*, 1999), nevertheless the two species are remarkably similar. In this study, phylogenetic analysis based on IEA data showed that *L. V. peruviana* strains and stocks formed a discrete clade nestled amongst the *L. V. braziliensis* clades; this observation contradicts that of Banuls *et al.*, (1999; 2000) who found that stocks of *L. V. peruviana* (not including those from Huánuco) formed a DTU.

The Huánuco population contained 26 putative *L. V. braziliensis / L. V. peruviana* hybrid stocks. In contrast to the *L. V. braziliensis / L. V. panamensis* hybrids from Nicaragua, the genotypes of these hybrids were not all simple combinations of the 'parental' genotypes present. At least 11 genotypes (A – K) were scored from these 26 stocks: these genotypes are summarized in *table 6.7.3*.

Table 6.7.3 **Summary of genotypes scored from putative *L. V. braziliensis* / *L. V. peruviana* hybrid stocks.**

Genotype	Zymodeme	Microdeme	Stocks	No. stocks
A	LON218	LMS43	HR399	1
B	LON218	LMS44	HR799	1
C	LON218	LMS45	LC2570	1
D	LON218	LMS46	HR108, HR110, HR413, HR424, HR434, LC2485, LC2576, LC2790, LC2841, LC2844, LC2901, LC2904, LC3150	13
E	LON218	LMS47	HR797	1
F	LON218	LMS48	LC2825	1
G	LON218	LMS52	HR80	1
H	LON218	LMS59	LC2422	1
I	LON219	LMS43	HR410	1
J	LON220	LMS45	LC2435	1
K	LON221	LMS46	LC2877	1
	LON218	ND	LC2511, LC2553	2
	LON218	NS	LC2902	1

Stocks in blue type were isolated from MCL lesions. ND indicates that microsatellite analysis was not done; NS indicates that a microdeme could not be assigned because Genotyper® analysis failed at 2/3 loci.

The majority of stocks (14/26) had genotype D. These stocks may have arisen from clonal expansion after a genetic exchange event between local strain(s) of *L. V. braziliensis* and *L. V. peruviana* and appear to have increased fitness compared to the parental strains. Increased fitness is evident from the excess of these heterozygous stocks compared to any genotypes scored from the 'parental' species. It is not known whether this increased fitness is due to greater virulence and/or pathogenicity or because it is transmitted with greater facility by the vector species in the area. It is noteworthy that, among hybrid stocks, MCL was associated only with genotype D. The other hybrid genotypes scored each contained a single stock. These stocks may have arisen by mutation from genotype D, or from separate genetic exchange events involving different combinations of loci.

CHAPTER 7 –
EXPERIMENTAL INVESTIGATION OF GENETIC EXCHANGE

7.1 Introduction

Genetic transformation involves introducing exogenous DNA into a host organism. The first transfection system for a trypanosomatid resulted in transient gene expression (Bellofatto & Cross, 1989). Stable transformation is dependent on genes which, when expressed, confer a constant selectable phenotype to the host organism. Stable transformation of a trypanosomatid was first described for *Leishmania* (Laban *et al.*, 1990; Kapler *et al.*, 1990).

A number of vectors that permit the stable expression of exogenous genes in *Leishmania* have now been described (Laban *et al.*, 1990; Cruz & Beverley, 1990; LeBowitz *et al.*, 1990, 1991; Coburn *et al.*, 1991; Panton., 1991; Kelly *et al*, 1992; Freedman & Beverley, 1993; Brooks *et al.*, 2000). Some of these vectors are also functional in other trypanosomatid genera e.g. *pTEX* functions in both *Leishmania* and *Trypanosoma cruzi* (Kelly *et al.*, 1992). Clayton (1999) has recently reviewed genetic manipulation of the Kinetoplastida.

Numerous selectable markers [parentheses give selective drug / method of selection] have been used in *Leishmania* including neomycin phosphotransferase II [G418], hygromycin B phosphotransferase [hygromycin B], *Sh ble* [phleomycin, zeocin], puromycin acetyltransferase [puromycin], N-acetyl glucosamine-1-phosphate transferase [tunicamycin], green fluorescent protein (GFP) of *Aequorea victoria* [fluorescence], thymidine kinase from the Herpes simplex virus, HSV [ganciclovir, negative selection] and blasticidin-S-deaminase [blasticidin S] (Kapler *et al.*, 1990; Laban *et al.*, 1990; Cruz *et al.*, 1991; Lee & van der Ploeg, 1991; Leowitz *et al.*, 1992, Liu & Chang, 1992; Freedman & Beverley, 1993; Ha *et al.*, 1996; Brooks *et al.*, 2000, respectively).

Transformation can be mediated by integration or by episomal (shuttle) vectors. For integration, specific targeting fragments are required in the vector to promote homologous recombination. Cruz & Beverley (1990) and Lee & van der Ploeg (1991)

showed that gene targeting is mediated exclusively by homologous recombination in kinetoplastid organisms, and that linearized constructs integrate with much higher efficiencies. Tobin *et al.* (1991) showed that *Leishmania* contain the enzymatic machinery necessary for homologous recombination.

Several research groups have attempted to demonstrate genetic exchange in trypanosomatids. Success has been achieved only in *Trypanosoma brucei* (Jenni *et al.*, 1986) and, despite continued research, the precise mechanism of exchange remains unclear (reviewed by Gibson & Stevens, 1999). The existence of "natural" hybrids has been reported in other trypanosomatid genera such as those described in the Nicaraguan and Huánuco *Leishmania* populations (Darce *et al.*, 1991; Belli *et al.*, 1994; Dujardin *et al* 1995b; see also chapters 5 and 6), indicating that exchange may occur in these organisms.

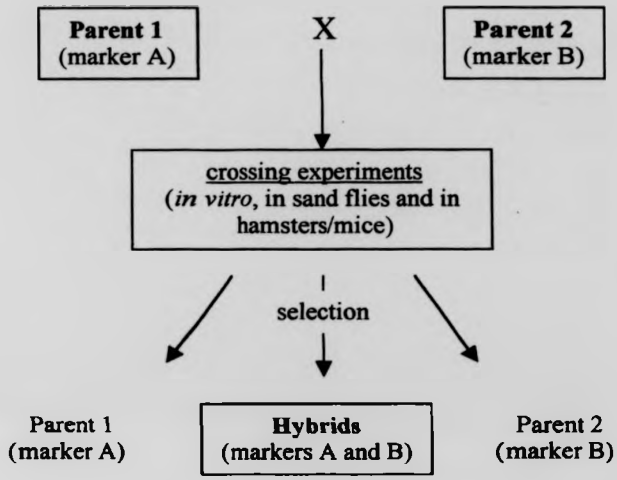
The aim of this experiment was to attempt to demonstrate genetic exchange in *Leishmania*. Previously, a single, unsuccessful attempt to demonstrate exchange using Old World species of the subgenus *Leishmania* had been reported (Panton *et al.*, 1991). These authors obtained a mixed infection in sand flies but did not find any hybrid progeny among 2500 scored. Other attempts have been made using Old World strains (David Evans, personal comm.), however, to the best of my knowledge, there are no reports of these experiments, presumably due to lack of success.

For reasons, some of which will be discussed later, the experiment described herein proved too ambitious given the time available, and was thus discontinued. This chapter has been included because some of the findings may be of use to future work and the discussion raises important points regarding the generation of diversity in *Leishmania*.

7.2 Strategy

In theory, the experiment appears relatively simple (see *figure 7.2*): stocks from two species (parents 1 and 2, respectively) would be transformed to different drug resistance phenotypes (markers A and B, respectively) and crossed. Any progeny that exhibited both drug-resistance phenotypes (i.e. having both markers) were likely to be the product of a genetic exchange event, and could be selected on media containing both selective drugs.

***Figure 7.2*: Proposed experimental scheme for demonstrating genetic exchange**
(figure adapted from Gibson & Stevens, 1999)



7.3 Selection of *Leishmania* strains for transformation

The Nicaraguan *Leishmania* population comprised stocks belonging to two *Viannia* species (*L. V. braziliensis* and *L. V. panamensis)* and putative hybrid stocks. As discussed in chapter 5, these hybrids exhibit markers from both Nicaraguan parental strains of these two species at all discriminatory loci studied. Since all these loci are unlikely to be located on a single chromosome, it is possible that these hybrids may be the product of an exchange event involving haploid chromosomes, one set from each parental strain. Hybrid stocks were independently isolated from humans living in areas separated by large distances and geographical barriers and, as such, it is possible that this genetic exchange event may have occurred more than once. Nicaraguan stocks of the two 'parental' species were selected for transformation because it was felt more likely that they would be capable of generating hybrid offspring from crossing experiments than would randomly isolated strains of the two species. In hindsight, it is likely that stocks from the Huánuco population would have been more suitable for this study in that they were isolated from an epidemic population in a small area: these stocks were, however, not available at the start of this experiment.

Nicaraguan "parental" *L. V. braziliensis* and *L. V. panamensis* stocks (XD28 and ZF01 respectively) were selected for transformation to different drug resistance phenotypes (G418 and hygromycin or phleomycin). Another *L. V. braziliensis* stock, M2903, was also selected as an "integration control" because it was the origin of the targeting fragments which were going to be incorporated into the vector.

7.4 Construction of transformation vectors

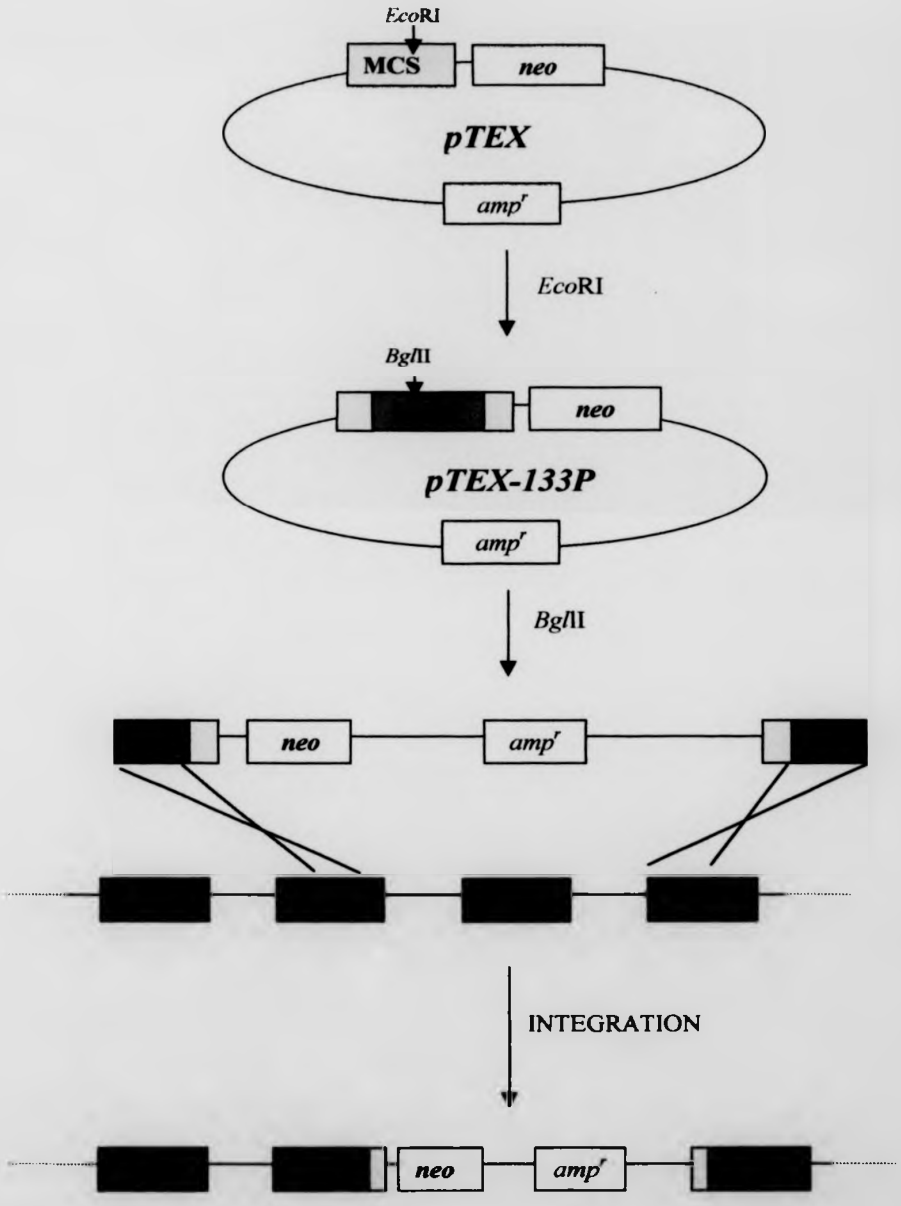
As described previously, trypanosomatids have unusual mechanisms for gene transcription. To ensure that transformation vectors function in the target cell, vectors in current use carry processing elements (spliced leader and polyadenylation signals) involved in mRNA maturation. To simplify the analysis of progeny, it was intended here to construct vectors incorporating specific targeting fragments to promote integration of the drug resistance gene into the *Leishmania* genome by homologous recombination.

7.4.1 Construction of the vector conferring neomycin resistance

The vector *pTEX-neo-133P* (see *figure 7.4.1*) was constructed from two existing plasmids: *pT3T719U-133P* (see *figure 2.12.2*, Chapter 2, Amorim *et al.*, 1996) and *pTEX* (*figure 7.4.1*; Kelly *et al.*, 1992). *pT3T719U-133P* contains 133P, a 2.5kb sequence spanning parts of the coding and untranslated regions of the 70kDa heat shock protein gene (*hsp70*) from *L. V. braziliensis* stock M2903. *pTEX* contains the neomycin resistance gene (neo^r) and multiple cloning site (MCS) flanked by *T. cruzi* processing elements derived from glyceraldehyde phosphate dehydrogenase (*gapdh*). *pTEX* is maintained episomally and has been shown to confer resistance to neomycin in *T. cruzi* and *Leishmania* spp. The vector also contains the ampicillin resistance gene (amp^r) for selection purposes.

Briefly, the 2.5kb 133P fragment was isolated from the *pT3T719U-133P* vector by agarose gel electrophoresis after digestion with *Eco*RI. After purification, 133P was ligated to *Eco*RI-linearized *pTEX*. *E. coli* cells were transformed with the construct. Plasmids containing the 133P insert (*pTEX-neo-133P*, *figure 7.4.1*) were selected by size after gel electrophoresis. At each stage of construction, the products were checked for correct size and orientation using restriction enzyme analysis and gel electrophoresis.

Figure 7.4.1*:** **Construction of *pTEX-neo-133P



Promastigote cultures of each stock (M2903, XD28 and ZF01) were electroporated (see Chapter 2 for details), both with and without the addition of vector DNA (DNA+ and mock-transfected controls, respectively).

In initial experiments, cultures of the *L. V. braziliensis* stocks M2903 and XD28 (both DNA+ and mock-transfected controls) appeared to have survived the electroporation procedure, but died after transfer to fresh liquid medium even in the absence of the neomycin analogue, G418. This may have occurred due to the over-dilution of electroporated cells or because the promastigotes were damaged by the electroporation procedure. A second experiment was successful: both DNA+ XD28 and M2903 stocks were transformed and grew well at concentrations of G418 up to 100ug/mL. Mock-transfected control cultures died after transfer to fresh liquid medium containing even low concentrations of G418. The presence of the neomycin gene in these stocks was confirmed by Southern analysis, however, the size of the band indicated that the construct was maintained episomally and had not integrated.

Cultures of the *L. V. panamensis* stock ZF01 (both DNA+ and mock-transfected controls) survived the electroporation procedure and grew well in liquid medium in the absence of G418. ZF01 DNA+ parasites grew well in the presence of 25μg/mL and 50μg/mL G418, suggesting that they too had been transformed to neomycin resistance. These parasites, however, failed to grow and subsequently died, in medium containing higher G418 concentrations (75 and 100μg/mL).

Southern analysis of DNA extracted from this "transformed" stock using ^{32}P-labeled neomycin gene probes (isolated from two different sources, *pGEM-Neo* and *pTEX*) showed that the *Neo*^{r} gene was absent. The probes were shown to be labeled and specific for the neomycin gene by their use on of a digest of a cosmid containing the neomycin gene (Martin Taylor, personal communication). It was concluded that the observed growth in medium containing G418 was not a result of transformation, but was indicative of natural resistance. Mock-transfected ZF01 control cultures died in the presence of even low concentrations of G418, indicating that the isolate as a whole was not resistant, however it is possible that a subpopulation of ZF01 grew out under G418 selection. A mutation may have occurred which conferred resistance to G418; any organism carrying such a mutation would obviously have a selective advantage and would thus expand rapidly. "Natural" resistance to G418 and related drugs has

been noted previously; such resistance may be conferred by as little as a single base change in rRNA genes (John Kelly, personal communication).

7.4.2 Construction of the vector conferring phleomycin resistance

The second vector was initially designed to contain the gene encoding resistance to hygromycin (*hygromycin B phosphotransferase, hyg^r)* however preliminary studies showed that ZF01, and 2 other *L. V. panamensis* stocks, exhibited natural resistance to this drug in addition to neomycin. It was subsequently decided to base the second vector on *pTEX-ble* (Nozaki & Cross, 1994), a plasmid vector containing the gene encoding resistance to phleomycin (*ble^r*), because all *L. V. panamensis* stocks tested (ZF01, ZE09, LS94) proved susceptible to this drug.

Initially, the same approach to construction was taken as for *pTEX-neo-133P*, i.e. with 133P being inserted into the *pTEX-ble* vector. This, however, proved impossible owing to the presence of unmapped restriction sites in *pTEX-ble*. A second approach was attempted whereby a 0.5kb fragment containing the phleomycin resistance (*ble^r*) marker from pTEX-ble was inserted into pT3T719U-133P.

In practice, construction of the vectors proved to be extremely difficult and only one of the vectors, *pTEX-neo-133P*, was successfully constructed. Construction of the *ble^r* vector was not completed.

It became evident, after encountering experimental difficulties, that this experiment was not going to be completed within an acceptable time. After the investment of many hours, this work was discontinued to allow time to concentrate on other areas of the project. Only one of the required vectors was successfully constructed. This vector was used to transform two *L. V. braziliensis* stocks, but failed to integrate and was maintained episomally. The *L. V. panamensis* stock exhibited natural resistance to G418 thus preventing the selection of transformants. The transformed stocks are stored, for future use, in the PMBBU cryobank at LSHTM.

7.5 Discussion of genetic transformation and genetic exchange

7.5.1 Vectors and integration by homologous recombination

For this study, integrative vectors were required to promote stable drug resistance phenotypes, thus facilitating the analysis of progeny from crossing experiments. To this end, constructs carrying hsp70 gene fragments from *L. V. braziliensis* were designed to promote homologous recombination into the *hsp70* gene array. Successful transformation was achieved using the linearized construct however the vector did not appear to integrate and was maintained episomally, even in the stock from which the *hsp70* targeting fragments were derived.

Failure of integration is, in hindsight and after the report of Papadopoulou & Dumas (1997), not surprising given the level of sequence homology required to promote homologous recombination. Papadopoulou & Dumas (1997) found that the frequency of homologous recombination between vector and chromosomal target DNA sequence was influenced by many factors. The most critical parameters for efficient homologous recombination in *Leishmania* were shown to be (1) the degree of homology between the vector and the genomic target (isogenic DNA or very high degree of homology critical) and (2) the length of homologous sequences (optimal length 1 – 2 kb on each side). The targeted locus was also shown to be important because (i) recombination frequency increased with increasing copies of the target locus and (ii) variable levels of targeting efficiency were observed at different chromosomal locations. In addition, variable targeting frequencies were exhibited by different strains of the same *Leishmania* species.

The *hsp70* targeting fragments incorporated into the targeting vector *pTEX-neo-133P* were approximately 1.25 kb in length on either side of the resistance marker which, according to Papadopoulou & Dumas (1997) is the optimal length required to facilitate homologous recombination. The *hsp70* targeting fragments, however, included part of the more variable *hsp70* array untranslated region (see *figure 2.13.2*, Chapter 2). According to Papadopoulou & Dumas (1997), sequence divergence drastically reduces the efficiency of gene targeting in *Leishmania*. It is seems likely that the sequence homology between the vector and *L. V. panamensis* and *L. V. braziliensis* strain XD28 were sufficiently divergent to prevent homologous recombination.

It is more difficult to explain why the vector did not integrate into the *hsp70* array of the *L. V. braziliensis* strain M2903 because this strain was the source of the *hsp70* targeting fragments. Again, however, failure to integrate might have been the result of sequence divergence: M2903 is a widely-used reference strain and its continued maintenance in culture in different laboratories may have resulted in accumulated sequence differences, presumably in the variable region, between our M2903 strain and that used by Amorim *et al.* (1996) who constructed *pT3T719U-133P*. It is also possible that the linearized construct did integrate in some cells. The presence of other cells transformed with multiple episomal copies would, however, in the presence of G418, likely have 'out-selected' cells containing a single *neo* copy.

Cross-species homologous recombination has recently been reported In *Leishmania* using a vector targeting a highly conserved gene (Krobitsch & Clos, 2000).

7.5.2 Crossing experiments and potential site of genetic exchange

Panton *et al.* (1991) performed experimental crosses in *Phlebotomus papatasi* using two cloned strains of *L. major*. The clones contained numerous restriction fragment length polymorphisms and were selected for resistance to methotrexate or tunicamycin by gene amplification (i.e. not by transfection). Promastigotes from the gut contents of flies shown to have mixed infections were plated onto selective media. Despite examining 2500 progeny for the presence of both drug resistance markers, no hybrid parasites were scored. The authors concluded that the frequency of genetic exchange in their cross was less than 4×10^{-4}.

One possible reason why these authors failed to generate hybrid offspring could have been that genetic exchange between *Leishmania* may not occur in the sand fly vector but in the host. Genetic exchange in the related kinetoplastid *Trypanosoma brucei* has been shown to occur in the tsetse vector (Jenni *et al.*, 1985). However, *T. brucei* subsp. parasites circulate in the blood of the host and do not have an intracellular stage: genetic exchange seems unlikely in this environment because the chances of encountering another parasite are low. By comparison, *Leishmania* species reside inside professional phagocytic cells such as macrophages, Langerhan's and Küppfer cells. Co-inoculation of more than one *Leishmania* species or strain could result in the co-localization of parasites inside the host phagocyte thereby allowing close proximity for genetic exchange events. Indeed, Kreutzer *et al.* (1994) reported data of nuclear

DNA content for amastigotes and promastigotes (measured using quantitative (Feulgen) microspectrophotometry), which suggested that sexual reproduction may take place inside the intracellular amastigote form.

7.5.3 Models for genetic exchange; ploidy

Based on observations of apparently triploid offspring from experimental crossing experiments using *T. brucei* (Gibson *et al.*, 1992; Gibson & Whittington, 1993; Gibson & Bailey, 1994), Gibson (1995) proposed 2 models for the mechanism of genetic exchange, both involving fusion and meiosis. The first model involves the fusion of diploids followed by meiosis to produce diploid progeny. In the second model, the processes are reversed with meiosis followed by fusion of haploids. In *T. brucei*, evidence for meiosis comes from the Mendelian inheritance patterns observed for housekeeping genes and the high frequency of triploid hybrids; evidence for cell fusion in *T. b*rucei rests on observations of the inheritance patterns of kinetoplast DNA (for a review, see Gibson & Stevens, 1999). To date, no haploid stage has been substantiated for *T. brucei*, or for any other trypanosomatid.

Similar evidence for genetic exchange has been documented in *Leishmania*. Lanotte & Rioux (1990) reported the apparent cell fusion of cultured *L. infantum and L. tropica* promastigotes. Indirect evidence of diploidy, genetic recombination and Mendelian inheritance in natural *Leishmania* populations comes from data generated by IEA, RAPD, RFLP and molecular karyotype analyses (Evans *et al.*, 1987; Darce *et al.*, 1991; Kelly *et al.*, 1991; Bonfante-Garrido *et al.*, 1992; Belli *et al.*, 1994; Dujardin *et al.*, 1995b; Banuls *et al.*, 1997). Diploidy is taken as the working hypothesis for *Leishmania*, however several groups have also reported evidence of aneuploidy, mostly from molecular karyotpye analysis (e.g. Bastien *et al.* 1990). Evidence of aneuploidy has also been reported in *T. cruzi* (Gibson & Miles, 1986; see Gibson & Stevens, 1999). *Leishmania* have been shown to have highly plastic genomes in which chromosomal rearrangements are common (reviewed in chapter 1). Bastien *et al.* (1990) suggested a mosaic structure for some strains of *L. infantum* after isolating clones with different karyotypes from the same strain. This finding is supported by clonal variation within an *L. V. braziliensis* isolate using isoenzyme analysis (Cuba-Cuba *et al.*, 1991).

7.5.4 Detection of heterozygotes

So, why are natural hybrids or products of genetic exchange not detected more often?

The detection of genetic exchange requires that molecular markers can be differentiated. If all markers examined are identical in all stocks (as in Três Braços, Brazil) it would not be possible to tell whether stocks had undergone genetic exchange (although clonal expansion seems more likely). In addition, genetic exchange may not necessarily result in the exchange of all markers studied (as in the Nicaraguan stocks); partial recombination involving sets of markers also occurs (as described here in the Huánuco stocks). Highly discriminatory techniques have only relatively recently been introduced. With the application of more markers, genetic exchange events may become more evident.

Genetic exchange requires the presence of mixed infections. Mixed *Leishmania* infections have been reported both in sand flies and in humans (Barrios *et al.*, 1994; and Silveira *et al.*, 1984; Mehbratu *et al.*, 1991; Ibrahim *et al.*, 1994; Hernandes-Montes *et al.*, 1998, respectively). Such reports are, however, relatively rare.

Many *Leishmania* species have restricted ranges due to suitable vector and host availability. Hence, even if a new strain or species is introduced into an area, it is possible that the vector species present may be refractory to infection or may not capable of transmitting the new parasite.

Genetic exchange may result in loss or gain of fitness. Offspring resulting from a genetic exchange event may be less fit than the "parental" strains and be lost as the result of selection processes.

Past studies have investigated strains which have been amassed from wide geographical areas over a large time scale. Often, the population size has been too small and has been biased towards human isolates. The ideal population in which to find evidence of genetic exchange would likely comprise many isolates from sand fly vectors, human, domestic and sylvatic hosts from a small geographical area in an epidemic situation.

CHAPTER 8 - CONCLUDING REMARKS

The *Viannia* subgenus is found only in the New World, where the vast range of habitats and diverse fauna therein are likely to have played a major role in the diversification of parasites. Species of the *Leishmania* subgenus *Viannia* were originally thought to exist in discrete transmission cycles and to be responsible for distinct clinical presentations. However, four *Viannia* species have now been associated with MCL, a clinical presentation previously attributed to infection with *L. V. braziliensis*. Recent reports describing putative species hybrids have fueled the debate regarding the taxonomic status and population structure of these parasites.

The aim of this project was to use molecular methods to reassess inter- and intra-specific relationships in the *Leishmania* subgenus *Viannia*. By exploring the extent of genetic diversity occurring in natural *Viannia* populations and the occurrence of genetic recombination across currently recognized species, it was hoped that this project would provide an insight into the changing epidemiology of cutaneous and mucocutaneous leishmaniasis (CL and MCL) in Latin America.

A panel of reference strains was selected, including representatives of the subgenus *Leishmania*, for initial comparisons of established and novel methods. The most discriminatory techniques were subsequently used to examine *Viannia* stocks from three endemic areas (Três Braços, Bahia State, Brazil; Nicaragua; and Huánuco, Peru) in detail. In addition, stocks isolated from widely separated geographical areas in Brazil were examined.

Isoenzyme analysis (IEA) was used as a gold standard to confirm the identity of isolates and, alongside other markers, to study diversity, gene flow and phylogenetic relationships. Overall, 27 zymodemes were scored from 125 *Viannia* strains and stocks (6 species and 1 potential new species) using 12 enzyme loci. Nineteen stocks belonging to eight zymodemes were associated with MCL, including one *L. V. peruviana* stock, a finding not previously reported.

Random amplification of polymorphic DNA (RAPD) data were of value in detecting and confirming the presence of *L. V. braziliensis / L. V. panamensis* hybrids among Nicaraguan stocks, although it was found to be difficult to reproduce these data on occasions. PCR-RFLP analysis of the rRNA ITS region was found to be highly informative and allowed distinction of reference species even within the *Viannia* species complexes.

During the course of this project, collaborators in Cambridge isolated microsatellites specific for the subgenus *Viannia*. Three PCR-based strategies were employed to examine all *Viannia* strains and stocks at three microsatellite loci. Non-denaturing PAGE analysis was found to be a sensitive method of typing stocks, although data interpretation was complicated by the presence of multiple bands and artefactual bands. The presence of heteroduplexes was a useful indicator of multiple alleles, not only in the putative inter-species hybrids.

Using a Genescan® / Genotyper® approach, it was possible to identify allele(s) present in *Viannia* stocks at each microsatellite locus. Moreover, putative hybrid stocks could be detected by the presence of double peaks at one or more of the loci examined. The term "microdeme" was adopted for a stock, or group of stocks, defined by a combination of microsatellite characters (Genotyper® data). Tremendous diversity was revealed, with at least 61 microdemes scored over all stocks.

DNA sequencing was performed on PCR products of the AC01 locus, generating data on the number of dinucleotide repeats and variation in the flanking regions. At least 33 AC01 sequence types were scored compared to 9 AC01 allele sizes identified using Genotyper®. There was general agreement between dinucleotide repeat number and allele size, as determined by sequencing and Genotyper®, respectively; although in several instances bias was noted in the resolution of alleles which could not always be related to the length of the microsatellite product.

Microsatellite data were exploited for both population genetics (Genotyper® data) and phylogenetics (AC01 sequence data).

Little genetic diversity was found among *L. V. braziliensis* stocks from Três Braços and Corte de Pedra, Brazil, although more than one microdeme was identified. In contrast, each of the four *L. V. braziliensis* stocks from Amariji, Pernambuco State, had a distinct genotype. Isoenzyme and microdeme heterogeneity among Brazilian *L. V. braziliensis* stocks was more common among stocks originating from different geographical areas.

Among the Nicaraguan stocks, 3 zymodemes were scored which corresponded to *L. V. braziliensis*, *L. V. panamensis* and putative *braziliensis / panamensis* hybrids. The presence of inter-species hybrids was supported by RAPD and microsatellite data.

In Huánuco, Peru, *L. V. braziliensis* and *L. V. peruviana* and *L. V. braziliensis / L. V. peruviana* hybrid forms were found sympatrically in the same locality. Furthermore, all three forms were found in humans and two of them in dogs. The presence of indistinguishable stocks occurring sympatrically in humans and dogs strongly implicates the dog as a potential reservoir of infection. Two other parasites were also described in the Huánuco population: a single human isolate was identified as *L. V. lainsoni* and three dog isolates were identified as a potential new species, previously described in a sand fly isolate from Manaus, Brazil. Mucocutaneous disease, usually associated with *L. V. braziliensis*, was also associated with *L. V. peruviana* and *L. V. braziliensis / L. V. peruviana* hybrids in Huánuco.

Isoenzyme and microsatellite data were used to provide an estimation of segregation (Hardy-Weinberg analysis) and linkage disequilibrium (I_A and D' / r^2 indices) in the Huánuco *Viannia* population. Analysis of combined data suggested that at least one, and probably several, recombination events had occurred among these stocks. However, the population did not conform to panmixia, implying that propagation appeared to be predominantly clonal. Interpretation of linkage disequilibrium indices was complicated by a lack of statistical power, a result of the large numbers of possible microsatellite alleles relative to the small number of stocks analyzed. The expansive clonal propagation of one of the putative hybrid types suggests an increased fitness of this parasite relative to the parental strains present.

Phylogenetic analysis of these stocks generally supported intuitive concepts of species groups and relationships, despite limitations in the range and number of samples, and despite equivocal models, e.g., for phylogenetic analysis of microsatellite data. In agreement with previous reports, the subgenus *Viannia* was, from IEA data, monophyletic.

It is apparent from pre-existing literature that the boundaries between species of the two major *Viannia* species complexes have doubtful validity in view of the description of hybrid forms between *L. V. braziliensis* and *L. V. panamensis*; *L. V. braziliensis* and *L. V. peruviana*, and *L. V. braziliensis* and *L. V. guyanensis*. Work reported in this thesis confirms these doubts. In particular, the status of *L. V. peruviana* as a species distinct from *L. V. braziliensis* was, in contrast to previous reports, not supported. *L. V. lainsoni* stocks, and those of a putative new species, were shown to be distinct from stocks of the *braziliensis* and *guyanensis* species complexes. A recently revised classification has suggested that the two most recently described *Viannia* species, *L. equatorensis* and *L. colombiensis* should, in fact, be considered as members of a new Trypanosomatidae section, Paraleishmania.

Genetic recombination in the subgenus *Viannia* has fundamental implications in terms of outbreaks of new pathogenic forms, e.g., as shown here with the *L. V. braziliensis* / *L. V. peruviana* hybrids which were associated with MCL, and for the spread of drug resistance. *L. V. braziliensis* strains in particular have shown themselves to be exceptionally adaptable to changing environments. Experimental studies of genetic recombination using transformed laboratory strains were discontinued owing to limited resources, however these studies are clearly of interest.

The insight gained into the epidemiology of these *Viannia* populations suggests a disease agent that can undergo genetic exchange and can exist in several distinct transmission cycles. Interruption of natural sylvatic cycles can lead to founder effects, clonal propagation, and subsequent widespread dissemination of biological clones.

In future work there is further scope for the application of PCR-RFLP analysis to molecular taxonomy, population genetics and phylogenetics of *Leishmania*. Additional targets suitable for PCR-RFLP analysis are likely to emerge from the *Leishmania* genome sequencing programme. The application of Genescan® / Genotyper® would facilitate high throughput scoring of PCR-RFLP data, although sequence comparisons of such targets would provide the ultimate approach to molecular phylogenetics.

The application of microsatellites to the population genetics and phylogenetics of *Leishmania* is in its infancy. Further microsatellite loci, which are amenable to both Genotyper® and DNA sequence analysis, are required. The chromosomal location of these markers should be investigated by Southern blotting, using labeled oligonucleotides and high stringency conditions. As yet, there are no microsatellite markers available for the subgenus *Leishmania*. Microsatellites are likely to be particularly valuable for studies of gene flow between closely related *Leishmania* populations, rather than for the definition of species specific profiles.

In retrospect, we would like to have had more isolates from the Huánuco region in order to detect predicted additional genotypes, thus strengthening the analyses of segregation and linkage. To exclude the possibility of polyploid or aneuploid genotypes, it would be useful to quantitate the DNA content per cell of Huánuco stocks representative of the putative parental and hybrid forms. More isolates are available from Três Braços in Brazil, but in view of their relative lack of diversity it seems less important to extend the study of that locality. In contrast, the extraordinary diversity scored in isolates from Amariji merits further investigation.

If the epidemiology of the subgenus *Viannia* were comparable to that of the African trypanosome, *T. brucei*, in Africa, an intensive study of genetic diversity in an undisturbed sylvatic transmission cycle would complement this project. Such a study would be very difficult logistically because it is likely that large numbers of mammalian reservoir hosts and vector species would need to be examined, although it might be possible to exploit existing isolate collections.

By analogy with the research on the geographical distribution of *T. cruzi* in Latin America, where approximately 500 isolates have now been examined by a variety of methods, it would be of interest to map *Viannia* genotypes in all major endemic areas. A history of emergence and spread of biological clones might then become apparent which could be linked to vector or reservoir host distribution. Among reservoir hosts, the dog may have a significant role in the dissemination of *Viannia* far beyond regions with natural, sylvatic reservoir hosts, a role which clearly needs clarifying. This would help to predict where vector distribution could encourage new suburban outbreaks, as have recently occurred in Brazil. Furthermore, if the importance of the dog as a reservoir host is confirmed, this should provide impetus for the development of new vaccines, drugs and diagnostic methods for canine leishmaniasis.

Molecular comparisons, e.g., through PCR-RFLP or DNA and amino acid sequence-based phylogenetics will also allow an insight into the link between mammal host species and *Leishmania* species, or between vector species and *Leishmania* species, and the possible phenomenon of co-evolution. This may reveal whether *Leishmania* was originally a parasite of insects which ventured into mammals, as is frequently proposed, or a parasite of mammals that hijacked arthropod vectors for transmission.

We remain convinced that genetic transformation should allow experimental studies of the mechanisms of hybridization and recombination in *Leishmania*.

There is no doubt that leishmaniasis is a major public health problem in Latin America and around the world, and that the *Viannia* subgenus is spreading in Latin America and encroaching on suburban areas. The epidemiology is thus highly dynamic. This project has expanded the molecular approaches to studying the epidemiology and taxonomy of the subgenus *Viannia* and has produced a new perception of the variety and complexity of *Viannia* populations.

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APPENDIX I - CULTURE MEDIA*

*All reagents were purchased from Sigma unless otherwise stated.

LEISHMANIA SUBGENUS *VIANNIA* CULTURE MEDIA

4N slopes (makes approx. 25 slopes)

2g	Blood agar (Difco)
5mL	Defibrinated rabbit blood (St. Georges' Hospital)
1mL	Gentamycin (50mg/mL) (Gibco BRL)

Blood agar was added to 50mL deionized-distilled water (ddH_2O). The mix was sterilized by autoclaving (121°C for 15 minutes) before storing at room temperature until required. When 4N slopes were required, the agar was melted at low heat in a microwave. The agar was cooled to 56°C: rabbit blood and gentamycin were added and the mixture was swirled gently. 2mL aliquots were poured into bijoux tubes, sealed and left to set at an angle to produce the slope. 4N slopes were stored (up to 1 month) at +4°C until required.

2.5X α-modified MEM stock (makes 2L of 2.5X stock)

5L equivalent	αMEM powder
11.00 g	$NaHCO_3$
2.95 g	L-glutamine
15.00 g	D-glucose
50.00 g	HEPES

The above reagents were dissolved in ~1900mL ddH_2O water. The pH was adjusted to 7.5 (+/- 0.5) using 10N NaOH. The solution was filter-sterilized through 2X 1L 0.22μM filter units (Sartorius) into sterile 1L Schott bottles.

200mg	Adenine
25mg	Folic Acid
10mg	D-biotin

The above reagents were dissolved in 2.5mL 1M NaOH. 17.5mL ddH_2O water was added: 10mL of this solution was filtered into each 1L bottle. Medium was stored (up to 3 months) at +4°C until required.

500X haemin

37.5mg	bovine haemin
15mL	50% triethanolamine

Haemin was dissolved in 50% ethanolamine by shaking vigorously. The solution was filter-sterilized through a 0.22μM filter unit (Sartorius) and stored at +4°C.

Liquid medium (makes 1 L)

400mL	2.5X α-MEM stock
2mL	500X haemin
100mL	Heat-inactivated foetal calf serum (FCS)
1 mL	Gentamycin solution (50mg/mL stock) (Gibco BRL)

α-MEM stock was diluted to working strength with 600mL autoclaved ddH_2O water. 100mL was decanted into a sterile container and stored at +4°C for later use. FCS, haemin and gentamycin were added. Small aliquots were removed for sterility testing: after overnight incubation at 23°C and 37°C these were examined microscopically for microbial growth Liquid medium was stored at +4°C until required (up to 1 month).

Proline-balanced salt solution (PBSS)

800mg	KCl
120mg	$Na_2HPO_4.12H_2O$
120mg	KH_2PO_4
370mg	$CaCl_2.2H_2O$
200mg	$MgSO_4.7H_2O$
200mg	$MgCl_2.6H_2O$
16g	NaCl
2g	L-proline
2mg	Phenol red indicator

Reagents were dissolved in 1950 mL ddH_2O water. The pH was adjusted to 7.2 with Trizma base and the solution made up to 2L with ddH_2O water. The solution was aliquoted into 4 x 500mL bottles and autoclaved. PBSS was stored (up to 6 months) at +4°C until required.

BACTERIAL CULTURE MEDIA

LB medium

5g	Bacto-tryptone (Difco)
2.5g	Bacto-yeast extract (Difco)
5g	NaCl

The reagents were dissolved in 300mL ddH_2O water, made up to 500mL with ddH_2O water and autoclaved. LB medium was stored at room temperature (up to 6 months) until required.

LB agar plates

7.5g	Bacto-agar (Difco)
500mL	LB medium

The reagents were mixed together and autoclaved. The agar solution was cooled to approximately 50°C before immediate pouring into petri dishes. Plates were left to set, uncovered, at room temperature. Once set, lids were replaced and plates were kept at 4°C until required.

APPENDIX II - GENERAL REAGENTS

10% AMPS

2g	ammonium persulphate (AMPS)
20mL	distilled water

Dissolve AMPS in water. Aliquot into 20 X 1mL and store at -20°C.

1M $CaCl_2$

5.4g	$CaCl_2.6H_2O$
20mL	MilliQ water

Dissolve $CaCl_2.6H_2O$. Filter-sterilize through a 0.2µM filter. Aliquot into 20 X 1mL and store at -20°C.

Cell lysis buffer

25 mL	1M NaCl
50 mL	0.5M EDTA
50 mL	10% SDS
25 mL	1M Tris-HCl, pH 8.0

Mix reagents thoroughly and divide into 20mL aliquots. Autoclave and store at room temperature until required.

DGGE electrophoresis buffer (MOPS - 50X)

209.3g	MOPS (3-[N-morpholino]propane-sulphonic acid)
18.6g	EDTA
36g	NaOH

Dissolve reagents in ~800mL ddH_2O. Make up to 1L. Filter-sterilize through a 0.2µM filter. Store at room temperature.

DGGE gel mix

8mL	50X DGGE (MOPS) buffer
42mL	Ultrapure bis-acrylagel* (2% N,N'methylene bis-acrylamide solution)
77.6mL	Ultrapure acrylagel* (30% acrylamide solution)
8mL	glycerol
192g	urea

Mix reagents thoroughly using heated magnetic stirrer, adding distilled water to 400mL. Store at +4°C until required. For each gel use 40mL gel mix and add 80µL TEMED and 260µL 10% AMPS. *(National Diagnostics, Hull, UK).

DGGE loading buffer

10mg	xylene cyanol FF
10mg	bromophenol blue
1g	sucrose
10mL	distilled water

Mix reagents thoroughly. Aliquot into 10 x 1mL. Store at +4°C.

PBS (10X)

80g	NaCl
2g	KCl
11.5g	Na_2HPO_4
2g	KH_2PO_4

Make up to 1L with ddH_2O, adjust to pH 7.4 and autoclave. Store at room temp. until required.

Plasmid extraction buffers

Buffer 1

9g	glucose
2.5g	EDTA
2mL	0.5M Tris-HCl, pH 8.0

Make up to 100mL with ddH_2O and autoclave. Store at room temperature.

Buffer 2

0.2N	NaOH
1%	SDS

Make up fresh as required.

Buffer 3

60mL	5M potassium acetate
1.5mL	glacial acetic acid

Make up to 100mL with ddH_2O. Store at room temperature.

Silver staining solutions and protocol (Vidigal *et al.*, 1994)

Buffer A

100mL	ethanol (Analar grade, BDH)
5mL	glacial acetic acid
895mL	distilled water

Mix reagents together thoroughly. Store at room temperature.

Buffer B

500mg	silver nitrate
500mL	distilled water

Mix reagents together thoroughly. Store at room temperature in a covered glass bottle. Buffer B can be reused 5-10X.

Buffer C

7.5g	NaOH
50mg	sodium borohydride ($NaBH_4$)
2mL	formaldehyde
500mL	distilled water

Denhardt's solution (50X)

5g	Ficoll (type 400, Pharmacia)
5g	Polyvinylpyrrolidone
5g	BSA (fraction V)

Make up to 500mL with ddH_2O. Aliquot and store at -20°C.

0.5M EDTA, pH 8.0

93.05g	EDTA

Dissolve EDTA in ~400mL distilled water by stirring on a magnetic stirrer. Adjust pH to 8.0 using NaOH pearls. Sterilize by autoclaving.

Electroporation buffer

46.55g	sucrose
3.5mL	1M sodium phosphate, pH 7.2
300mL	distilled water

Make up to 500mL with ddH_2O. Aliquot and store at -20°C.

Enzyme stabiliser (stock)

0.5mL	200mM EDTA, pH 7.0
15.4mg	dithithreitol (DTT).
13.1mg	ε-amino-caproic acid (□-ACA)

Dissolve DTT in EDTA solution. Add this to the ε-ACA and pipette gently to dissolve. Store at +4°C for up to 2 months (discard if turns yellow). Make up working strength solution (1/1000 in ddH_2O) fresh for preparing stabilates.

Ethidium bromide

0.1g	ethidium bromide
10mL	distilled water

Stir on a magnetic stirrer for 2 hours. Store in dark bottle at room temperature.

Gel electrophoresis (agarose and polyacrylamide) loading buffer

0.05g	xylene cyanol FF
0.05g	orange G
15mL	glycerol
35mL	distilled water

Mix reagents and aliquot into 5 x 10mL. Autoclave and store at room temperature.

PAGE gel mix (non-denaturing)

40mL	10X TBE buffer
42mL	Ultrapure bis-acrylagel* (2% N,N'methylene bis-acrylamide solution)
77.6mL	Ultrapure acrylagel* (30% acrylamide solution)
20mL	glycerol
220.4mL	distilled water

Mix reagents thoroughly using magnetic stirrer. Store at +4°C until required. For each gel use 40mL gel mix, filtered through a 1μM filter, and add 80μL TEMED and 260μL 10% AMPS. *(National Diagnostics, Hull, UK)

PBS (10X)

80g	NaCl
2g	KCl
11.5g	Na_2HPO_4
2g	KH_2PO_4

Make up to 1L with ddH_2O, adjust to pH 7.4 and autoclave. Store at room temp. until required.

Plasmid extraction buffers

Buffer 1

9g	glucose
2.5g	EDTA
2mL	0.5M Tris-HCl, pH 8.0

Make up to 100mL with ddH_2O and autoclave. Store at room temperature.

Buffer 2

0.2N	NaOH
1%	SDS

Make up fresh as required.

Buffer 3

60mL	5M potassium acetate
1.5mL	glacial acetic acid

Make up to 100mL with ddH_2O. Store at room temperature.

Silver staining solutions and protocol (Vidigal *et al.*, 1994)

Buffer A

100mL	ethanol (Analar grade, BDH)
5mL	glacial acetic acid
895mL	distilled water

Mix reagents together thoroughly. Store at room temperature.

Buffer B

500mg	silver nitrate
500mL	distilled water

Mix reagents together thoroughly. Store at room temperature in a covered glass bottle. Buffer B can be reused 5-10X.

Buffer C

7.5g	NaOH
50mg	sodium borohydride ($NaBH_4$)
2mL	formaldehyde
500mL	distilled water

Mix reagents together thoroughly. Buffer C must be made up immediately before use, ensuring that the $NaBH_4$ is dissolved.

Buffer D

3.75g	sodium carbonate
500mL	distilled water

Mix reagents together thoroughly. Store at room temperature.

Protocol

1. Incubate gel in buffer A with agitation (i.e. on rocker) for 2 X 3 mins.
2. Incubate gel in buffer B, as previously, for 10 mins.
3. Wash gel twice for 15s in distilled water.
4. Incubate gel in buffer C, as previously, for 20 mins or until bands are visible.
5. Discard buffer and rinse gel briefly in distilled water.
6. Soak gel in buffer D for 20 mins. Discard buffer D
7. Seal gel in plastic bag with a little buffer D. Store flat.

SSC (20X)

175.3g	NaCl
88.2g	Trizma base

Make up to 1L with ddH_2O and adjust to pH 7.0. Sterilize by autoclaving.

TAE buffer (50X)

242g	Trizma base
57.1mL	glacial acetic acid (BDH)
100mL	0.5M EDTA, pH 8.0

Make up to 1L with ddH_2O.

TBE buffer (10X)

108g	Trizma base
55g	boric acid (BDH)
40mL	0.5M EDTA, pH 8.0

Make up to 1L with ddH_2O.

TE buffer

5mL	1M Tris-HCl, pH 7.2
1mL	0.5M EDTA, pH 8.0

Make up to 500mL with ddH_2O.

APPENDIX III - IEA REAGENTS AND CONDITIONS

I. THIN-LAYER STARCH GEL ELECTROPHORESIS (TSGE)

Thin-layer starch-gel electrophoresis (TSGE) was carried out essentially as described by Godfrey & Kilgour (1976) and Harris & Hopkinson, (1976). Conditions for electrophoresis and enzyme development were based on those of Miles *et al.* (1980a, 1980b, 1981) and Evans *et al.* (1984).

TSGE electrophoresis conditions for *Leishmania Viannia* species

Enzyme	Tank Buffer† (see below for recipes)	Gel buffer (tank buffer : water)	Volts	Running Time (hours)
MPI	6	1 : 4	300 V	2.5
NHi	1	1 : 4	200 V	2.0
NHd	1	1 : 4	200 V	2.0
ES	1	1 : 9	300 V	2.5
PEPD	7	1 : 7	250 V	2.0
PGM	2	1 : 4	250 V	2.5
6PGD	1	1 : 9	300 V	2.5
GPI	1	1 : 9	300 V	2.5
G6PD	9	1 : 4	150 V	3.5
ASAT	3	1 : 9	300 V	2.0
ALAT	3	1 : 9	300 V	2.0
MDH	4	2 : 1	250 V	4.0
ICD	5	1 : 1	250 V	3.0
SOD	8	1 : 9	300 V	2.5
PK	3	1 : 9	300 V	2.5
ME	8	1 : 4	200 V	3.0

†TSGE Tank Buffer Stock Recipes

All TSGE buffer stocks were made up and stored at room temperature for up to 6 months. The day before use, buffers were made up to 1X strength using distilled water and stored overnight at 4°C.

1. TMEM (5X stock) GPI, 6PGD, ES (pH 7.4); NHi/NHd (pH 8.0)

60.55g	Trizma base
58.05g	Maleic Acid
10.7g	Magnesium acetate
18.6g	EDTA

Reagents were dissolved ~800mL distilled water. The solution was adjusted to the required pH with 10N NaOH and made up to 1L.

2. TBEM (5X stock) PGM (pH 9.0)

151.35g	Trizma base
17.0g	Boric Acid
8.0g	Magnesium acetate
18.6g	EDTA

Reagents were dissolved ~800mL distilled water. The solution was adjusted to pH 9.0 with 10N NaOH and made up to 1L.

3. TRIS - CITRIC ACID (10X stock) ASAT, ALAT, PK (pH 9.0)

90.85g	Trizma base (0.15M)
7.85g	Citric Acid (0.0075M)

Reagents were dissolved ~400mL distilled water. The solution was adjusted to pH 9.0 with 10N NaOH and made up to 500mL.

4. TRIS - CITRIC ACID (10X stock) MDH (pH 7.0)

166.4g	Trizma base (0.25M)
94.2g	Citric Acid (0.09M)

Reagents were dissolved ~400mL distilled water. The solution was adjusted to pH 7.0 with 10N NaOH /conc. HCl and made up to 500mL.

5. TRIS - CITRIC ACID (10X stock) ICD (pH 8.8)

106.2g	Trizma base (0.4M)
52.3g	Citric Acid (0.05M)

Reagents were dissolved ~400mL distilled water. The solution was adjusted to pH 7.0 with 10N NaOH and made up to 500mL.

6. TRIS-PHOSPHATE (10X stock) MPI (pH 7.6)

60.55g	Trizma base (0.1M)
78.0g	$NaH_2PO_4.2H_2O$ (0.1M)

Reagents were dissolved ~400mL distilled water. The solution was adjusted to pH 7.6 with 10N NaOH and made up to 500mL.

7. TRIS- PHOSPHATE (5X stock) PEPD (pH 8.2)

90.85g	Trizma base (0.15M)
78.0g	$NaH_2PO_4.2H_2O$ (0.1M)

Reagents were dissolved ~800mL distilled water. The solution was adjusted to pH 8.2 with 10N NaOH and made up to 1L.

8. PHOSPHATE (5X stock) ME, SOD (pH 7.4)

136.28g	Na_2HPO_4 (0.192M)
6.24g	$NaH_2PO_4.2H_2O$ (0.08M)

Reagents were dissolved ~800mL distilled water. The pH of the solution was checked and adjusted to pH 7.4 with 10N NaOH and made up to 1L.

9. PHOSPHATE (10X stock) G6PD (PH 7.0)

57.5g	Na_2HPO_4 (0.081M)
1.48g	$NaH_2PO_4.2H_2O$ (0.019M)

Reagents were dissolved ~400mL distilled water. The pH of the solution was checked and adjusted to pH 7.0 with 10N NaOH and made up to 500mL.

II. CELLULOSE ACETATE ELECTROPHORESIS (CAE)

CAE was carried out according to the methods described by Lanham *et al.* (1981), Evans *et al.* (1989) and according to the manufacturer's handbook (Helena Laboratories, Beaumont, Texas, USA). Conditions for electrophoresis were adapted from those used for TSGE.

CAE conditions for *Leishmania Viannia* species

Enzyme	Tank Buffer$^{\infty}$ (see below for recipes)	Further dilution of 1X tank buffer : water)	Volts	Running Time (mins.)
MDH	1.1	-	160 V	45
ME	1.1	-	160 V	45
PEPD	1.2	-	275 V	20
ICD	2	-	200 V	50
NHi	3	3:37	275 V	30
SOD	4	1:2	275 V	30

$^{\infty}$CAE Tank Buffer Recipes

Tris barbital buffer stock was made up and kept refridgerated, without sucrose, for up to 1 week. All other CAE buffer stocks were made up and stored at room temperature for up to 6 months. The day before use, buffers were made up to 1X or working strength using distilled water and stored overnight at 4°C. Sucrose was added to a final concentration of 15% to all buffers immediately before electrophoresis. Sucrose was added in order to reducing the current across the plate, thus improving resolution of isoenzyme bands.

1. TRIS BARBITAL / SODIUM BARBITAL (stock, pH 9.0)

Tris-barbital buffer was obtained in preweighed powdered form from Helena Laboratories (Beaumont, Texas, USA). 1 sachet was dissolved in 500mL distilled water to make the stock buffer [ionic strength, I = 0.1] for use in making tank buffers 1.1 and 1.2.

1.1 TRIS BARBITAL / SODIUM BARBITAL / MAGNESIUM ACETATE MDH, ME (pH 9.0 [I = 0.075])

300mL	Stock buffer
100mL	distilled water
86mg	magnesium acetate

Reagents were mixed until dissolved and stored overnight at +4°C before use.

1.2 TRIS BARBITAL / SODIUM BARBITAL PEPD (pH 9.0 [I = 0.02])

100mL	Stock buffer
400mL	distilled water

Reagents were mixed and stored overnight at +4°C before use.

2. TRIS-GLYCINE (10X stock) ICD (pH 8.5)

30g	Trizma base
144g	glycine

Reagents were dissolved in ~400mL distilled water and made up to 500mL.

3. PHOSPHATE (5X stock) NHi (pH 8.0)

68.13g	Na_2HPO_4 (0.192M)
3.12g	$NaH_2PO_4.2H_2O$ (0.08M)

Reagents were dissolved in ~400mL distilled water. The pH of the solution was adjusted, if necessary, to pH 8.0 with 10N NaOH and made up to 500mL.

4. TBEM 2 (5X stock) SOD (pH 9.0)

75.69g	Trizma base
8.5g	Boric Acid
4.02g	Magnesium acetate
4.65g	EDTA

Reagents were dissolved ~400mL distilled water. The solution was adjusted to pH 9.0 with 10N NaOH and made up to 500mL.

III DEVELOPMENT CONDITIONS FOR *VIANNIA* SPECIES (TSGE and CAE+)

Enzyme Code	Developer Mix		Visualization Method*
MPI	0.3M Tris-HCl, pH 7.4	12 mL	1
	0.1M $MgCl_2$	2 mL	
	NADP (10 mg/mL)	0.6 mL	
	G6PD (1000 U/mL)	20 µL	
	PGI (2U/µL)	15 µL	
	M6P	10 mg	

Enzyme Code	Developer Mix		Visualization Method*
NHi	0.3M Tris-HCl, pH 7.0	16 mL	
	Xanthine Oxidase	50 µL	1
	Inosine	20 mg	
NHd	0.3M Tris-HCl, pH 7.0	16 mL	
	Xanthine Oxidase	50 µL	1
	Deoxyinosine	20 mg	
ES	0.1M PO_4, pH 7.4	6 mL	
	4-MUB†	1 mg	5
PEPD	0.3M Tris-HCl, pH 7.4	14 mL	
	0.5M $MnCl_2$	0.4 mL	3
	L-leucyl L-proline	20 mg	
	Peroxidase	2 mg	
	Snake venom	2 mg	
PGM	0.3M Tris-HCl, pH 8.0	12 mL	
	0.1M $MgCl_2$	2 mL	1
	NADP (10 mg/mL)	0.6 mL	
	G6PD (1000 U/mL)	10 µL	
	G1P + G1,6diP (20 mg/mL)	1.5 mL	
6PGD	0.3M Tris-HCl, pH 7.4	12 mL	
	0.1M $MgCl_2$	2 mL	1
	NADP (10 mg/mL)	0.6 mL	
	6PG (10 mg/mL)	1 mL	
G6PD	0.3M Tris-HCl, pH 7.0	14.5mL	
	1M $MgCl_2$	0.5 mL	1
	NADP (10 mg/mL)	1.0 mL	
	G6P (10 mg/mL)	1.0 mL	
GPI	0.3M Tris-HCl, pH 8.0	13.4mL	
	1M $MgCl_2$	0.4 mL	1
	NADP (10 mg/mL)	1 mL	
	G6PD (1000 U/mL)	10 µL	
	F6P (10 mg/mL)	1.6 mL	
ASAT	0.1M PO_4, pH 7.4	6 mL	
	NADH	5 mg	4
	MDH	6 µL	
	α-ketoglutaric acid	12 mg	
	Aspartic acid	20 mg	
ALAT	0.1M PO_4, pH 7.4	6 mL	
	NADH	5 mg	4
	LDH	6 µL	
	α-ketoglutaric acid	12 mg	
	L-alanine	80 mg	
MDH	0.3M tris-HCl, pH 7.4	6.6 mL	
	water	8 mL	1
	NAD (10mg/mL)	1.0 mL	
	1M L-malate, pH 7.0	1.5 mL	
SOD	0.1M PO_4, pH 8.0	18.8mL	
	200mM EDTA, pH 8.0	0.2 mL	1, 2
	riboflavin	0.6 mg	

Enzyme Code	Developer Mix		Visualization Method*
ICD	0.3M tris-HCl, pH 7.0	14 mL	1
	1M $MgCl_2$	1 mL	
	NADP (10 mg/mL)	1 mL	
	dl-isocitric acid (Na_3 salt)	40 mg	
PK	0.3M tris-HCl, pH 7.4	13.3mL	6
	0.1M $MgCl_2$	4 mL	
	ADP (15mg/mL)	2.7 mL	
	1M KCl	3 mL	
	LDH	10 µL	
	NADH	5 mg	
	F1,6 diP	100 mg	
	PEP	22 mg	
ME	0.3M tris-HCl, pH 7.4	13.4mL	1
	1M $MgCl_2$	0.4mL	
	NADP (10 mg/mL)	0.4mL	
	1.0M L-malate, pH 7.0	1.2 mL	

§ 25 mg/mL in ethanol, filtered

† 4-methylumbilliferryl butyrate (4-MUB), dissolved in 200µL acetone

***Visualization Methods**

1. PMS (2 mg/mL) - 1.0 mL
   MTT (5 mg/mL) - 1.0 mL
   Agar (1.2%) - 20mL.
   Incubate at 37°C and monitor for purple/black bands.
2. NBT (5mg/mL) - 1.0 mL
   Agar (1.2%) - 20mL.
   Expose to light for 10 mins., incubate at 37°C and monitor for purple/black bands.
3. 3-amino, 9-ethyl carbazole (25mg/mL in ethanol) - 1.0mL (filtered)
   Agar (1.2%) - 20mL.
   Incubate at 37°C and monitor for red/brown bands.
4. Pour developer onto Whatman No. 1 filter paper and place soaked filter paper over starch gel, avoiding bubbles between the gel and filter paper. Incubate at 37°C and monitor under UV illumination (λ = 340nm) for dark bands on a fluorescent background.
5. Agar (1.2%) - 20mL.
   Incubate at 37°C and monitor under UV illumination (λ = 340nm) for fluorescent bands on a dark background.
6. Agar (1.2%) - 20mL.
   Incubate at 37°C and monitorunder UV illumination (λ = 340nm) for dark bands on a fluorescent background.

+ For visualization of CAE plates, the volumes and weights listed above were halved and 15% (w/v) sucrose was added (Lanham *et al.*, 1981). Staining reactions were carried out in square petri dishes. The developer was poured into the dish and allowed to set (carried out in the dark). The CAE plate was then placed face down onto the agar-based developer. Reactions were stopped by removing the plate from the developer and immersing it in distilled water.

APPENDIX IV - PCR PRIMERS AND CYCLING PARAMETERS

RAPD ANALYSIS

PCR primers

Primer	Primer sequence (5' - 3')
A1*	TCA CGA TGC A
A2	GAA ACG GGT G
A3*	AGT CAG CCA C
A4*	AAT CGG GCT G
A5	GTG ACG TAG G
A6	GTG ATC GCA G
L1*	CGG CCC CTG T
L2	CGG ACG TCG C
L3*	CCC GCC ATC T
L4*	GTG GAT GCG A
L5*	AAG AGC CCG T
L6	AAG GAT CAG A
H1*	CGC GCC CGC T
H2*	TTC CCC CGC T

Primer	Primer sequence (5' - 3')
H3	CAT CCC CCT G
H4	TGC CGA GCT G
H5	TAG GAT CAG A
H6	CAC ATG CTT C
D1	CAG GCC TTC
D2	AGG GGT CTT C
D3*	TTC CGA ACC C
D4*	GGT CCC TGA C
D5	GGG TAA CGC C
D6*	CAA TCG CCG T
D7*	TCG GCG ATA G
D8	AGC CAG CGA A
D9*	AGG TGA CCG T
D10*	GTT GCG ATC C

Primers indicated with the symbol "*" were found to clearly distinguish between the *L. V. braziliensis* and *L. V. guyanensis* species complexes. Primers in **bold** type were found to distinguish putative parental and hybrid strains from Nicaragua.

Cycling parameters

Temp. (°C)	Time (mins)	No. of cycles
94	5	1
37	1	
72	1	
94	1	40
37	1	
72	1	
72	10	1

RIBOPRINTING (SSUrRNA) (Uliana *et al.*, 1991)

PCR primers

Primer	Primer sequence (5' - 3') and Tm
DN-SSU1	GAT CTG GTT GAT TCT GCC AG Tm = 51.6°C
DN-SSU2	GAT CCA GCT GCA GGT TCA CC Tm = 55.7°C

Cycling parameters

Temp. (°C)	Time (mins)	No. of cycles
95	5	
58	1	1
72	2	
95	1	
58	0.5	25
72	1.5	
72	10	1

PCR-RFLP ANALYSIS (rRNA ITS) (Cupolillo *et al.*, 1995; Stothard *et al.*, 1996)

PCR primers

Primer	Primer sequence (5' - 3') and Tm
DN-IR1	GCT GTA GGT GAA CCT GCA GCA GCT GGA TCA TT Tm = 65.5°C
DN-IR2	GCG GGT AGT CCT GCC AAA CAC TCA GGT CTC Tm = 66.9°C

Cycling parameters

Temp. (°C)	Time (secs.)	No. of cycles
95	15	
58	30	37
72	90	
72	10 min.	1

MICROSATELLITE ANALYSIS

(Russell *et al.*, 1999)

PCR primers

Primer	Primer sequence (5' - 3') and Tm
AC01 A	GAG AGG CCA CCA GAC ACG TCA GCA CAC Tm = 71.4°C
AC01 B	CCC CCT TCC TTC GCC TTC AAC ACC TTT AC Tm = 70.1°C
AC16 A	CTT CTT CTC ATG CTG CAC GGT CTC CTC CTT Tm = 70.2°C
AC16 B	CCA TGG GCG GGC TTG TTT CGT TAC TTT TTA Tm = 70.1°C
AC52 A	CCA CCG CCG GCT TCA CTA C Tm = 63.4°C
AC52 B	GCG GCA ATC GTC TGG CTA AA Tm = 62.6°C

Cycling parameters

Temp. (°C)	Time (mins)	No. of cycles
95°C	30 s.	
62°C (60°C)	30 s.	35 (38)
72°C	60 s.	
72°C	10 min.	1

An annealing temperature of 60°C was used for amplification of the AC16 locus. This temperature was also used to amplify the AC01 and AC52 loci for isolates which gave poor yield at the higher annealing temperature; in addition, for these isolates, the number of amplification cycles was increased to 38.

STANDARD CODING SYSTEM FORMAT FOR NUCLEOTIDES AND NUCLEOTIDE COMBINATIONS:

Symbol	Meaning
A	Adenine
C	cytosine
G	guanine
T	thymine
Y	C or T (pyrimidine)
R	A or G (purine)
W	A or T
S	C or G

Symbol	Meaning
K	T or G
M	C or A
B	C or G or T
D	A or G or T
H	A or C or T
V	A or C or G
X or N	unknown

APPENDIX V - POPULATION GENETIC ANALYSIS

Table V-1 **Allele and genotype frequency results based on IEA data from Huánuco stocks±**

Locus	No. alleles scored	Allele	Allele frequency	No. possible genotypes	Genotype	Genotype frequency	
						Observed	Expected
MPI	4	5	0.0259	10	5 / 5	0	0.026
		6	0.6552		6 / 5	3	1.983
					6 / 6	22	24.783
		7	0.2931		7 / 5	0	0.887
					7 / 6	26	22.470
					7 / 7	4	4.878
		9	0.0259		9 / 5	0	0.078
					9 / 6	3	1.983
					9 / 7	0	0.887
					9 / 9	0	0.026
NHi1	2	2	0.6983	3	2 / 2	23	28.174
					3 / 2	35	24.652
		3	0.3017		3 / 3	0	5.174
NHd	2	4	0.7759	3	4 / 4	32	34.826
					5 / 4	26	20.348
		5	0.2241		5 / 5	0	2.826
ES	2	4	0.8103	3	4 / 4	39	38.009
					5 / 4	16	17.983
		5	0.1897		5 / 5	3	2.009
PEPD	2	2	0.7845	3	2 / 2	36	35.609
					4 / 2	19	19.783
		4	0.2155		4 / 4	3	2.609
PGM	2	2	0.0536	3	2 / 2	3	0.135
					3 / 2	0	5.730
		3	0.9464		3 / 3	53	50.135
					null	2	-
GPI	2	2	0.0517	3	2 / 2	3	0.130
					4 / 2	0	5.722
		4	0.9483		4 / 4	54	51.118
					null	1	-
G6PD	2	2	0.9483	3	2 / 2	55	52.130
					3 / 2	0	5.739
		3	0.0517		3 / 3	3	0.130
NHi2	1	7	1.0000	1	7 / 7	58	-
6PGD	1	5	1.0000	1	5 / 5	58	-
ASAT	1	5	1.0000	1	5 / 5	58	-
ALAT	1	4	1.0000	1	4 / 4	58	-

± analysis excludes *L. V. lainsoni* stock LC2679.

Table V-2 **Allele and genotype frequency results based on AC01 & AC16 microsatellite data from Huánuco stocks±**

Locus	No. alleles scored	Allele	Allele frequency	No. possible genotypes	Genotype	Genotype frequency	
						Observed	Expected
		225	0.0189		225 / 225	1	0.010
AC01	8	227	0.4057	36	227 / 225	0	0.819
					/ 227	0	8.600
		229	0.0377	9 scored	229 / 225	0	0.076
					/ 227	0	1.638
					/ 229	0	0.057
		231	0.3774		231 / 225	0	0.762
					/ 227	39	16.381
					/ 229	2	1.524
					/ 231	0	7.429
		233	0.0377		233 / 225	0	0.076
					/ 227	1	1.638
					/ 229	3	0.152
					/ 231	0	1.524
					/ 233	0	0.057
		235	0.0377		235 / 225	0	0.076
					/ 227	0	1.638
					/ 229	0	0.152
					/ 231	0	1.524
					/ 233	0	0.152
					/ 235	2	0.057
		237	0.0660		237 / 225	0	0.133
					/ 227	3	2.867
					/ 229	0	0.267
					/ 231	0	2.667
					/ 233	0	0.267
					/ 235	0	0.267
					/ 237	2	0.200
		241	0.0189		241 / 225	0	0.038
					/ 227	0	0.819
					/ 229	0	0.076
					/ 231	0	0.762
					/ 233	0	0.076
					/ 235	0	0.076
					/ 237	0	0.133
					/ 241	1	0.010
		240	0.0769		240 / 240	3	0.272
AC16	10	244	0.4231	55	244 / 240	2	3.417
					/ 244	17	9.184
		246	0.0289	14 scored	246 / 240	0	0.233
					/ 244	3	1.282
					/ 246	0	0.029
		248	0.2885		248 / 240	0	2.330
					/ 244	1	12.816
					/ 246	0	0.874
					/ 248	13	4.223
					/ 244	0	1.282

Table V-2 **Allele and genotype frequency results based on AC01 & AC16 microsatellite data from Huánuco stocks± (cont'd)**

Locus	No. alleles scored	Allele	Allele frequency	No. possible genotypes	Genotype	Genotype frequency	
						Observed	Expected
					248 / 246	0	0.087
AC16				55	/ 248	3	0.874
		250	0.0865		250 / 240	0	0.699
				14 scored	/ 244	2	3.845
					/ 246	0	0.262
					/ 248	0	2.621
					/ 250	1	0.350
		252	0.0385		252 / 240	0	0.311
					/ 244	0	1.709
					/ 246	0	0.117
					/ 248	0	1.165
					/ 250	5	0.350
					/ 252	0	0.058
		254	0.0096		254 / 240	0	0.078
					/ 244	0	0.427
					/ 246	0	0.029
					/ 248	0	0.291
					/ 250	1	0.087
					/ 252	0	0.039
					/ 254	0	0.000
		262	0.0289		262 / 240	0	0.233
					/ 244	0	1.282
					/ 246	0	0.087
					/ 248	3	0.874
					/ 250	0	0.262
					/ 252	0	0.117
					/ 254	0	0.029
					/ 262	0	0.029
		266	0.0096		266 / 240	0	0.078
					/ 244	1	0.427
					/ 246	0	0.029
					/ 248	0	0.291
					/ 250	0	0.087
					/ 252	0	0.039
					/ 254	0	0.010
					/ 262	0	0.029
					/ 266	0	0.000
		274	0.0096		274 / 240	0	0.078
					/ 244	1	0.427
					/ 246	0	0.029
					/ 248	0	0.291
					/ 250	0	0.087
					/ 252	0	0.039
					/ 254	0	0.010
					/ 262	0	0.029
					/ 266	0	0.010
					/ 274	0	0.000

The Index of Association (I_A) (from Maynard Smith *et al.*, 1993).

n = number of individuals

p_{ij} = frequency of the *i*th allele at *j*th locus

h_j = probability that two individuals are different at the *j*th locus

$$h_j = 1 - \sum p_{ij}^2$$

K = "distance" between two individuals (i.e. no. of loci at which they differ)
= mean difference between two individuals

$$K = \sum h_j$$

V_E = expected variance of K (assuming no linkage disequilibrium)

$$V_E = \sum h_j \ (1 - h_j)$$

V_O = observed variance of K (estimated, for assumptions made , see Maynard-Smith *et al.*, 1993)

$$V_O = K + \frac{1}{3K^2}$$

I_A = Index of association

$$I_A = \frac{V_O}{V_E} - 1$$

Table V-3 **Allele frequencies of whole Huánuco population and of subgroups therein*, by locus.**

		Allele frequencies of whole Huánuco population and subgroups therein*					For D' & r^2 calculations†	
Locus	**Allele**	**All (58)**	**Lb (25)**	**Lpe (4)**	**Lb/Lpe (26)**	**IM (3)**	A_1	A_2
MPI	5	0.026	-	-	-	0.500	0.655	0.345
	6	0.655	0.940	-	0.500	0.500		
	7	0.293	-	1.000	0.500	-		
	9	0.026	0.060	-	-	-		
NHi1	2	0.698	0.820	-	0.500	1.000	0.698	0.302
	3	0.302	0.180	1.000	0.500	-		
NHd	4	0.776	1.000	1.000	0.500	1.000	0.776	0.224
	5	0.224	-	-	0.500	-		
ES	4	0.810	0.680	1.000	0.981	-	0.810	0.190
	5	0.190	0.320	-	0.019	1.000		
PEPD	2	0.784	0.620	1.000	1.000	-	0.784	0.216
	4	0.216	0.380	-	-	1.000		
PGM[a]	2	0.054	1.000	1.000	1.000	-	§ not done	
	3	0.946	-	-	-	1.000		
GPI[a]	2	0.052	1.000	1.000	1.000	-		
	4	0.948	-	-	-	1.000		
G6PD	2	0.948	1.000	1.000	1.000	-		
	3	0.052	-	-	-	1.000		
NHi2	7	1.000	1.000	1.000	1.000	1.000		
6PGD	5	1.000	1.000	1.000	1.000	1.000		
ASAT	5	1.000	1.000	1.000	1.000	1.000		
ALAT	4	1.000	1.000	1.000	1.000	1.000		
AC01	225	0.019	-	-	-	0.500	0.406	0.594
	227	0.406	0.420	-	0.458	-		
	229	0.038	0.006	-	0.021	-		
	231	0.377	0.340	-	0.479	-		
	233	0.038	0.008	-	-	-		
	235	0.038	-	0.500	-	0.500		
	237	0.066	0.100	-	0.042	-		
	241	0.019	-	0.500	-	-		
AC16	240	0.077	-	-	0.174	-	0.423	0.577
	244	0.423	0.229	0.250	0.700	-		
	246	0.029	0.063	-	-	-		
	248	0.289	0.604	-	0.022	-		
	250	0.087	0.021	0.500	0.065	0.500		
	252	0.039	-	-	0.043	0.333		
	254	0.010	-	-	-	0.167		
	262	0.029	0.063	-	-	-		
	266	0.010	-	0.250	-	-		
	274	0.010	0.021	-	-	-		

* - Allele frequencies of whole Huánuco population and subgroups therein (see text).

† - **For D' & r^2 calculations**: A_1 = frequency of most common allele at locus; A_2 = combined frequencies of all other alleles at locus. § - not done because frequency of the most common allele greater than 0.90 (see text).

Table V-4 **I_A values calculated from isoenzyme and microsatellite data for the Huánuco stocks±†**

		All data (10 polymorphic loci)		IEA data (8 polymorphic loci)			Microsatellite data (2 polymorphic loci†)		
		No. ET & MSTs	I_A value		I_A values		No.	I_A values	
Population	**No. stocks examined**	identified	All stocks	No. ETs identified	All stocks	ETs only	MSTs identified	All stocks	MSTs only
All	**58**	**23**	**0.9670**	**11[a]**	**0.5891**	0.9110	**16[♦]**	**2.7663**	5.9910
SG1	25	8	**1.1210**	5[a]	0.9581	0.7580	7[♦]	2.2460	3.3669
SG2	4	2	**1.8662[*]**	1	0[*]	0	2	1.8662	2.2665
SG3	26	11	**1.0437**	4[a]	1.1355	10.7124	7[♦]	1.7120	2.5192
SG4	3	2	**1.3585**	1	0[*]	0	2	1.8326	1.8662

Notes to table V-4: I_A values calculated from IEA and microsatellite (MS) data for the Huánuco stocks. ±analysis excludes *L. V. lainsoni* stock LC2679 and data from microsatellite locus †AC52 (see text). Population subgroups (SG) identified using IEA: SG1 – *L. V. braziliensis*; SG2 – *L. V. peruviana*; SG3 – putative *L. V. braziliensis* / *L. V. peruviana* hybrids; SG4 – IM2832-types. ET - electrophoretic type; MST – microsatellite type. *Both SG2 and SG4 presented only one ET, the I_A value of which was zero. ♦One stock from both SG1 and SG3 had an identical MST. Values of I_A increased for all stocks and all subgroups when using data from ETs and MSTs only, except for SG1 (*L. V. braziliensis*) ET data (see text).

Linkage disequilibrium calculation based on maximum-likelihood estimates of gametic frequencies (taken from Hedrick, 1985)

With 2 polymorphic loci (such as enzyme loci), the possible gametes arising from an inferred genotype (consisting of 2 loci, A and B) are shown in the table below:

Phenotype	Inferred genotype	Possible gametic types
A_1B_1	$A_1A_1B_1B_1$	A_1B_1
A_1B_2	$A_1A_1B_2B_2$	A_1B_2
A_2B_1	$A_2A_2B_1B_1$	A_2B_1
A_2B_2	$A_2A_2B_2B_2$	A_2B_2
$A_1A_2B_1$	$A_1A_2B_1B_1$	A_1B_1 or A_2B_1
$A_1A_2B_2$	$A_1A_2B_2B_2$	A_1B_2 or A_2B_2
$A_1B_1B_2$	$A_1A_1B_1B_2$	A_1B_1 or A_1B_2
$A_2B_1B_2$	$A_2A_2B_1B_2$	A_2B_1 or A_2B_2
$A_1A_2B_1B_2$*	$A_1A_2B_1B_2$	A_1B_1 or A_2B_2 (coupling) A_1B_2 or A_2B_1 (repulsion)

Conventional analyses can be used in the case of loci with alleles showing dominance because all possible gametic types can be identified. In the case of co-dominant loci however, as here with enzyme data, it is not possible to determine the origins of gametic types in the double heterozygote*. In this case the phenotype could have arisen from union between parental-type ("coupling") or recombinant-type ("repulsion") gametes.

For 2 co-dominant loci, therefore, a maximum-likelihood (ML) approach is taken to circumvent this lack of information regarding gametic types. The following table is completed and then used in subsequent analysis:

	Observed phenotype at locus A			
Observed phenotype at locus B	**A_1A_1**	**A_1A_2**	**A_2A_2**	**Total**
B_1B_1	N_{11}	N_{12}	N_{13}	N_1
B_1B_2	N_{21}	N_{22}	N_{23}	N_2
B_2B_2	N_{31}	N_{32}	N_{33}	N_3
Total	$N_{.1}$	$N_{.2}$	$N_{.3}$	N

where: A_1 = most common allele of locus A
A_2 = all other alleles of locus A
B_1 = most common allele of locus B
B_2 = all other alleles of locus B

N_{11} = number of isolates with phenotype $A_1A_1B_1B_1$
N_{12} = number of isolates with phenotype $A_1A_2B_1B_1$ etc.

To calculate the maximum-likelihood estimation of $\times_1$ ($\times_1$ is the frequency of the A_1B_1 gamete), an arbitrary starting point is taken, where:

$$\times_1 = 2N_{11} + \frac{N_{12} + N_{21}}{2(N - N_{22})}$$

This starting figure of $\times_1$ is then used in the equation below, which is reiterated until the value of $\times_1$ does not change from the previous iteration:

$$\times_1 = \frac{1}{2N}\left[2N_{11} + N_{12} + N_{21} + \frac{N_{22}\times_1(1 - p_1 - q_1 + \times_1)}{\times_1(1 - p_1 - q_1 + \times_1) + (p_1 - \times_1)(q_1 - \times_1)}\right]$$

The maximum-likelihood estimate of $\times_1$ is then used to calculate the disequilibrium parameter, where:

$$D = \times_1 - p_1q_1$$

D' is calculated as follows:

$$D' = \frac{D}{D_{max}}$$

where D_{max} is the maximum D possible for a given set of allelic frequencies at the 2 loci. D_{max} is equal either to the lesser of p_1q_2 or p_2q_1 if D is positive, or to the lesser of p_1q_1 or p_2q_2 if D is negative.

A test of significance (Q) can be calculated for each D score from which probability (P) values are assigned:

$$Q = Nr^2 = \frac{ND^2}{p_1p_2q_1q_2}$$

r^2 is calculated as follows:

$$r^2 = \frac{D^2}{p_1p_2q_1q_2}$$

Table V-5 **Linkage disequilibrium parameter results (complete).**

2-locus combination	N	D	Q	D'	P value	r^2
MPI^{6} x $NHi1^{2}$	58	0.136	22.199	**0.687**	**Sig. P < 0.005**	**0.383**
MPI^{6} x NHd^{4}	58	0.147	31.907	1.000	Sig. P < 0.005	0.548
MPI^{6} x ES^{4}	58	- 0.038	2.378	**- 0.579**	**Not Sig.**	**0.041**
MPI^{6} x $PEPD^{2}$	58	- 0.048	3.492	**- 0.644**	**Not Sig.**	**0.060**
$NHi1^{2}$ x NHd^{4}	58	0.156	38.512	1.000	Sig. P < 0.005	0.664
$NHi1^{2}$ x ES^{4}	58	- 0.056	5.607	- 0.976	Sig. P < 0.025	0.097
$NHi1^{2}$ x $PEPD^{2}$	58	- 0.064	6.655	- 0.981	Sig. P < 0.01	0.115
NHd^{4} x ES^{4}	58	- 0.043	3.927	- 1.000	Sig. P < 0.05	0.068
NHd^{4} x $PEPD^{2}$	58	- 0.048	4.613	- 1.000	Sig. P < 0.05	0.080
ES^{4} x $PEPD^{2}$	58	0.141	44.244	0.946	Sig. P < 0.005	0.763
$AC01^{227}$ x MPI^{6}	53	0.127	16.600	1.000	Sig. P < 0.005	0.313
$AC01^{227}$ x $NHi1^{2}$	53	0.127	16.600	1.000	Sig. P < 0.005	0.313
$AC01^{227}$ x NHd^{4}	53	0.101	12.761	1.000	Sig. P < 0.005	0.241
$AC01^{227}$ x ES^{4}	53	0.083	9.805	1.000	Sig. P < 0.005	0.185
$AC01^{227}$ x $PEPD^{2}$	53	0.089	10.269	1.000	Sig. P < 0.005	0.194
$AC16^{244}$ x MPI^{6}	52	- 0.061	3.662	**- 0.333**	**Not Sig.**	**0.070**
$AC16^{244}$ x $NHi1^{2}$	52	- 0.087	7.566	**- 0.489**	**Sig. P < 0.01**	**0.146**
$AC16^{244}$ x NHd^{4}	52	- 0.074	6.685	**- 0.576**	**Sig. P < 0.01**	**0.129**
$AC16^{244}$ x ES^{4}	52	0.075	7.514	0.882	Sig. P < 0.01	0.145
$AC16^{244}$ x $PEPD^{2}$	52	0.079	7.431	0.805	Sig. P < 0.01	0.143
$AC01^{227}$ x $AC16^{244}$	51	0.044	1.652	**0.191**	**Not Sig.**	**0.032**

N = number of stocks analyzed in calculation