PCR BASED APPROACHES TO THE IDENTIFICATION AND CLASSIFICATION OF *LEISHMANIA*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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LEISHMANIASIS IN NICARAGUA, A CASE REPORT

"I said to Flavio our guerrilla doctor, "I'm all screwed up- here, on my calves." "What you have got there's an infection."

This so-called infection started growing then like crazy; it was as big as a córdoba piece. It hurt so much I couldn't sleep. I had to fold over the top of my rubber boot, since if it even touched my skin it about killed me. They had given me some antibiotic capsules to knock it out , but I said to Flavio, "This bullshit isn't going away. Flavio, I'm starting to smell something bad, this shit is really starting to stink." Flavio leaned over for a whiff. " You're right brother, this is putrid. .... I'm going to give you a shot of benzetacil"

I spent four days crippled, off my feet. We were all of us weak, just skin and bones; a shot like that just wiped me out. Four days passed and they began my treatments. It was horrible, because they stuck pincers wrapped in cotton right in the open sore and dug into it with the cotton swab. I gritted my teeth and clenched my fists....*aiii, brother!* I yanked my foot back, and Flavio sat down on it and kept hold of me.

... We were undernourished and reduced to shit. I had to stay off my feet, and the treatments continued every day because every day there was pus, and the ulcer got bigger and bigger. He gave me three benzetacil shots, and the thing was getting worse; it was eating me alive, and that pain.....I couldn't get up, not even to get my food. It was all I could do to get up and go shit or take a bath.

It took three people for my treatment. A compañero would cut a couple of branches. They put one in each of my hands and another in my mouth so I wouldn't yell out when they started swabbing out the holes in my legs with gauze. I could feel them digging right down to the quick.....The came out all covered with pus and blood and chunks of flesh a bit bigger than a bean or a kernel of corn. I was losing little chunks of flesh. Now I just have the scars, but the lesions were much bigger, about five inches in diameter, the size of your hand and all eaten out in side.

... Poor Flavio was always in his hammock, depressed because he couldn't figure out what it was. One afternoon he came running up: "It's lesymaniasis, lesymaniasis!" Like somebody crying out "Land, land!" "What's lesymaniasis?" "Brother it's what you've got, it's mountain leprosy, that's lesymaniasis." I remembered in a course I had taken before coming to the mountains we had studied tropical medicine: "That's ...that's...Reprodral, Reprodral! That's the cure Reprodral!"

(Omar Cabezas, 1985, Fire from the mountain, the making of a Sandinista, translated by K. Weaver, Jonathan Cape, London, 107-108)

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ABBREVIATIONS

ACL	American Cutaneous Leishmaniasis
AP-PCR	Arbitrarily Primed PCR
ATCC	American Type Culture Collection
AVL	American Visceral Leishmaniasis
bp	base pairs
cDNA	copy DNA
CHEF	Contour Clamped Homogenous Electric Field electrophoresis
CL	Cutaneous Leishmanisis
DAF	DNA Amplification Fingerprinting
DCL	Diffuse Cutaneous Leishmaniasis
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
ELISA	Enzyme Linked Immuno Absorbent Assay
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin
kDNA	Kinetoplast DNA
LSU	Large Sub-unit
MCL	Mucocutaneous Leishmaniasis
MIE	Multi-locus Isoenzyme electrophoresis
MYA	Million Years Ago
NAD	Nicotinamide adenine diphosphate
NBT	Nitro Blue Tetrazolium
OTU	Operational Taxonomic Unit
PAHO	Pan American Health Organisation
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PMS	Phenazine methosulphate
RADES	Randomly Amplified Differentially Expressed
RAPD	Randomly Amplified Polymorphic DNA
rDNA	ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	ribosomal RNA
SNOPAD	Standardised Nomenclature on Parasitic Diseases
SSCP	Single Stranded Conformation Polymorphism Sequences
SSU	Small Sub-unit
TE	Tris-HCl 10mM, EDTA 1mM
Th	Thymus-dependent helper
VL	Visceral Leishmaniasis

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PREFACE

This study was conducted as a part of a larger collaborative investigation of the genetic diversity of *Leishmania* and sandflies in Central America. The project was carried out with the support of the European Union and involved workers at the Liverpool School of Tropical Medicine and subsequently the University of Keele together with leading researchers of *Leishmania* from Panama, Costa Rica, Nicaragua, Honduras and Guatemala. They are Dr R. Maingon and Prof. R. Ward (LSTM and Keele University, UK), Dr B. Arana and F. Arana (Universidad del Valle, Guatemala), Dr C. Ponce and Dr E. Ponce (Ministry of Health, Tegucigalpa, Honduras), A. Belli (Ministry of Health, Managua, Nicaragua), Dr R. Zeledon (Universidad Nacional, Costa Rica), Dr P de Carreira and Dr O. Sousa (Universidad de Panamá, Panamá).

The object of the project was to promote the integration of research into leishmaniasis in Central America and to introduce new techniques where appropriate. Specifically, strains of *Leishmania* from each country were exchanged so that the different methods of identification used at each centre could be compared and workshops were conducted in which training in the PCR and schizodeme analysis was provided. Equipment for the PCR was supplied to the participating laboratories in Panamá and Nicaragua and staff at these laboratories were trained in the use of the equipment.

In addition to the dissemination of existing PCR based methods for the identification of *Leishmania* strains it was also proposed to test RAPD for the analysis of putative hybrid strains of *Leishmania* from Nicaragua and *L. chagasi* strains from cases of visceral and cutaneous leishmaniasis from Costa Rica and Honduras.

The work presented here is based upon the strains received from Central American colleagues and the issues that arose out of their identification.

LIST OF PUBLICATIONS

The following papers were published or accepted or submitted for publication during the course of this study.

Published:

Carreira, P.F., Maingon, R., Ward, R.D., Noyes, H., Ponce, C., Belli, A., Arana, B., Zeledon, R. and Sousa, O.E. (1995). Molecular techniques in the characterisation of *Leishmania* isolates from Central America. *Annals of Tropical Medicine and Parasitology* **89** Suppl. 1 31-36.

Accepted:

Hamilton, J.G.C., Ward, R.D., Dougherty, M.J., Ponce, C., Ponce, E., Noyes, H., Zeledon, R. and Maingon, R. (In Press). Comparison of sex pheromone components of *Lutzomyia longipalpis* (Diptera: Psychodidae) from areas of visceral and atypical cutaneous leishmaniasis in Honduras and Costa Rica. *Annals of Tropical Medicine and Parasitology*

Motazedian, H., Noyes, H. and Maingon, R. (In Press). *Leishmania* and *Sauroleishmania*: the use of random amplified polymorphic DNA for the identification of parasites from vertebrates and invertebrates. *Experimental Parasitology*

Noyes, H., Belli, A. and Maingon, R. (In Press) Appraisal of various RAPD-PCR primers for *Leishmania* identification. *American Journal of Tropical Medicine and Hygiene.*

Submitted:

Noyes, H,. Chance, M., Ponce, C., Ponce, E. and Maingon, R. Genotypically similar *Leishmania chagasi* parasites cause both visceral and cutaneous leishmaniasis in Honduras.

Noyes, H.A., Perez Camps, A.M. and Chance, M.L. *Leishmania herreri* (Kinetoplastida; Trypanosomatidae) is more closely related to *Endotrypanum* (Kinetoplastida; Trypanosomatidae) than to *Leishmania*

A NOTE ON NOMENCLATURE

No proposed nomenclature for either *Leishmania* or the diseases that parasites of this genus cause has been universally adopted, and the subject remains controversial. In the present work the nomenclature of Lainson and Shaw (1987) as adopted by the WHO (Anon., 1990a) has been used with the exception of the nomenclature of the lizard parasites. Lainson and Shaw (1987) allocated the lizard *Leishmania* to the genus *Sauroleishmania*, as had Killick-Kendrick *et al.* (1986). However this classification has not been adopted by a number of specialists in this group (Frech et al., 1995; Fu & Kolesnikov, 1994; Landweber & Gilbert, 1993). Evidence for the inclusion of the lizard parasites in the genus *Leishmania* was discovered in the course of the studies presented here, and consequently lizard parasites have been referred to as *Leishmania* to maintain consistency.

Sub-specific names have not been used and therefore all references to *L. braziliensis* and *L. mexicana* denote isolates and strains of those species alone and not other members of the complexes to which they lend their names, unless specifically stated.

SNOPAD's proposal for naming the diseases caused by *Leishmania* "leishmanioses" has not been taken up in the present work as it has not yet been adopted by any major English language publication (Ashford, 1994; Burt, 1994; Kassai & Burt, 1994).

ABSTRACT

PCR BASED APPROACHES TO THE IDENTIFICATION AND CLASSIFICATION OF *LEISHMANIA*

H.A.Noyes

Random amplified polymorphic DNA (RAPD) was tested for the identification and classification of *Leishmania*. RAPD was found to be useful for the identification of species of *L. (Leishmania)* and *L. (Viannia)* and for the classification of *L. (Viannia)* species.

The polymerase chain reaction (PCR) was tested for the identification of *Leishmania* from mammals and lizards, using both published primers and new primers which amplify kinetoplast minicircle DNA. The size of the PCR product was found to be useful for discriminating between some sympatric pairs of species such as *L. braziliensis* and *L. mexicana*. Isotopically labelled probes prepared from the variable region of the kinetoplast minicircle were tested for specificity for the identification of New and Old World species of *Leishmania*. The specificity was dependent on the concentration of target DNA and was manipulated to investigate relationships between *Leishmania* species. Restriction digests of kinetoplast DNA (schizodemes) prepared by PCR and by centrifugation through 20% sucrose were compared for the identification of strains of *L. infantum* and *L. chagasi*.

Twenty three strains of *L. chagasi* from cases of visceral and cutaneous leishmaniasis in Honduras were examined by RAPD, schizodemes, differential display, isoenzymes, RFLPs and PFGE to discover whether genetic differences existed between parasites causing the two different pathologies. The parasites were found to be unusually homogeneous and no differences were found which correlated with pathology by any of these methods.

Restriction digests of PCR amplified small subunit ribosomal DNA (SSU rDNA) (ribodemes) were tested to find markers specific for the genus *Leishmania*. A classification of the *Leishmania* based on the restriction fragments indicated that *L. hertigi* and *L. herreri* were more closely related to *Endotrypanum* than to *Leishmania*, and that the lizard *Leishmania* could not be placed in separate genus from the *Leishmania*.

Ribodemes were used to identify two strains of parasites supplied by colleagues in Central America that could not be identified by existing methods for the identification of *Leishmania*. One of these strains appeared to be identical to a *C. luciliae* reference strain. The other strain produced a fingerprint unlike any of the available reference strains. A variable region of the SSU rRNA gene was identified that was suitable for classifying trypanosomatids and the sequence of this region was used to classify the strain that could not be identified by fingerprinting.

1. INTRODUCTION

1.1. TAXONOMIC POSITION

1.1.1. The classification of the Trypanosomatidae

Leishmania are dimorphic and heteroxenous parasites of the trypanosomatid family. The most recent authoritative revision of the classification of the Trypanosomatidae was by Levine *et al.* (1980) and is as follows:-

Kingdom:- **Protista Haeckel, [1866]**

Subkingdom **Protozoa (Goldfuss, 1817) von Siebold, [1845]**. Single celled, eukaryotic organisms. Not a natural group. Most with single vesicular nucleus. Sexual and asexual reproduction are present depending on group.

Phylum:- **Sarcomastigophora Honigberg & Balamuth, 1963.** Flagella, pseudopodia, or both types of locomotory organelles: usually single type of nucleus; sexuality, when present, essentially syngamy.

Subphylum:- **Mastigophora Diesing, [1866].** One or more flagella typically present in trophozoites; asexual reproduction basically by intrakinetal (symmetrogenic) binary fission; sexual reproduction known in some groups.

Class:- **Zoomastigophora Calkins, [1909].** Chloroplasts absent; one to many flagella; amoeboid forms, with or without flagella, in some groups; sexuality known in a few groups; a polyphyletic group.

Order:- **Kinetoplastida (Honigberg, 1963) Vickerman, 1976.** One or two flagella arising from depression; flagella typically with paraxial rod in addition to axoneme; single mitochondrion (nonfunctional in some forms) extending length of body as single tube, hoop, or network of branching tubes usually containing a Feulgen-positive (DNA-containing) kinetoplast (nucleoid) located near flagellar kinetosomes; Golgi apparatus typically in region of flagellar depression, not connected to kinetosomes and flagella; parasitic (majority of known species) and free living.

Suborder:- **Trypanosomatina Kent [1880]** Single flagellum either free or attached to body by undulating membrane; kinetoplast relatively small and compact; parasitic.

Family:- **Trypanosomatidae (Döflein, 1901) Grobben, [1905]**. Only family within trypanosomatina.

The recent availability of large quantities of sequence data particularly for rRNA has led to phylogenies of these sequences that differ radically from previous classifications. Cavalier-Smith (1993) used SSU rRNA sequences as well as a range of morphological, biochemical, and genetic data to designate 18 phyla within the protozoa and placed *Leishmania* as follows:- Empire, Eukaryota; Superkingdom, Metakaryota; Kingdom, Protozoa; Infrakingdom, Euglenoza; Phylum, Euglenoza; Subphylum, Kinetoplasta; Order, Trypanosomatida. Cox (1993) raised the Kinetoplasta to phylum status. As the present flood of data subsides a new consensus may emerge which should offer interesting insights into the evolution of the Trypanosomatidae.

1.2. THE TRYPANOSOMATID FLAGELLATES

1.2.1. Morphology

The life cycles of trypanosomatids are characterised by morphological changes involving repositioning of the flagellum base-kinetoplast complex with respect to the nucleus and to the anterior end of the body.

Seven morphologies were recognised by Vickerman (1976) (Fig 1.1).

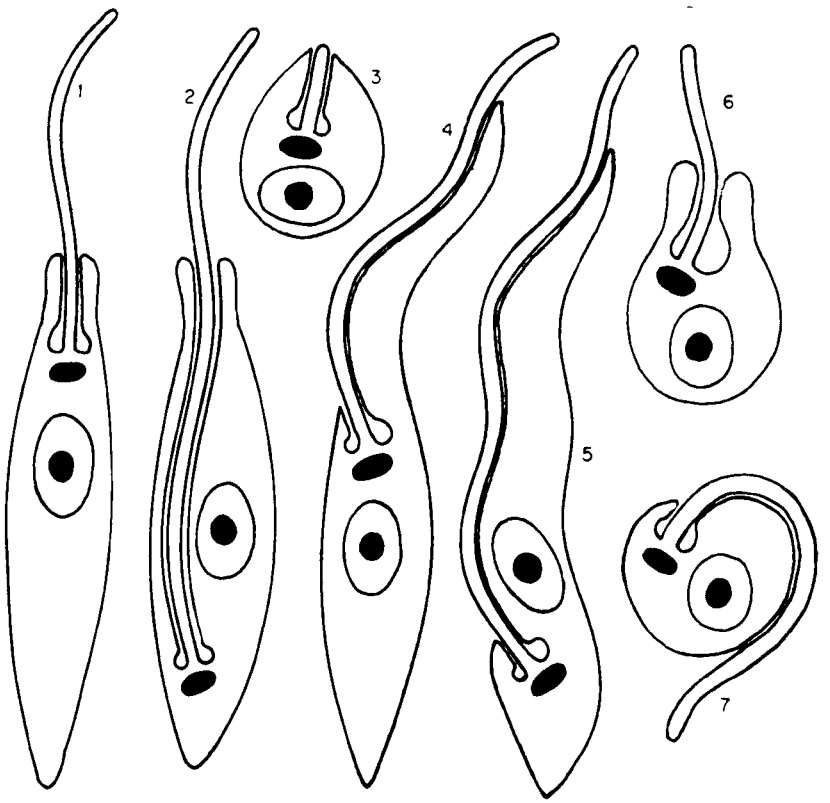


Figure 1.1 Diagrams of developmental stages of trypanosomatids named according to the scheme of Hoare and Wallace (1966) (1-6) and Brack (1968) (7): 1, promastigote; 2, opisthomastigote; 3, amastigote; 4, epimastigote; 5, trypomastigote; 6, choanomastigote; 7, sphaeromastigote. (From Vickerman, 1976)

Promastigote: flagellar base near anterior end of body from which the flagellum emerges.

Amastigote: flagellar base in front of nucleus but no emergent flagellum.

Opisthomastigote: flagellar base behind the nucleus with a long flagellar pocket leading to the anterior end.

Choanomastigote: flagellum base lies just in front of the nucleus with the flagellum emerging anteriorly from a broad flagellar pocket.

Epimastigote: flagellar base lies in front of the nucleus, the flagellum emerges laterally to form an undulating membrane as it runs along the body to the anterior end.

Trypomastigote: flagellar base behind the nucleus, flagellum emerges laterally to form an undulating membrane along the body to the anterior end.

Sphaeromastigote: forms whose spherical shape precludes recognition of the anterior end and hence classification in the above scheme.

1.2.2. Genera of trypanosomatid flagellates.

Vickerman (1976) recognised nine genera within the Trypanosomatidae:-

Blastocrithidia Laird 1959. Monogenetic parasites of Diptera, Hemiptera, Siphonoptera and ixodid ticks. The epimastigote is the characteristic form but amastigotes may be produced as flagellar cysts in some species.

Crithidia Leger 1902. Monogenetic parasites of Diptera, Hemiptera, Trichoptera and Hymenoptera possessing the characteristic choanomastigote form.

Endotrypanum Mesnil & Brimont 1908. Digenetic parasites with trypomastigote and epimastigote stages inside erythrocytes of edentates and promastigotes and amastigotes in phlebotomine sandflies.

Herpetomonas Kent 1880. Monogenetic parasites of Diptera and possibly other insect orders with promastigote and opisthomastigote forms.

Leptomonas Kent 1880. Monogenetic parasites with promastigote stages and cysts.

Leishmania Ross 1903. Digenetic parasites multiplying in the mononuclear phagocytic cells of mammals and in the gut lumen of sandflies (Diptera, Psychodidae, Phlebotominae); characterised by intra cellular amastigotes in the mammal and free or attached promastigotes in the vector.

Phytomonas Donovan 1909. Digenetic parasites of plants and insects retaining the promastigote form throughout although amastigotes have been reported.

Rhynchoidomonas Patton 1910. Monogenetic parasite of Diptera characterised by trypomastigotes without conspicuous undulating membrane or free flagellum.

Leishmania incertae sedis. Digenetic parasites of reptiles and phlebotomine sandflies. In the reptile host some species are found as promastigotes in the gut whilst other species may be promastigotes or amastigotes in the blood or in mononuclear phagocytic cells. In the phlebotomine vector promastigotes are found in the hind gut.

Trypanosoma Gruby 1843. Digenetic parasites in the blood of vertebrates and the gut of leeches and arthropods; trypomastigote and epimastigote stages are common to nearly all trypanosome lifecycles but amastigotes and rarely promastigotes may also be present.

Large subunit (LSU) and small subunit (SSU) rRNA gene sequences from representatives of the Trypanosomatidae have been used to generate phylogenies of the family that are in reasonably good agreement with each other (Fernandes, et al., 1993; Marché et al., 1995; Maslov et al., 1994). These phylogenies indicate a very early separation of the African and American trypanosomes perhaps 600 million years ago, whilst the divergence time of the *Endotrypanum* and of the Old and New World *Leishmania* correspond more closely to the separation of the continents about 80 million years ago. More detailed studies of the Trypanosomatidae and its constituent genera suggest that the currently recognised taxa may not be holophyletic. SSU rRNA sequences shows that the *Crithidia* may not be monophyletic (Du & Chang, 1994). *Dimastigella trypaniformis* is generally considered a member of the Bodonidae but 16S rRNA sequence data indicates a closer relationship with the Trypanosomatidae (Berchtold et al., 1994). kDNA and rDNA sequences reveal a phylogenetic cluster of species of *Herpetomonas* and *Phytomonas* separate from their parent genera (Nunes et al., 1994).

1.3. CLASSIFICATION OF *LEISHMANIA*

1.3.1. Extrinsic classifications

The classification of the American *Leishmania* has changed considerably since their discovery. Early classifications accorded each *Leishmania* taxon species status (Lainson & Shaw, 1987). Biagi (1953) introduced the use of subspecies, he named the agent of chiclero's ear *L. tropica mexicana* and suggested that the agent of muco-cutaneous leishmaniasis and the less pathogenic parasite that rarely affects the mucosa and that is found in Peru, Guyana and Central America should both be *L. braziliensis* subspecies but did not name them.

Floch (1954) adopted Biagi's proposal for the use of subspecies but used *L. tropica* subspecies. He created *L. tropica guyanensis* and divided the American cutaneous *Leishmania* into three subspecies of *L. tropica*:- *L. tropica braziliensis*, *L. t. mexicana* and *L. t. guyanensis*. In 1961 Pessoa considered all ACL to be due to subspecies of *L. braziliensis* whilst in 1962 Garnham raised the agent of Chiclero's ulcer to specific rank as *L. mexicana* (Lainson & Shaw, 1987). Garnham (1971) introduced the behaviour of the parasite in the vector as an additional taxonomic character and raised all the New World *Leishmania* to specific rank.

Lainson and Shaw (1972) created the *L. braziliensis* and *L. mexicana* complexes based on epidemiology, clinical appearance, geographic distribution and behaviour in hamsters and culture. They made the agents of ACL subspecies of *L. braziliensis* and *L. mexicana*, and included *L. enriettii* in the *L. mexicana* complex whilst retaining its species rank.

Lainson and Shaw (1979) revised their classification of the New World *Leishmania*. Development in the vector was added as the primary characteristic for allocating parasites to complexes. They recognised three major subdivisions, the suprapylaria for *L. mexicana*, the peripylaria for *L. braziliensis* and the hypopylaria for the lizard *Leishmania*.

In 1982 Saf'janova created two subgenera:- *Leishmania (Sauroleishmania)* for the majority of the reptilian *Leishmania* and *Leishmania (Leishmania)* for the mammalian *Leishmania*. In 1986 Killick-Kendrick *et al.* adopted a proposal by Ranque and raised the *Sauroleishmania* to genus rank and included all the reptilian species (Lainson & Shaw, 1987).

Lainson and Shaw (1987) again revised their classification using the development in the vector, pathogenicity in the hamster, *in vitro* development and geographic distribution. They raised the *L. braziliensis* complex to the subgenus *L. (Viannia)* and switched *L. hertigi* into Safjanova's *Leishmania (Leishmania)* from the *L. braziliensis* complex owing to its suprapylarian development. All other mammalian *Leishmania* were placed in *L. (Leishmania)*.

1.3.2. Intrinsic classifications

Since 1981 a number of authors have produced classifications of the *Leishmania* based on biochemical criteria (Beverley et al., 1987; Cupolillo et al., 1994; Kreutzer et al., 1987; Lanotte et al., 1984; Rioux et al., 1990; Thomaz Soccol, 1993). All used enzyme electrophoresis except Beverley *et al.* who used RFLPs.

With some exceptions (see species listing below) biochemical methods have confirmed the validity of previously described taxa and their attribution to complexes. However a number of new species have been described that are indistinguishable from previously known species except by biochemical analysis (Grimaldi, G. et al., 1992; Kreutzer et al., 1991; Lainson & Shaw, 1989; Lainson et al., 1989; Silveira et al., 1987; Yoshida et al., 1993).

The status of *L. chagasi* remains controversial on two counts:- firstly whether it is *L. infantum* imported after the conquest from the Mediterranean and secondly whether the differences in the two taxa warrant separation at the species level. Evidence from pathology, enzyme electrophoresis, serology and DNA fragment patterns all indicate near identity between *L. chagasi* and *L. infantum* stocks (Beverley et al., 1987; Cupolillo et al., 1994; Momen & Grimaldi, 1987). However (Lainson et al., 1987) argued that *L. chagasi* appears to be better tolerated by indigenous animals such as foxes and opossums than by imported dogs and that therefore the parasite is also likely to be indigenous. If this view is correct the biochemical data represents the product of parallel or convergent evolution of long separated species. However the evidence for better toleration by foxes than dogs is not conclusive (Courtenay et al., 1994). In the present work the specific name *L. chagasi* will be retained despite the doubtful status of this species.

1.3.3. New World *Leishmania* species

The following species have been described from the New World. Species in bold have been reported from Central America.

A Subgenus *Leishmania (Leishmania)*.

Characteristic: Suprapylarian development in the vector, promastigotes are found in the midgut which migrate to the foregut.

Type species: *L. (L.) donovani* Ross 1903.

Distribution: Worldwide in the tropics and subtropics.

Leishmania (L.) donovani complex

***L. (L.) infantum* (Nicolle 1908) Lainson & Shaw 1987**

***L. (L.) chagasi* da Cunha & Chagas 1937**

Agents of visceral and cutaneous disease. Reservoirs in dogs and foxes

Leishmania (L.) mexicana complex

***L. (L.) mexicana* (Biagi 1953) Lainson & Shaw 1987**

Agent of cutaneous disease, particularly Chiclero's ear in Mexico and parts of Central America.

***L. (L.) amazonensis* Lainson & Shaw, 1972**

Parasite of forest rodents in South America, rarely infects humans, usually with single or limited cutaneous lesions. Can cause anergid "diffuse" cutaneous leishmaniasis and occasionally visceral leishmaniasis.

***L. (L.) aristidesi* (Lainson & Shaw 1979) Lainson & Shaw 1987**

Parasite of rodents and marsupials in Panama

L. (L.) forattinii Yoshida et al., 1993

Three strains have been recovered from rodents and opossums from primary forest on the Atlantic coast of Brazil.

Possible members of the *L. (L.) mexicana* complex

L. (L.) pifanoi Medina & Romero 1959 emend Lainson & Shaw 1972

Originally associated with anergid diffuse leishmaniasis.

L. (L.) garnhami Scorza et al., 1979

Associated with ACL in Venezuelan Andean Highlands. Some strains of both *L. pifanoi* and *L. garnhami* are indistinguishable from *L. mexicana* or *L. amazonensis* (Cupolillo et al., 1994; Momen & Grimaldi, 1984)

L. (L.) venezuelensis Bonfante-Garrido, 1980

Associated with the single nodular cutaneous lesions occurring in northwestern Venezuela, but is identical to *L. mexicana* by MIE (Cupolillo *et al.* (1994).

Other *Leishmania (Leishmania)* species

L. (L.) enriettii Muniz & Medina, 1948

Parasite of *Cavia porcellus* in Brazil

L. (L.) major Yakimof & Schokor [1914]

Traditionally the agent of rural cutaneous leishmaniasis in the Old World, isoenzymatically similar parasites have been reported from Brazil. (Momen et al., 1985)

Leishmania hertigi complex

***L. (L.) hertigi* (Herrer, 1971) Lainson & Shaw 1987**

Parasite of the porcupine *Coendu rothschildi* in Panama and Costa Rica.

L. (L.) deanei Lainson & Shaw, 1977

Parasite of *Coendu* sp in Brazil

B Sub-genus *L. (Viannia)* Lainson and Shaw 1987

Characteristic: Peripyloric development. Promastigotes in the hind gut of the sandfly with a migration to the mid- and foregut.

Type species: *Leishmania (V.) braziliensis* Vianna, 1911.

Distribution: New World tropics

Leishmania (V.) braziliensis complex

***L. (V.) braziliensis* (Vianna 1911),Lainson & Shaw 1987**

Widespread agent of cutaneous and mucocutaneous disease.

L. (V.) guyanensis Floch, 1954

Usually associated with "pian-bois" in Guyana and Venezuela.

***L. (V.) panamensis* Lainson & Shaw, 1972.**

Agent of cutaneous leishmaniasis from Colombia north to Honduras, rarely associated with MCL

L. (V.) peruviana Velez, 1913

The agent of cutaneous leishmaniasis (uta) in western Peru.

***L. (V.) colombiensis* Kreutzer *et al.*, 1991**

Isolated from humans (2 strains), sandflies (6 strains) and sloths (1 strain) in Colombia, Venezuela and Panama.

L. (V.) naiffi Lainson & Shaw, 1989

Isolated from the armadillo *Dasypus novemcinctus*

L. (V.) lainsoni Silveira *et al.*, 1987

Isolated from the "paca" *Agouti paca* and cases of ACL in the Amazon area of Brazil.

L. (V.) shawi Lainson *et al.*, 1989

Parasite of sloths and monkeys, occasional human infections.

L. (V.) equatoriensis Grimaldi *et al.*, 1992

Parasite of sloths and squirrels in Ecuador.

C. *Incertae sedis*

***L. herreri* Zeledon, Ponce & Murillo, 1979** isolated from *Lu. ylephiletor, Choloepus hoffmanni, Bradypus griseus.*

A number of other strains which may be new species have been referred to by various authors but have not been formally described (Beverley et al., 1987; Cupolillo et al., 1994; Lainson & Shaw, 1987).

1.4. THE DISCOVERY OF LEISHMANIASIS

1.4.1. Old World Cutaneous Leishmaniasis

Copies of Accadian texts from the second or third millennium BC in the library of King Ashubanipal of Assyria (c650BC) described a system of prophecy based on the location of ulcers on the face. Bray (1987) argued that such ulcers would be painless and common and that in a non-urban civilisation these could be attributed to *L. major*. Good descriptions of "Balkh sore" from several towns in Central Asia date from the first century AD and Avicenna (980-1037AD) in the Qânûn fi t-tibb described it in sufficient detail for modern authors to be confident that Avicenna was referring to *Leishmania* (Elgood, 1934).

Leishmania parasites from cutaneous lesions were first observed by Cunningham (1885) in India though he believed the infected macrophage to be a fungal cell with spores. Borovsky (1898) accurately described the agent of "Sart sore" and placed it in the protozoa, however Borovsky's paper was in a little known Russian journal and the aetiology of the various tropical sores only became widely known with the publication of Wright's micrographs in 1903. Wright believed the organism to be a microsporidian and named it *Helcosoma tropicum*, Lühe included them in the *Leishmania* in 1906 whilst following Laveran and Mesnil in believing it to be a piroplasm (Hoare, 1938).

1.4.2. New World Cutaneous Leishmaniasis

There is no documentary evidence of leishmaniasis in the Americas prior to the Spanish invasion. The Moche of Peru (AD 100 - AD 750) left numerous ceramic representations of diverse pathologies some of which are widely believed to represent espundia (Viola & Margolis, 1991), however the aetiology of espundia is frequently confused and includes *Blastomycosis* as well as *Leishmania* (Escomel, 1922). The Nahuatl of Costa Rica refer to both the sandfly and leishmaniasis as "papalomoya" - butterfly-mosquito suggesting a long standing association of the two (Zeledon, 1985).

Lesions similar to oriental sore were recognised by Adeodato and others in Brazil in the second half of the nineteenth century (Laveran, 1917), however proof that some tropical sores had a similar aetiology throughout the world depended on identification of the parasite.

American *Leishmania* parasites were first seen in Brazil where railway construction labourers were frequently infected (Carini & Paranhos, 1909; Lindenberg, 1909), they were

identified as *L. tropica*. *Leishmania* parasites were first seen in Central America by Darling (1910) who identified them as *L. tropicum*. Darling's first case may not have been indigenous as the patient was from the Caribbean, had been in Panama for eight months, and the infection arose on the site of a lesion caused by scratching a tabanid bite. However several further cases were soon found by the same author (Laveran, 1917). Darling's parasites were presumably *L. panamensis* which is the commonest agent of American cutaneous leishmaniasis (ACL) in Panama. *L. mexicana* the agent of chiclero's ulcer was first described by Seidelin (1912) as *L. tropica* whilst on a yellow fever expedition from the Liverpool School of Tropical Medicine to the Yucatan.

1.4.3. Visceral leishmaniasis

The agent of visceral leishmaniasis was first seen in London at autopsy by Leishman (1903), who believed the amastigote to be a degenerate trypanosome. Similar observations were made almost simultaneously in Bengal by Donovan (1903) who had believed the amastigotes to be the long sought after resting stage of *Plasmodium* or possibly degenerate spleen cells until he read Leishman's article. Ross (1903) created the genus *Leishmania* and species *L. donovani* to accommodate this organism. *L. infantum* the agent of infantile visceral leishmaniasis in the Mediterranean was first described by Nicolle (1908). Da Cunha & Chagas (1937) named the agent of visceral leishmaniasis from the New World (AVL) *L. chagasi*. The first confirmed case of visceral leishmaniasis in Central America was found in Guatemala in 1949 by Cabrera and Leon (Zeledon, 1985).

1.5. DISEASES CAUSED BY *LEISHMANIA*

Seven species of *Leishmania* have been isolated from humans in the Old World and 14 species in the New World (Shaw, 1994). These 21 pathogenic species cause a wide spectrum of diseases, the most important of which are visceral, cutaneous and mucocutaneous leishmaniasis. Although particular parasites have become associated with particular pathologies in humans these are not necessarily indicative of the behaviour of parasites in reservoir animals (Lainson & Shaw, 1987). Furthermore the pathologies caused by a given species of *Leishmania* may vary from cutaneous to visceral or mucocutaneous disease.

The standard treatment for all leishmaniases is with the pentavalent antimonials sodium stibogluconate or meglumine antimonate 20mgkg^{-1} per day daily for ten days, although many variations exist. The second line drug is amphotericin (Marsden & Jones, 1985). The drugs

are expensive to buy (£6 per gramme Sb^V for sodium stibogluconate. Anon., 1990b) and must be administered intravenously or intramuscularly. Due to the difficulty and expense of treatment many cases of cutaneous disease go untreated. Unless secondarily infected, cutaneous lesions usually resolve spontaneously within a few months to one year (Herwaldt et al., 1992).

1.5.1. Visceral disease

Visceral leishmaniasis is usually caused by members of the *L. donovani* complex. *L. donovani* and *L. infantum* replicate well in macrophages within tissue throughout the human body leading to enlargement and marked alterations in function of the liver, spleen, bone marrow and lymph nodes. An enhanced non-specific and ineffective antibody response is accompanied by a marked depression of cellular immune responses during the illness. The primary symptom of early disease is fever, after a period lasting a few weeks to several months the characteristic illness known as kala-azar develops. The illness includes prolonged fever and massive hepatosplenomegaly and unless treated is usually fatal. Death is caused by secondary bleeding or bacterial sepsis. There are no characteristic symptoms of kala-azar to distinguish it from several other fevers including malaria and misdiagnosis may be common. *L. infantum* infections are usually found in young children around the Mediterranean and in Latin America, whilst *L. donovani* frequently infects adults in India and East Africa (Marsden & Jones, 1985).

1.5.2. Cutaneous disease

All species of *Leishmania* which are pathogenic to humans may cause cutaneous disease. Although the symptoms of cutaneous leishmaniasis are very variable the most typical lesion is an ulcer, which is usually open, surrounded by a raised erythematous border (Fig. 1.3). Parasites may be recovered most easily from this border region. If the immune response fails the parasites may become disseminated and cause diffuse cutaneous leishmaniasis. Erythematous nodules containing large numbers of amastigotes appear on the skin and grow slowly without ulceration. Alternatively well defined skin granulomas may occur with evidence of a strong cell mediated immune response. In such cases amastigotes are difficult to find or isolate from the lesions which are chronic and progressive (Marsden & Jones, 1985).

1.5.3. Mucocutaneous disease

Mucosal lesions are most frequently associated with *L. braziliensis* infections in the New World but are also occasionally reported from the Middle East and Sudan. The mucocutaneous lesion is usually a metastasis of an earlier healed cutaneous lesion. The time from the initial cutaneous lesion to the development of mucocutaneous disease varies from a few months to thirty years. The proportion of cutaneous cases that progress to mucocutaneous disease varies from area to area, but was found to be between 3 and 5% in Central America rising to 18% in Bolivia (Anon., 1994).

The initial lesion is often a small granuloma on the nasal septum. Ulceration follows and the cartilaginous septum is often perforated. Sometimes necrosis results in the loss of the cartilaginous nose and the buccal cavity, pharynx and larynx may become involved. Parasites are often very difficult to detect in the lesions. The disease is generally chronic and frequently refractory to treatment and several courses of treatment at high dosage (28mg kg^{-1} Sb^{V}) may be required. Death is rare but may be caused by suffocation or secondary aspiration pneumonia (Marsden & Jones, 1985).

Figure 1.2 Map of Central America, showing the principal foci of *Leishmania* from which parasites used in this study were isolated. The region from which parasites from Panamá were isolated is not known. (From Philip's Great World Atlas, 5th ed. 1995, George Philip Ltd, London) Scale 1:10,000,000.

1.6. *LEISHMANIA* IN CENTRAL AMERICA

1.6.1. Introduction

No systematic national survey of *Leishmania* has been made in any Central American country, incidence rates are only known from passive case reporting and consequently are believed to be widely underestimated. The distribution and ecology of the different species is not reliably known in detail and new species have been reported infecting humans as recently as 1991. There follows a brief description of each of the known species from the Central American countries from which isolates have been studied.

1.6.2. *L. (Viannia)*

1.6.2.1. *L. panamensis* is found in Panama, Costa Rica, Nicaragua, eastern Honduras and rarely in Guatemala (Arana et al., 1991; Grimaldi, et al., 1989). In Panama and Costa Rica it is the cause of the majority of cases of leishmaniasis. In Nicaragua and Honduras where its distribution overlaps with that of *L. braziliensis* the relative importance of the two parasites are not known and putative hybrids between the two species have been isolated (Belli et al., 1994; Darce et al., 1991; Kreutzer et al., 1994). The incidence and prevalence of disease due to *L. panamensis* have been estimated at various foci. 20 cases per 100,000 inhabitants were recorded in Bocas del Toro, Panama in 1970 (Zeledon, 1985) and infection rates as high as 20% in three days have occurred amongst military personnel in the Panama Canal Zone (Sanchez et al., 1992). Intensity of transmission varies considerably and may be very local. In Costa Rica a survey using the Montenegro skin test in three rural communities indicated that 54% of subjects were or had been infected with *Leishmania*. Agricultural workers and people over fifteen years old were more at risk and there was a higher prevalence rate (71%) amongst Amerindians living in close contact with dense forest (Marrano et al., 1989).

L. panamensis typically causes single or multiple cutaneous lesions, mucosal involvement is rare and may be due to a primary lesion near the nasal mucosa rather than a metastasis of a lesion elsewhere (Fig 1.3)(Saenz et al., 1989).

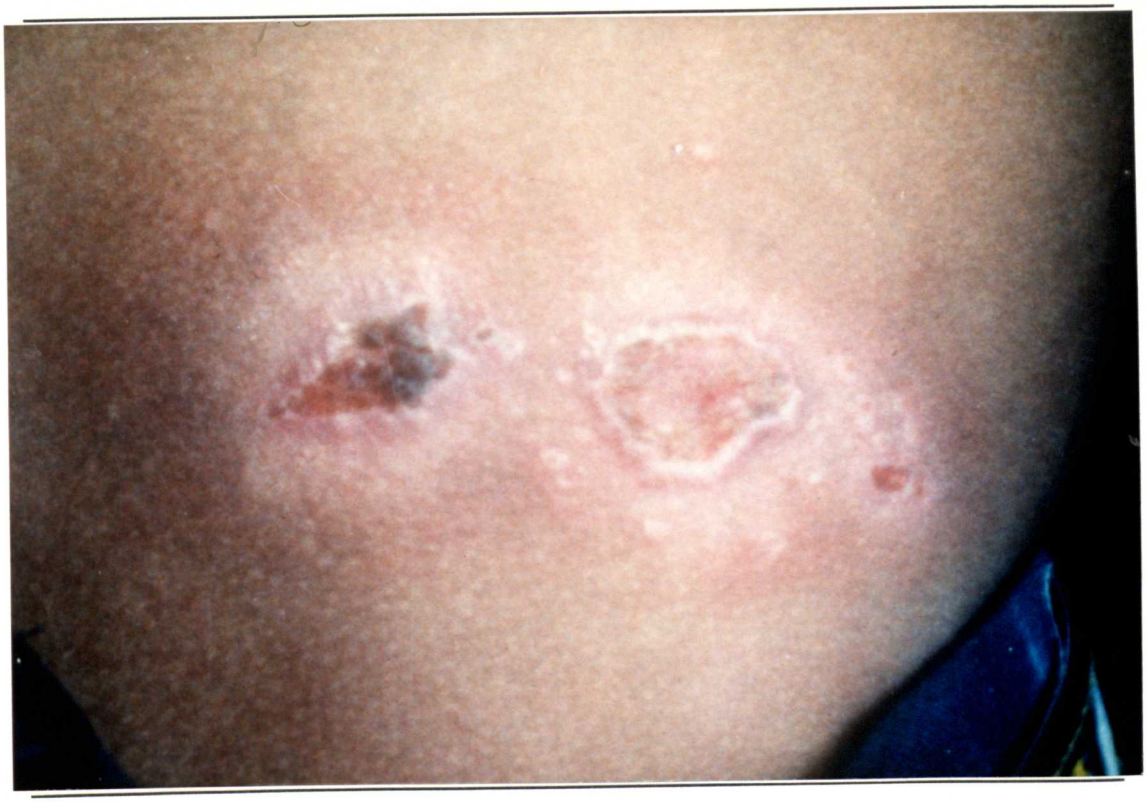


Figure 1.3 Lesion caused by *L. panamensis* on the back of a three year old boy. Central Panama.

Herrer and Christensen (1976a) characterised two distinct epidemiological patterns: firstly transitory epidemics affecting entire communities and characteristic of newly settled forest; and secondly, endemic leishmaniasis only affecting the men who enter the forest for work. However the same authors also described one community where the disease had affected all age groups but particularly children for seventy five years, this apparently unusual epidemiology was ascribed to the scattered nature of the community amongst coffee plantations with trees shading many houses. This pattern of settlement is found throughout the extensive coffee growing districts of Central America and may be the most common setting for endemic leishmaniasis. In Costa Rica the presence of poultry around the house was found to be associated with a lower risk of contracting the disease. Schlein and Jacobson (Schlein & Jacobson, 1994) found that avian blood inhibited *Leishmania* development in the sandfly and suggested that this would account for these observations.

The main reservoirs are believed to be arboreal mammals, principally the sloths *Choloepus hoffmanni* and *Bradypus griseus*, though dogs may be an incidental reservoir and possibly contribute to peridomestic transmission (Herrer & Christensen, 1976b; Shaw, 1969).

The following sandflies have been found naturally infected in Panama:- *Lu. trapidoi; Lu. ylephiletor; Lu. gomezi; Lu. sanguinarius* and *Lu. panamensis*. The majority of these species were more often found infected in the canopy at 11 metres than at ground level implicating arboreal mammals as the reservoir (Johnson et al., 1963). However *Endotrypanum schaudinni*, an intraerythrocytic parasite of sloths, is also found as a promastigote in sandflies, therefore crude sandfly infection rates are insufficient to indicate which are the most important vectors (Christensen & Herrer, 1979). The only proven vector in Costa Rica is *Lu. ylephiletor* (Zeledon et al., 1981) but *Lu. youngi* and *Lu. trapidoi* are also suspected vectors (Feliciangeli & Murillo, 1987; Grimaldi, et al., 1989).

1.6.2.2. *L. braziliensis* has only recently been identified in Central America and is principally found in Guatemala, Honduras and northern and central Nicaragua. *L. (Viannia) spp.* were not believed to be found north of Costa Rica until Honduran isolates were characterised by G6PDH mobility, DNA buoyant density, and growth *in vivo* in 1979 (Zeledon et al., 1982), but *L. braziliensis* was not specifically identified in that country until 1987 (Grimaldi, et al., 1987).

L. (Viannia) spp. were assumed to be the agent of leishmaniasis in Nicaragua at least from 1982 when ketoconazole was tested against cutaneous and mucocutaneous disease (Gomez Urcuyo & Zaias, 1982). *L. braziliensis* was first specifically identified in 1984 when a survey of 259 cases of leishmaniasis at two health centres in Matagalpa, Nicaragua found 8.5% with mucocutaneous lesions which the authors attributed to *L. braziliensis* on grounds of the pathology (Missoni & Morelli, 1984). Its presence in the region was confirmed when parasites isolated from British troops training in southern Belize were identified by isoenzyme electrophoresis (Evans et al., 1984). The parasites from the Nicaraguan focus were initially identified as *L. panamensis* (Missoni et al., 1986) but later studies using eleven enzymes found both *L. panamensis* and *L. braziliensis* (Darce et al., 1991). Some putative hybrids between *L. panamensis* and *L. braziliensis* were subsequently identified in the same focus (Belli et al., 1994). *L. braziliensis* was not identified in Guatemala until 1988 (Navin et al., 1988), the "extraordinary rarity" of mucosal involvement in Guatemala

(Herwaldt et al., 1992) may partially account for the parasite being overlooked for so long. However Lainson and Shaw (1987) have suggested that the *L. (Viannia)* parasite from Guatemala should have separate species status owing to the absence of mucocutaneous disease and the high nDNA buoyant density of 1.720g ml^{-1} (cf 1.716-1.717g ml^{-1} for *L. panamensis* and *L. braziliensis* (Chance et al., 1974)).

Incidence and prevalence of disease due to *L. braziliensis* are not known, however a comparison of active and passive case detection of cutaneous leishmaniasis in Guatemala found that of 2,938 persons interviewed 5% had had leishmaniasis, 1.3% reported the onset of the disease in the last twelve months and 0.5% had parasitologically confirmed cases. The Ministry of Health statistics showed 64 passive case reports for the previous year for the population of approximately 10 million which may consequently be an approximately 40 fold underestimate. 4067 cases of cutaneous leishmaniasis were reported from 1982-4 in Nicaragua under a more rigorous reporting regime and ACL ranked fifth amongst all infectious diseases in the country (Darce et al., 1991). In an earlier study in Nicaragua 37% of cases were in the 0-5 year age group with a high rate of positive family history (45.6%) suggesting peridomestic transmission (Missoni & Morelli, 1984).

A placebo controlled trial of drug treatments for *L. braziliensis* and *L. mexicana* in Guatemala found that for subjects on placebo 68% of those with *L. mexicana* lesions healed spontaneously, with a median lesion age of 4 months at healing, whereas only 6% of those with *L. braziliensis* lesions self-cured. *L. braziliensis* lesions were larger than *L. mexicana* with median areas of 1.5-3.1 cm^2 for the former and $< 1cm^2$ for the latter. Sixty percent of ear lesions were due to *L. braziliensis* although these lesions are classically associated with *L. mexicana* (Herwaldt et al., 1992).

No descriptions of the reservoirs of *L. braziliensis* in Nicaragua, Honduras and Guatemala have been published. The sloth is not found in these countries, other reservoirs which have been found infected in Costa Rica and Panama are rodents, opossums (*Didelphis* spp) the kinkajou (*Potus flavus*), coati (*Nasua nasua)* and various monkeys (Zeledon, 1985). Although domestic animals including pigs, dogs, asses and horses have been found infected with *L. braziliensis* in South America the epidemiological significance of such infections is not clear (Aguilar et al., 1987; Brazil et al., 1987).

L. braziliensis has on rare occasions been isolated in Panama (Kreutzer et al., 1987) and Costa Rica (Zeledon et al., 1981) from cases of mucocutaneous disease. This species has not always been distinguished from *L. panamensis* (Kreutzer & Christensen, 1980) consequently its epidemiological significance is uncertain, however it has been suggested that cases of true metastasising mucosal leishmaniasis in Panama and Costa Rica may be due to *L. braziliensis* and not *L. panamensis* (Walton, 1987).

1.6.2.3. ***L. colombiensis*** was first described from Colombia and Panama by Kreutzer *et al.* (1991). Nine strains were originally described and two more were subsequently found in Venezuela (Delgado et al., 1993). The parasite has been isolated from a sloth *Choloepus hoffmanni* and the following sandflies:- *Lu. hartmanni*; *Lu. gomezi* and *Lu. panamensis*. As *L. colombiensis* can only be distinguished from other members of *L. (Viannia)* by biochemical means it may have been mistaken for *L. panamensis* in earlier studies. It can cause single or multiple ulcerating skin lesions in humans but attempts to infect rodents in the laboratory have failed.

1.6.3. *L. mexicana* complex

1.6.3.1. Until recently *L. mexicana* was only considered to be a significant human pathogen in northern Guatemala, Belize and Mexico, however a new focus was discovered in southern Honduras in 1993 (Dr C. Ponce, Ministry of Health, Tegucigalpa, personal communication) which suggests that its distribution may be wider than previously thought.

The very particular epidemiology of leishmaniasis amongst the Chicleros was well known by the 1930's. There was a close association with the men who entered the forest to gather the chiclé but not the inhabitants of the small villages around the forest (Shattuck, 1938). Lainson and Strangeways-Dixon (1963, 1964) found 40% of cases to be localized to the ear where the lesion tends to particularly persistent and destructive. However the parasite can cause lesions on any part of the body but those lesions frequently spontaneously resolve within 6-8 weeks. It is suggested that the apparent association with the ear is due to the persistence of lesions there and not a higher infection rate at that site. Two alternatives are that sweat discourages the flies from biting elsewhere or that *L. mexicana* might be better adapted to the lower temperature of the ears. Three genera of forest rodents were found naturally infected with *Leishmania*,- *Ototylomys phyllotis, Nyctomys sumichrasti* and *Heteromys desmarestianius*. Both *Ototylomys* and *Nyctomys* are largely nocturnal and

arboreal but come down to the ground to forage at night and appear to be unaffected by *L. mexicana* which causes non-ulcerating lesions at the base of the tail. The single isolate obtained from *Nyctomys sumichrasti* was used as the *L. mexicana* reference strain (M379) in the present study.

The most important vector of *L. mexicana* is *Lu. olmeca* which was identified by Disney (1968), who captured infected flies in Belize using a rodent baited trap. Biagi confirmed the role of this insect as the vector in Yucatan using human baited traps (Lainson, 1982). *Lu. olmeca* is the main rat biter at ground level, it rests in the forest litter during the day where it can be disturbed by humans. Other more anthropophilic sandflies have been caught naturally infected such as *Lu. cruciata* and may be secondary vectors (Zeledon, 1985).

The ecology of the Honduran focus is characterised by dry tropical forest and shifting agriculture and is quite distinct from that of the Petén and Belize. The reservoirs and vectors are not known but rodents must be suspected as the reservoir. No autochthonous cases of *L. mexicana* have been confirmed in Costa Rica, *Lu. olmeca* the vector of *L. mexicana* is found in all seven provinces but is highly zoophilic so if *L. mexicana* is endemic in Costa Rica it may exist exclusively in the rodent reservoir as does *L. aristidesi* in Panama (Zeledon et al., 1981).

1.6.3.2. *L. aristidesi* was first described in Panama. It was initially classified as *L. mexicana* (Herrer et al., 1971, 1973) until Lainson and Shaw (1979) created *L. aristidesi* to accommodate it. It has been isolated from five species of rodents and opossums in the Sasardi region of Panama but no human cases have been recorded. As with *L. mexicana* it causes non ulcerating lesions at the base of the tail and on the ears of its natural hosts. Most sandflies captured in rodent baited traps were *Lu. olmeca* (95.5%) the remainder were *Lu. gomezi* (4.3%) and *Lu. panamensis* (0.2%) (Christensen et al., 1972).

1.6.3.3. *L. amazonensis* has occasionally been reported in Panama including one mixed infection with *L. mexicana*. The rarity of human infection with *L. mexicana, L. amazonensis* and *L. aristidesi* in Panama may be due to the rodentophilic feeding habits of *Lu. olmeca bicolor* the implicated vector of these species (Petersen et al., 1990).

1.6.4. *L. donovani* complex

1.6.4.1. *L. chagasi* was first described in Central America in 1949 from Guatemala (Zeledon, 1985) but only six cases have subsequently been identified in that country. It appears to be endemic along the semi-arid pacific coast from El Salvador through Honduras and Nicaragua to the province of Guanacaste in northern Costa Rica where the vector *Lu. longipalpis* is abundant. Cases of visceral leishmaniasis are comparatively rare but are probably under reported or misdiagnosed for malaria.

In Honduras 69 cases of VL were diagnosed between 1975 and 1983, 96% from the three southern provinces of Valle, El Paraiso and Choluteca which includes the island of El Tigre in the Gulf of Fonseca. 95% of cases were children under three years old, and poorly constructed wood stick houses were a significant risk factor (Navin et al., 1985). In another study using a dot ELISA the prevalence of infection amongst family members of cases was 29%, compared with 3% in a randomly selected group of children (Walton et al., 1986). The first case of VL in Nicaragua was identified in 1991 (Duarte et al., 1994), although Le Pont *et al.* (1987) suggested that the disease was probably present in view of the large numbers of *Lu. longipalpis* that they captured in peridomestic traps. The first case of VL in Costa Rica was reported in 1995 (Prof. Zeledon, Universidad Nacional de Costa Rica, personal communication) however non-ulcerating papular cutaneous lesions that were mistaken for leprosy for many years have been found to be caused by *L. chagasi* (Zeledon et al., 1989). Identical cases were subsequently found in Honduras in the same provinces as VL and on El Tigre island where the prevalence was 1% amongst children under three by passive case finding (Ponce et al., 1991).

L. chagasi has also been isolated occasionally (Kreutzer et al., 1987) in Panama but little is known of its epidemiology or ecology. There are no semi arid regions in Panama which would form a suitable habitat for *Lu. longipalpis* and it appears to be restricted to one island in the gulf of Panama (PAHO, 1994).

1.6.5. *L. hertigi* complex

L. hertigi was first described in Panama from tree porcupines (*Coendu rothschildi*) (Herrer, 1971). 83 out of 94 porcupines collected from sites throughout the central part of the country were found infected. *L. hertigi* has also been isolated from *Coendu mexicana laenatum* in Costa Rica (Zeledon et al., 1977). The slightly larger species *L. deanei* has

been isolated from 11 out of 18 porcupines in Brazil (Lainson & Shaw, 1977). The parasite does not appear to be pathogenic to the porcupine in which it is distributed widely in the skin and viscera. The particular cell type parasitised in the porcupine has not been reported. Rodents have been experimentally infected with *L. hertigi*. The parasite causes no gross tissue alterations and can only be detected by culture of the site of inoculation (Herrer, 1971; Zeledon et al., 1977). Other workers have been unable to infect hamsters with *L. hertigi* unless the animals were first immunosuppressed with cyclophosphamide (Croft, 1977).

The classification of *L. hertigi* has been problematic. Lainson and Shaw (1974) initially included it in the *L. braziliensis* complex on the basis of its growth *in vitro* and *in vivo* in hamsters but later created the *L. hertigi* complex which was provisionally included in the subgenus *L. (Leishmania)* on the basis of its supra-pylarian development in laboratory infected sandflies (Lainson & Shaw, 1987). The nDNA buoyant density (1.714-1.715g ml^{-1}) is intermediate between *L. braziliensis* (1.716g ml^{-1}) and *Endotrypanum* (1.712g ml^{-1}) (Chance et al., 1974; Croft et al., 1980). In one isoenzyme based study of the phylogeny of the New World *Leishmania* it could be included either in *L. (Viannia)* or *L. (Leishmania)* depending on the method used to analyze the data and both methods of data analysis indicated that *L. hertigi* arose soon after the divergence of the two subgenera (Thomaz Soccol, 1993; Thomaz Soccol et al., 1993a).

1.6.6. *Incertae sedis*

A possible new species *L. herreri* has been isolated from two toed sloths (*Choloepus hoffmanni*), three toed sloths (*Bradypus griseus*) and three sandfly species in Costa Rica (Zeledon et al., 1979). However this record is regarded as dubious by Lainson and Shaw (1987) on the grounds that the description includes drawings of sphaeromastigotes in hamster lesions. Zeledon *et al.* distinguished *L. herreri* from *Endotrypanum spp.* and *L. braziliensis*, which were sometimes found as mixed infections, by differential growth in cell culture and hamsters. *Endotrypanum* species were characterised as those that grew in Senekjie's medium but not in tissue culture. *L. herreri* were those parasites that grew in Senekjie's medium, and as intracellular amastigotes in primary hamster embryo tissue culture but not did not survive more than four days in hamsters. *L. braziliensis* were those that grew, in Senekjie's medium, hamster tissue culture and established chronic infections in hamsters. *L. herreri* has not been

reisolated and the only extant stabilates are held at the Liverpool School of Tropical Medicine.

1.7. THE *LEISHMANIA* OF LIZARDS

Leishmania like parasites have been isolated from the blood and viscera of a number of lizard genera in Africa, Asia, and Europe. Certain species of lizards naturally infected in the Old World (*Hemidactylus turcicus* and *Hemidactylus brookii*) are also found in the New World but they have not been found infected with *Leishmania*. Some New World phlebotomines feed predominantly on reptiles and amphibians, and leptomonads have been found together with nucleated red blood cells in the hindgut of sandflies. There have also been three reports of *Leishmania* like parasites isolated from the blood of lizards in Central America and the Caribbean, however extensive searches have failed to confirm these observations (Dollahon & Janovy, 1974). The inclusion of *Leishmania* like parasites of lizards in the genus *Leishmania* is controversial. A number of workers with interests in the classification of *Leishmania* believe that they should transferred to a new genus - the *Sauroleishmania* (Killick-Kendrick *et al.* 1986; Lainson & Shaw 1987; Vickerman 1976). Other workers who use them as models for the study of genetic processes in *Leishmania* and the trypanosomatidae refer to the lizard parasites as *Leishmania* (Frech et al., 1995; Fu & Kolesnikov, 1994; Landweber & Gilbert, 1993).

Killick-Kendrick *et al.* (1984) listed 14 species of lizard *Leishmania*. A fifteenth species, *Leishmania guliki*, has been referred to in a paper about guide RNA genes (Fu & Kolesnikov, 1994) but no formal description has been found (Table 1.1).

Killick-Kendrick *et al.* (1984) emphasized three important biological differences between the *Leishmania* of lizards and mammals:- the class of the vertebrate host, the genus of the invertebrate host and the site of development in the invertebrate host. The lizard *Leishmania* are found in sandflies of the genus *Sergentomyia*, whilst mammalian *Leishmania* are transmitted by *Phlebotomus*, *Lutzomyia* and *Psychodopygus*. Lizard *Leishmania* are hypopylarian or peripylarian in the sandfly whilst mammalian *Leishmania* are suprapylarian or peripylarian. The same authors also noted differences in the size of kDNA maxicircles and the distance between sub-pellicular microtubules. These biological and genetic differences were considered sufficient to warrant taxonomic recognition of the two groups at the generic level.

Leishmania henrici (Leger, 1918) Wenyon, 1920
Leishmania chamaeleonis Wenyon, 1920
Leishmania tarentolae Wenyon, 1920
Leishmania hemidactyli Mackie, Das Gupta and Swaminath, 1923
Leishmania davidi Strong, 1924
Leishmania agamae David, 1929
Leishmania ceramodactyli Adler and Theodor, 1929
Leishmania nicollei Khodukin and Sofiev, 1940
Leishmania gymnodactyli Khodukin and Sofiev, 1947
Leishmania zmeevi Andrushko & Markov, 1955
Leishmania adleri Heisch, 1958
Leishmania sofieffi Markov, Lukina and Markova, 1964
Leishmania hoogstraali McMillan, 1965
Leishmania senegalensis Ranque, 1973
Leishmania guliki

Table 1.1 List of lizard *Leishmania* species. References as cited in Killick Kendrick *et al.* 1986.

In addition to the debate about the taxonomic rank of the lizard *Leishmania* there are also problems with the generic affiliation of individual species or groups of species, particularly those species such as *L. henrici* and *L. davidi* which have only been described once and for which there are no reference strains available.

Of the lizard *Leishmania* species for which information is available three were isolated from the intestine, cloaca or rectum of lizards (*L. henrici*, *L. chamaeleonis* and *L. davidi*) and the remainder were cultured from the blood. Amastigotes of *L. tarentolae*, *L. gymnodactyli* and *L. agamae* have been found in blood mononuclear cells, other species are only known from promastigotes in culture.

The intestinal promastigotes of lizards have a range of morphologies including leptomonad, crithidial and herpetomonad forms (Vickerman, 1965). These forms are typical of monoxenous trypanosomatids of insects and it is suspected that lizards acquire these parasites

by eating infected flies (Killick-Kendrick et al., 1984; Wenyon, 1920). Vickerman (1965) therefore concluded that the intestinal promastigotes of lizards were more likely to be *Herpetomonas* species than *Leishmania*. Choanomastigote trypanosomatids from naturally infected Diptera have been recovered from the guts of *Cnemidophorous sexlineatus* by catheter at least six days after the infective feed, demonstrating that apparently monoxenous trypanosomatids can survive for short periods, at least, in the lizard gut (Dollahon & Janovy, 1971).

Amastigotes are difficult to find in lizard blood and have only rarely been recorded (David, 1929; Pozio et al., 1986), consequently six out of eleven species of haemoflagellates of lizards are only known from promastigotes in culture (Killick-Kendrick *et al.*, 1986). *Trypanosoma platydactyli* also infects *Tarentola mauritanica,* the type host of *L. tarentolae*. *T. platydactyli* is a trypomastigote or epimastigote in primary cultures but there are contradictory reports concerning its subsequent development. Wallbanks *et al.* (1985) found eight out of 43 *T. mauritanica* infected with trypomastigotes and primary cultures from these animals produced amastigotes, epimastigotes and trypomastigotes. The primary cultures transformed into promastigotes in the tertiary subculture, despite double cloning of trypomastigotes. The promastigote cultures retained the isoenzyme profile of type strains of *T. platydactyli*. Pozio *et al.*, (1986) examined 69 *T. mauritanica*, and found mixtures of amastigotes and trypomastigotes in the blood of six and only trypomastigotes in the blood of 11. Pure cultures of promastigotes were derived from lizards infected with mixtures of amastigotes and trypomastigotes. Cultures from lizards which had only trypomastigotes in the blood produced either only trypomastigotes in culture, or mixtures of trypomastigotes and epimastigotes, these cultures were stable and did not transform into promastigotes within twenty weeks. Cultures of promastigotes were typed as *L. tarentolae* by isoenzyme electrophoresis using the *L. tarentolae* LV414 reference strain used in the present study. Cultures of epimastigotes and trypomastigotes were typed as *T. platydactyli*. The reasons for these differences of behaviour in culture have yet to be elucidated and, until they are, the generic affiliation of promastigotes isolated from lizard blood cannot be assumed on the basis of morphology alone.

1.8. *L. CHAGASI* TROPISMS.

1.8.1. Introduction

In Honduras visceral leishmaniasis is found almost exclusively in the arid south of the country (Fig. 2.1), where 210 cases of have been detected between 1974 and 1990 (Dr Ponce. Ministry of Health, Tegucigalpa, personal communication). Distinctive cutaneous non-ulcerating papular lesions caused by *L. chagasi* have been recently described from the same area of the country (Fig. 1.4)(Ponce *et al.* 1991). The majority of cases of cutaneous leishmaniasis have been found to the east of the Rio Grande de Nacaome in Southern Honduras (Fig. 2.1). In some parishes to the east of the river, such as San Juan Bautista and Orocuina, only cases of cutaneous disease have been found. The partially focal nature of the diseases in Honduras suggests that differences in the parasite may be responsible for the two types of pathology. However as the region to the east of Rio Grande de Nacaome has a greater number of health centres and has been more intensively studied the apparently focal nature of cutaneous disease may not reflect its real distribution.

L. infantum has also been found to cause both cutaneous and visceral disease around the Mediterranean. In the Mediterranean region there is some correlation between *L. infantum* strain and pathology, and it has been proposed that the parasite has a genetically determined tropism (Gradoni & Gramiccia, 1994).

In Brazil cutaneous lesions have been found in cases of VL and in areas where VL is the principal form of leishmaniasis since at least the 1930s in Brazil. Many of these lesions were ascribed to *L. chagasi* but the possibility of double infections with *L. chagasi* and an agent of CL could not be ignored and some such infections have been identified (Deane & Grimaldi, 1985). In the Old World *L. donovani* has been suspected of being at least occasionally the agent of cutaneous leishmaniasis but isoenzyme electrophoresis provided the first biochemical confirmation of this when a cutaneous lesion from a patient in Kuwait was found to be caused by a parasite identical to an *L. donovani* isolate from a case of VL in the Sudan (Al-Taqi & Evans, 1978). In southern France the aetiology of VL was well established but there were also sporadic cases of mucocutaneous and cutaneous disease. The epidemiology of the cutaneous form could not be reconciled with *L. major* or *L. tropica*, the known agents of CL in the Mediterranean area. Eventually isoenzyme electrophoresis showed *L. infantum* to be the parasite responsible for CL in France (Rioux et al., 1980). Subsequently *L. infantum* has been identified from cases of CL in Italy, Tunisia and Greece

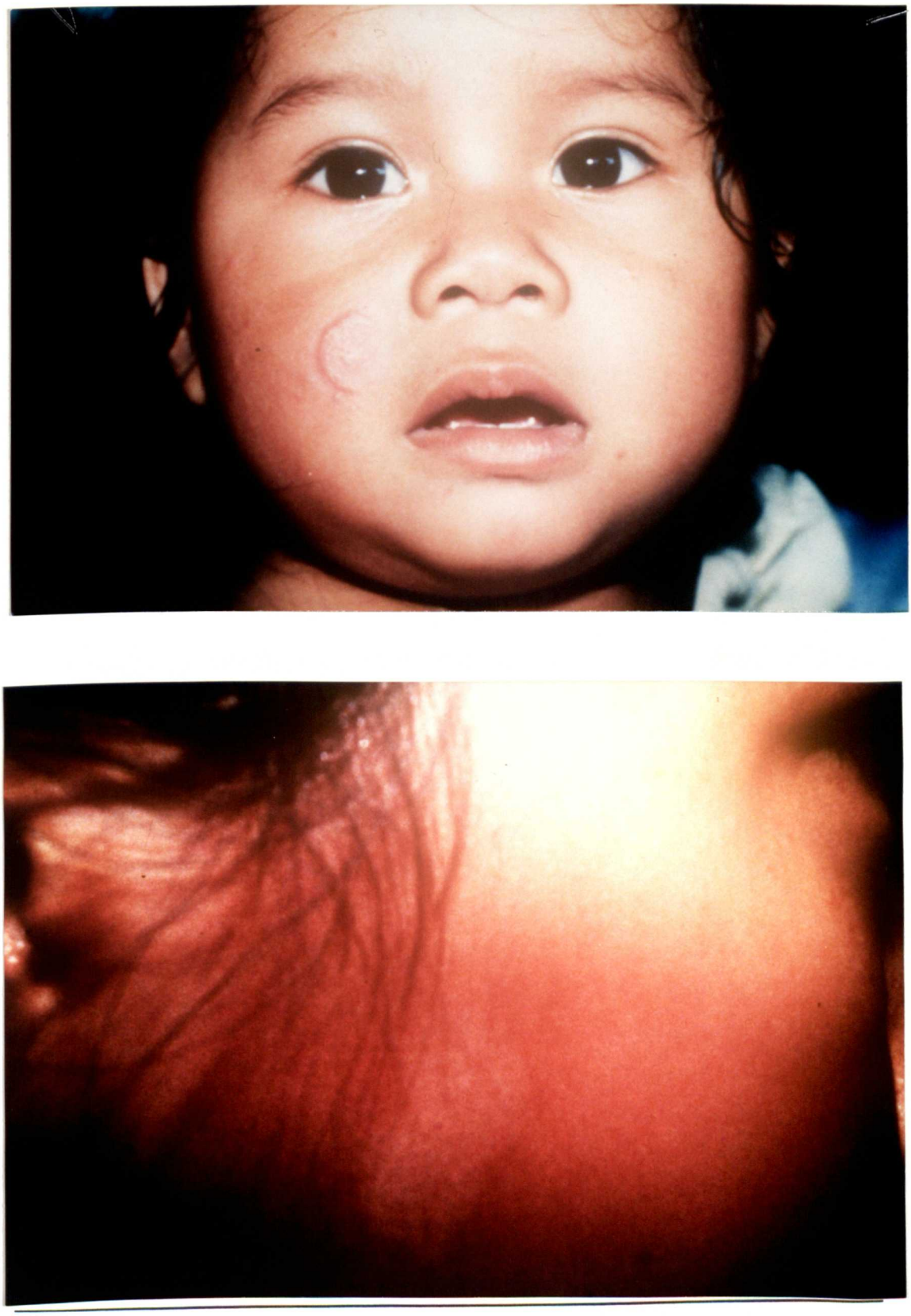


Figure 1.4 Cutaneous lesions caused by *L. chagasi*

(Ben-Ismail et al., 1992; Frank et al., 1993; Gramiccia et al., 1987) and in Kenya *L. donovani* was found to cause CL (Mebrahtu et al., 1993). The first confirmed case of CL due to *L. chagasi* in the New World was from Brazil where parasites isolated from an ulcer were identified as *L. chagasi* by isoenzyme electrophoresis, schizodemes and monoclonal antibodies (Oliveira Neto et al., 1986). At the end of the 1980's papular non ulcerating lesions due to *L. chagasi* were found in cases from Costa Rica and Honduras (Fig. 1.4)(Ponce et al., 1991; Zeledon et al., 1989).

Conversely there have been a number a reports of *L. tropica* causing VL (Bhattacharyya et al., 1993; Kreutzer et al., 1993; Magill et al., 1994; Marsden & Jones, 1985).

Differences in the parasite, the vector and the host are all known to effect the outcome of *Leishmania* infection.

1.8.2. The parasite

None of the strains of *L. chagasi* from cutaneous cases have been distinguished from *L. chagasi* causing VL by MIE (Ponce *et al.* 1991), however in the Mediterranean there is a strong correlation between particular zymodemes and the two different pathologies. Rioux's group at Montpellier had identified at least eighteen *L. infantum* zymodemes by 1990, many of which are quite rare, however the commonest, MON1 (=LON49), is usually associated with VL whilst CL is frequently found to be caused by MON24. All *L. chagasi* strains characterised at Montpellier to date have been MON1 (Professor Dedet, Montpellier, personal communication). MON1 and MON24 are distinguished by the difference in mobility of a single enzyme - nucleoside purine phosphorylase (NP_1) (Rioux et al., 1990), however this enzyme has not been used to characterise any of the New World isolates of *L. chagasi* causing CL (Ponce et al., 1991). A Greek study using NP_1 found that all cases of CL were due to MON1 and MON24 did not occur (Frank et al., 1993). Numerical analysis of schizodeme banding patterns has also clustered *L. infantum* VL and CL strains into distinct groups with little overlap (Angelici et al., 1989). A strain specific kDNA probe for dermotropic *L. infantum* is being developed in Italy (Angelici et al., 1992). Apparent antigenic differences have also been found between dermotropic and viscerotropic strains. 95% of sera from VL cases in Tunisia reacted with a 32kd membrane antigen from visceralizing parasites whilst sera from CL cases due to *L. infantum* did not react (Tebourski et al., 1994). However the difference in the reactivities of VL and CL sera may be due to

the absence of antibody in CL sera rather than absence of antigen in CL parasites, since VL is characterised by a strong humoral response in contrast to the strong cell mediated response of CL. Karyotype analysis shows almost strain specific variation in *L. infantum* and the polymorphisms do not correlate with isoenzyme variation or pathology (Pages et al., 1989).

Although it may be reasonable to assume that *L. donovani* complex parasites have a particular adaption to invade the viscera the nature of such an adaption is not known. Many other species of *Leishmania* can be isolated from the spleen and liver of their natural hosts, often with little evidence of pathology, but as these parasites cause cutaneous lesions in humans it is possible that additional factors are significant for the outcome of disease.

1.8.3. The vector

In Italy VL and CL due to *L. infantum* tend to be found in distinct foci, CL cases are mostly found to the east of the Apennines and in Sicily whilst VL is mostly found in the west with some cases in Sicily. *Phlebotomus perfiliewi* is the main vector of CL and is found in the eastern areas and is the most important human biting species in Sicily, whilst *P. perniciosus* is the main vector of VL and is found in the west. Nevertheless *P. perfiliewi* has been found infected with *L. infantum* of the MON1 visceralising zymodeme in the Abruzzi region in the east and may be capable of transmitting visceral disease. (Gramiccia et al., 1987; Maroli et al., 1987, 1988, 1991, 1994). In Tunisia however the two pathologies are sympatric (Ben-Ismail et al., 1992). In the New World *Lu. longipalpis* is the only proven vector of *L. chagasi*, although *Lu. evansi* has also been implicated. There is strong evidence that *Lu. longipalpis* is a species complex. This was first suggested when flies were found with either a pale spot on their fourth (one spot) or on their third and fourth (two spot) abdominal segments. Attempted crosses between the one and two spot forms showed lowered fertility (Ward et al., 1983). Sex pheromone differences have also been found between different groups which would form an effective barrier to mating between populations (Hamilton & Ward, 1991). Hybridization experiments between colonies from different countries have produced vigorous but infertile F_1 generations and isoenzymes show large differences between populations (Lanzaro et al., 1993). Warburg *et al.* (1994) found that the saliva of *Lu. longipalpis* from Costa Rican had two distinctive properties, it contained less of the vaso dilator maxadilan than *Lu. longipalpis* from Brazil and Colombia and the saliva of the Costa Rican flies was more effective in enhancing experimental cutaneous lesions in mice due to

L. major than the flies from Brazil and Colombia. Warburg *et al.* (1994) propose that the saliva of the Costa Rican flies causes the parasites to multiply rapidly at the site of infection but that the low level of maxadilan limits their dispersal, giving the host time to mount an effective immune response that can contain the infection.

1.8.4. The host

The immune response to leishmaniasis is well characterised although its generation and control are not well understood. CL is usually associated with a marked cell-mediated immune response detectable as a delayed-type hypersensitivity reaction (DTH), whereas in VL the cell mediated reaction is profoundly impaired but there is a strong humoral response (Liew & O'Donnel, 1993). The tropism of the infection may therefore be determined by events occurring early in the infection which switch the immune response between cell mediated and humoral responses.

The immune response to *L. donovani* has been most intensively studied in the mouse where it has been found that resolution of infection in resistant mice is associated with the activation of T helper 1 (Th1) cells resulting in interferon-γ and interleukin-2 (IL-2) production and subsequent activation of macrophages to a leishmanicidal state. Conversely, in susceptible mice, *L. major* provokes a progressive infection with visceral dissemination which is associated with a biased Th-2 cell induction and expansion. Here IL-4 and IL10 production plays the major role in mediating progressive disease. A single gene *Lsh* was found to be responsible for innate resistance to *L. donovani* whereas at least three genes are involved in acquired resistance. A study of families with cases of *L. chagasi* has indicated that there may also be a genetic component to *Leishmania* susceptibility in humans (Cabello et al., 1995). There is evidence that visceralising disease in humans is also associated with a Th-2 type response. VL patients fail to respond to *L. donovani* antigen in terms of DTH, lymphocyte proliferation, IL-2 and IFN-γ production *in vitro* but these immune responses are restored after successful chemotherapy (Gradoni & Gramiccia, 1994; Liew & O'Donnel, 1993; Marsden & Jones, 1985; Reiner, 1994).

The human host's immune status is known to significantly effect the outcome of *Leishmania* infection which can give rise to a wide range of outcomes from sterile self healing through subclinical infection to cutaneous and visceral disease (Bettini et al., 1983). The mothers immune status may also be significant particulary in cases of infant disease (Carlier &

Truyens, 1995). The existence of a reservoir of subclinical cases has been highlighted by the development of leishmaniasis in AIDS patients. These infections are sometimes at sites, such as the throat or gut, not normally associated with leishmaniasis or with previously unknown and presumably avirulent zymodemes (Gradoni et al., 1993; Gramiccia et al., 1992; Hernandez et al., 1993; Pratlong et al., 1993).

Nutritional status may be a factor influencing the outcome of *Leishmania* challenge (Dye & Williams, 1993) and Zeledon (1991) has proposed that the low levels of malnutrition in Costa Rica may account for the rarity of visceral disease in that country. However malnutrition may also be an indicator of a range of pressures which can generate stress. In humans stress causes the hypothalamus to stimulate the release of the glucocorticoid cortisol from the adrenal glands. Cortisol inhibits cell division in the skin leading to poor wound healing and down regulates the spleen and thymus leading to increased risk of infection. In the recent severe epidemic of VL in the Sudan malnutrition has gone hand in hand with the severe stresses of war and displacement and it may be difficult to assess their relative importance (Zijlstra et al., 1991). In mice it has been found that a pheromone from footshock stressed mice can modulate the Th-1/Th-2 response in other mice, this effect can be abrogated by glucocorticoid antagonists which inhibit these stress hormones' effects (Moynihan et al., 1994). The rural poor who are most at risk of leishmaniasis live a precarious existence over which they have little control, nevertheless individual responses to these difficulties vary enormously and those responses may in turn modify the outcome of infection with *Leishmania*.

1.9. GENETIC EXCHANGE IN *LEISHMANIA*

Reproduction of *Leishmania* is by binary fission and no evidence has been found of any morphological forms related to a sexual process, however there is some evidence for genetic exchange. There is considerable interest in determining the existence and frequency of genetic exchange in order to be able to predict the spread of drug and vaccine resistance.

For all species of *Leishmania* the most important zymodemes, such as MON1 in *L. infantum*, are widely distributed and there is strong evidence from isoenzyme electrophoresis that the population structure is essentially clonal (Tibayrenc, 1993; Tibayrenc et al., 1990). Tibayrenc *et al.* (1990) propose that if reproduction in *Leishmania* is sexual then, as there is no reason to presume that all isoenzyme loci are closely linked, genetic exchange and recombination would generate a very large number of different isoenzyme profiles or zymodemes. For example with 15 independent enzyme loci, each with two alleles, and panmictic recombination, 32,768 (2^{15}) different zymodemes would be expected with approximately equal frequencies. However by 1990 only 113 zymodemes of all *Leishmania* species had been identified at Montpellier despite having examined at least 1777 strains with 15 enzymes each of which may have numerous isozymes (Rioux et al., 1990), genetic exchange is therefore either non existent or very rare.

The evidence from isoenzymes for asexuality has been contested with results from karyotype analysis using pulsed field gel electrophoresis (PFGE). The variability of the *Leishmania* karyotype depends on the species under study and the locality. A considerable amount of intraspecific variation is found in *L. amazonensis* but very little in *L. major* (Lightall & Giannini, 1992), *L. infantum* strains from some regions have a higher degree of variability than from others and populations defined by karyotype do not always correlate with those defined by isoenzyme electrophoresis (Pages et al., 1989). *Leishmania* is probably mostly diploid, however the karyotype is unusual in that it appears that almost all pairs of homologous chromosomes are of equal size and presumably identical, if this is the case genetic exchange within a strain would generate identical offspring which could not be distinguished from the parents. As mixed infections between different zymodemes, in either the mammalian host or the vector, are probably rare, genetic exchange could occur quite frequently without disturbing the linkage disequilibrium and therefore there is not necessarily any contradiction between frequent genetic exchange and an essentially clonal population structure (Bastien et al., 1992a).

Kreutzer *et al.* (1994) found two populations of amastigotes with respect to nuclear DNA content in cultured macrophages, one population having double the DNA content by Feulgen staining of the other. The authors suggest that this is evidence of nuclear fusion between amastigotes in the macrophage and consequently of sexual reproduction. Whilst this hypothesis is consistent with the data, an alternative interpretation is that infection of the macrophages has effectively synchronised the cell cycle and the 4N amastigotes are dividing and not fusing (Dr P. Bates, Liverpool School of Tropical Medicine, personal communication). The synchronisation of the cell cycle in trypanosomes has recently been demonstrated (Galanti et al., 1994), if this can be extended to *Leishmania* then more detailed studies of the *Leishmania* cell cycle may shed further light on Kreutzer's interesting observations.

Trypanosoma brucei hybrids have been generated by passage of mixed zymodemes through *Glossina*, these crosses have generated large proportions of hybrids which permit a number of hypotheses for the mode of genetic exchange (Gibson, 1989; Gibson & Garside, 1990; Jenni et al., 1986; Schweizer et al., 1994). The initial experiments of Jenni et al., (1986) suggested the fusion of haploid gametes following meiosis with Mendelian inheritance. Later studies found subtetraploids in some hybrids which were best explained by the fusion of diploid trypanosomes with subsequent loss of chromosomes either before or after nuclear fusion. Schweizer *et al.* found no evidence of tetraploids and concluded that the large chromosomes are inherited in a Mendelian fashion after meiosis of diploid parents, however the smaller chromosomes did not fit into this model and appeared to be inherited independently. *Trypanosoma* mitochondrial DNA appears to be inherited in an unusual fashion in that all the maxicircles are inherited from one or other parent but the minicircles are inherited from both. This has been explained by stochastic effects, the maxicircles could also be inherited from both parents but the loss of the small number of maxicircles derived from one of the parents could occur easily in a small number of divisions whereas the large number of minicircles would prevent such loss from occurring (Gibson, 1995).

The results from African trypanosomes have prompted *Leishmania* workers to look for hybridisation events in sandflies. *L. major* parasites have been observed fusing in *Lu. longipalpis* (Walters et al., 1993), but attempts to produce biochemical evidence for this by passaging two strains of *L. major* with different antibiotic resistances through *Phlebotomus papatasi* failed to produce any parasites with a combined antibiotic resistance after screening

2,500 independent progeny. Cell fusion *in vitro* has been observed in *Herpetomonas* and recorded by video microscopy in both *L. infantum*, and *L. tropica* but as these records are of homogenous strains no biochemical evidence for consequent genetic exchange is available (Lanotte & Rioux, 1990; Sousa, 1994).

At present the strongest evidence for genetic exchange comes from a number of apparent natural hybrids that have been identified in both Old and New worlds. The existence of natural intraspecific hybrids was first suggested by Maazoun *et al.* (1981) who interpreted the presence of heterozygotes for one enzyme (*L. infantum* and *L. tarentolae*) and three enzymes (*L. aethiopica*) as evidence for intraspecific hybridisation and genetic exchange. These were uncloned stocks and it was possible that they were mixtures. Kreutzer *et al.* (1987) found no heterozygotes in 300 isolates with 20 enzymes despite high frequencies of different allomorphs of some enzymes particularly in *L. braziliensis*. Later apparent interspecific hybrids were found between *L. major* and *L. arabica* (Evans et al., 1987; Kelly et al., 1991), *L. braziliensis* and *L. guyanensis* (Bonfante-Garrido et al., 1992), *L. braziliensis* and *L. panamensis* (Belli et al., 1994; Kreutzer et al., 1994; Larsen, 1994) and *L. braziliensis* and *L. peruviana* (Dujardin et al., 1995). These hybrids have been identified by heterozygous isoenzyme patterns containing allozymes of the same mobility as both the homozygous parents. The putative hybrids between *L. panamensis* and *L. braziliensis* from the Ma'agalpa region of Northern Nicaragua where the two species are endemic were also identified by RFLP which produced characteristic heterozygous patterns (Belli et al., 1994).

The evidence for the mode of inheritance of kDNA in the *L. arabica* / *L. major* hybrid MD26 is contradictory, Evans *et al.* (1987) found that a *L. arabica* kDNA probe hybridised weakly to MD26 whilst Kelly *et al.* (1991) found that it did not. Nevertheless there is perhaps no contradiction between the inheritance of minicircles from both parents in experimental *Trypanosoma* crosses and the possible uniparental origin of minicircle kDNA in natural *Leishmania* hybrids. If the maxicircles rapidly become uniparental by stochastic forces then the minicircles encoding the guide RNAs for the eliminated maxicircle become redundant. In such circumstances the number of minicircle classes may slowly be reduced as has occurred in *T. evansi* and *T. lewisi* outside the tsetse belt where it is mechanically transmitted by tabanids (Borst et al., 1987).

1.10. THE MITOCHONDRIAL GENOME OF *LEISHMANIA*

The mitochondrion of all trypanosomatids consists of a single connected network. The mitochondrial genes are found in 20-50 catenated maxicircles each 20,000 - 40,000bp in length. Additionally there are 10,000 to 20,000 minicircles which are each 600-900bp in length in *Leishmania*, and up to 2500bp in length in other genera (Barker, 1987). The minicircles code for guide RNAs that control the extensive editing of mitochondrial mRNA.

Mitochondrial editing principally involves the posttranscriptional insertion and deletion of U residues. This process is most extensive in *T. brucei* producing open reading frames that may be twice as long as the original RNA. Editing is less extensive in *Leishmania* and mainly restricted to the 5' region of genes (Maslov et al., 1994). Editing may introduce frameshift mutations that generate greater variability in the edited protein than in the unedited mRNA (Landweber & Gilbert, 1993; Piller et al., 1995).

The total minicircle population is distributed between various minicircle classes. Within each minicircle class sequence variation may be of the order of 0.3%. Each minicircle has a number of distinctive regions. There are three short conserved sequence blocks (CSB) in which the sequence is conserved across all *Leishmania*. CSB1 a dodecamer GGGGTTGGTGTA represents an origin of replication of the minicircle and is conserved across all trypanosomatids so far examined. The CSBs lie within a conserved region of approximately 120bp which is highly conserved across all minicircle classes in a strain but does exhibit some variation within a species. There is only one conserved region in *Leishmania* but there are up to four conserved regions in other genera arranged symmetrically around the minicircle. The area of the minicircle outside the conserved region is known as the variable region and contains a sequence coding for guide RNA. Also within the minicircle there are runs of oligo dA which generate a conformational bend. The most extreme bend found in DNA from any organism is in the *C. fasciculata* minicircle which contains 16 successive oligo dA tracts which generate a 360° bend. The bend in *L. tarentolae* is less extreme consisting of regular repeats of $CA_{5-6}T$ with a ten base pair periodicity and is located adjacent to the conserved region. The bending of the DNA can cause migration abnormalities of minicircle fragments in polyacrylamide gels leading to underestimates of fragment size (Ryan, K.A. et al., 1988; Simpson, 1987).

There are approximately ten different minicircle classes in *Leishmania* although *Trypanosoma* species have up to 300 different sequence classes. The distribution of the total minicircle population between the various sequence classes is very variable and not necessarily related to the steady state concentration of the mature mRNA which it is involved in editing. In the *L. tarentolae* UC strain 26% of the total number of minicircles are in one minicircle class (Kidane et al., 1984). Considerable variation between strains in the distribution of minicircle classes is found in *C. fasciculata* in which 90% of minicircles belong to one sequence class in the Cf-C1 strain whilst a much more even distribution over 13 minicircle classes was found in the Borst laboratory strain (Simpson, 1987).

1.11. THE RIBOSOMAL GENES OF *LEISHMANIA*

There are approximately 166 copies of the *Leishmania* ribosomal genes located on the large diploid chromosomes and more than 10^4 ribosomal RNA molecules in the cytoplasm (Leon et al., 1978). The high copy number of the ribosomal DNA and RNA makes them a suitable target for DNA probes and PCR tests for diagnosing and identifying *Leishmania* (Guevara et al., 1992; Meredith et al., 1993; Uliana et al., 1991, 1994; van-Eys et al., 1992). The slow rate of evolution of the ribosomal genes has also led to their use for constructing phylogenies of the Kinetoplastida (Fernandes et al., 1993; Marché et al., 1995) and the rapid evolution of the intergenic region has been exploited to develop a classification of the *L. (Viannia)* subgenus (Cupolillo et al., 1995). The organization of the trypanosomatid rRNA locus is shown in figure 1.5.

Figure 1.5 Diagram of a repeat unit of rDNA. The 16S, 5.8S and 26S gene regions are depicted by heavy bars, whilst the spacer sequences are denoted by a thin line.

1.11.1. Ribosomal genes for the diagnosis and identification of *Leishmania*

The ribosomal genes are highly conserved, a comparison of the 16S SSU rRNA genes of *T. brucei* and *Leishmania* which are almost the most distantly related of the trypanosomatids show a structural similarity value of 0.900, the similarity of the 26S LSU rRNA genes between the same species is 0.949. The similarity within the *Leishmania* is 0.997 (Fernandes, A.P. et al., 1993). Consequently large fragments of the gene that might make stable and sensitive probes tend to cross react with other trypanosomatids, however oligonucleotide probes have been developed with various levels of specificity. A genus specific probe for *Leishmania* was developed based on a *PvuII* restriction site in the SSU rRNA that discriminated between *Leishmania* and *T. cruzi* (Uliana et al., 1991). However comparison of the sequence of this oligonucleotide with the SSU rRNA gene of various kinetoplastids show complete homology to the corresponding sequence in *Herpetomonas samuelpessoai*. Consequently although this probe may be useful for diagnostic purposes it could not be relied upon for identification of *Leishmania*. A group of 18 and 19mer

oligonucleotides have been developed which can discriminate between the *L. braziliensis*, *L. donovani*, *L. tropica* and *L. mexicana* species complexes. These oligonucleotides hybridise to sequences containing a single point mutation and consequently share 95% homology with heterologous sequences. Excellent specificity was obtained when using these probes against the complete PCR amplified SSU rRNA gene, but it was found that hybridisation had to be done at a specific temperature for each probe and that that temperature could not be varied by more than two degrees centigrade (Uliana et al., 1994).

Point mutations that discriminate between *Leishmania* species have also been used to develop complex specific PCR primers. The 3' end nucleotide of these primers anneal to the site of the point mutation and consequently should not amplify heterologous sequences (van-Eys et al., 1992). Large excesses over commonly used concentrations of $MgCl_2$ (4mM), dNTPs (100mM); and primer (50μM) were used to drive these reactions (*cf* standard concentrations Para. 2.4.1). The same workers also used restriction digests of PCR products generated using primers that amplified SSU rDNA from all kinetoplastids. *RsaI* and *HhaI* gave restriction patterns that discriminated between the *L. braziliensis* and *L. donovani* complexes and was said to be the easiest method for the identification of these groups.

1.11.2. Ribosomal genes for the classification of Trypanosomatidae

The complete sequence of the 16S SSU rRNA and a 1kb portion of the 26S LSU rRNA genes of a number of kinetoplastids have been used to prepare a phylogeny of this group. SSU rRNA distances between *Trypanosoma* and the *Crithidia/Leishmania* lineage was 6.5-9.7% compared with the 3.2% difference between mammals and amphibians, indicative of the ancient origin of the Trypanosomatidae. The phylogeny suggested that digenetic parasitism may have involved independently on four occasions in the trypanosomatids (Fernandes, A.P. et al., 1993).

Restriction digests of the spacer region between the 16S and the 26S subunits have been used to prepare a phylogeny of *L. (Viannia)* (Cupolillo et al., 1995). The method was named intergenic region typing (IRT) and clearly resolved different species within the subgenus, however distances between a number of species or groups of species exceeded 0.8 on a scale from 0 to 1 and the relationship between them could not be resolved with confidence in all cases.

1.12. THE POLYMERASE CHAIN REACTION AND ITS DERIVATIVES FOR IDENTIFICATION AND CLASSIFICATION OF *LEISHMANIA*

1.12.1. The polymerase chain reaction

The polymerase chain reaction (PCR) for the exponential amplification of DNA was first described by Saiki *et al.* (1985). The PCR can amplify target DNA ten million fold making it possible to detect and isolate even single copy sequences from a whole genome or detect the presence of alien DNA in the presence of a large excess of host DNA.

The PCR consists of three steps repeated 25-40 times:-

1) Denaturing of double stranded DNA at about 94°C to generate single stranded DNA.
2) Annealing of oligonucleotide primers to flanking regions of the target sequence.
3) Extension of the primer with a suitable DNA polymerase.

At first the Klenow fragment of *Escherichia coli* DNA polymerase I was used. The Klenow fragment is degraded at 94°C and so had to be added afresh for each cycle. This procedure was both tedious and expensive and therefore impractical for routine use. The introduction of the thermostable *Taq* polymerase from *Thermus aquaticus* made the reaction both practical and economical, leading to an exponential growth in applications, variations and acronyms (Saiki et al., 1988). For reviews see (Arnheim & Erlich, 1992) and (Erlich & Arnheim, 1992).

There are however a number of difficulties with the PCR. Because of the enormous power of the PCR to amplify DNA it is very vulnerable to contamination and false positives. It is also sensitive to changes in the many parameters associated with the reaction making careful optimisation of, and strict adherence to, protocols essential for reproducible results.

For identification and diagnostic purposes the ideal PCR target is a highly repeated sequence unique to that taxon, however such sequences are not known for many closely related species. In the *Leishmania* there are PCR primers based on multicopy kDNA or genomic DNA sequences for all the pathogenic complexes, however none of these primers permit identification down to the species level (de Bruijn et al., 1993; Degrave et al., 1994; Guevara et al., 1992; El Hassan et al., 1993; Ibrahim et al., 1994; Lopez et al., 1993; Perez

et al., 1994; Piarroux et al., 1993; Rodgers et al., 1990; Smyth et al., 1992; van-Eys et al., 1992).

One method for resolving species or populations is the restriction enzyme digestion of PCR products (Avila et al., 1990; Clark & Pung, 1994; Gage et al., 1994). When applied to kDNA this is essentially the same as the well established schizodeme analysis which is the restriction digestion of kDNA that has been separated from genomic DNA (Angelici et al., 1989; Jackson et al., 1986; Pacheco et al., 1990a; Spithill et al., 1984). Avila *et al.* (1990) describe the application of PCR based schizodemes to *T. cruzi* and Degrave *et al.* (1994) report that the same technique has been applied to *Leishmania*, although no experimental details were presented. The *HaeIII* digestion of PCR amplified *L. infantum* minicircles has been described in a poster by Lambson et al., (1995).

Detailed epidemiological studies are at present hampered by the difficulties and expense of isolating and culturing parasites from large numbers of hosts for the standard high resolution techniques of schizodeme and isoenzyme analysis. Furthermore whilst many *Leishmania* are readily isolated into culture others, such as some strains of the *L. (Viannia)* subgenus and some dermotropic strains of *L. infantum*, are frequently difficult or impossible to isolate at all, or, if growth is achieved, to sustain beyond the first passage (Ben-Ismail et al., 1992; Bray & Munford, 1967; Gramiccia et al., 1987; Walton & Shaw, 1977), Consequently there is often concern that the parasites that have been isolated and studied may not be representative of the populations in the host from which the parasites were isolated or of the range of parasites in circulation.

1.12.2. Randomly amplified polymorphic DNA (RAPD)

1.12.2.1. Introduction

The acronym RAPD was coined by Williams *et al.* (1990) to describe the random amplification of a number of DNA fragments of unknown origin and sequence. RAPD has come to be used to describe a family of closely related techniques. Williams *et al.* used a single arbitrarily chosen decamer primer and an annealing temperature of 36°C. Arbitrarily primed PCR (AP-PCR) uses primers of up to 35 nucleotides and higher annealing temperatures (Welsh & McClelland, 1990) and DNA amplification fingerprinting (DAF) uses hexanucleotide primers (Caetano-Anolles, 1994). The method has also been applied

to RNA (Welsh et al., 1992a) and total cDNA (randomly amplified differentially expressed sequences, RADES) (Murphy & Pelle, 1994).

1.12.2.2. The RAPD reaction

The reaction mechanisms of RAPD are not well understood. It has been shown that the probability b of producing a product between 500 and 2500bp in length from a genome of size C with a primer size n is $b = C(2000 \times 4^{-2n})$ (Williams, et al., 1993). For *Leishmania* where $n = 5 \times 10^7$, the probability of producing a product between 500 and 2500bp is 0.09 when using decamer primers. Nevertheless the number of products produced does not appear to be related to the size of the genome and it is evident that many of the products must be the consequence of mispriming events. Caetano-Anolles *et al.* (1993) found that the matching of the first six nucleotides are much more important than the nucleotides nearer the 5' end of the primer. However Williams *et al.* (1993) found that substitutions in any of the positions from 1-9 on a decamer completely changed the banding pattern and suggested that RAPD is capable of detecting single base changes.

The target sequence may be either single copy or highly repeated. N'Goran *et al.* (1994) found approximately equal numbers of products from both types of target in a study of *Theobroma cacoa* whilst others have found mainly repeated sequences (Kazan et al., 1992).

The number of products obtained is proportional to the primer length for primers of between six and twelve nucleotides, possibly because the primer has to compete with the 3' end of the fragment to be amplified. The 3' end of the template is necessarily the same sequence as the primer and it is possible that longer primers can compete more effectively with the 3' end for the annealing site (Caetano-Anolles et al., 1992). As a high degree of mismatching occurs the technique is sensitive to small changes in reagent concentrations and annealing temperature and it has been shown that standard protocols will not necessarily generate the same results in different laboratories (Penner et al., 1993; Williams, et al., 1993).

However most workers find that, by using standard conditions, RAPD generates sufficiently reproducible results to make it a very useful tool for elucidating a number of problems (Caetano-Anolles, 1994).

1.12.2.3. Applications of RAPD

RAPD has found its principal applications in the fields of plant breeding, and population studies of arthropods and microorganisms. In plant breeding RAPD has been used to construct linkage maps (Al-Janabi et al., 1993; Kennard et al., 1994), to detect hybrids (Baird et al., 1992; Wang et al., 1994), and to produce species specific DNA probes for pathogens (Manulis et al., 1994). Parasitologists and entomologists have used RAPD for the identification of members of arthropod species complexes (Adamson et al., 1993; Wilkerson et al., 1993) and for the identification of schistosomes and various parasitic protozoa (MacPherson & Gajadhar, 1993; Neto et al., 1993; Waitumbi & Murphy, 1993). RAPD has also been used for the classification of arthropods and parasites (Ballinger-Crabtree et al., 1992; Kambhampati et al., 1992; Kaukas et al., 1994; Welsh et al., 1992b). Most primers tested discriminate between species and many will discriminate between populations, but meaningful comparisons cannot usually be made between genera as the numbers of polymorphism are usually too high. An unrooted tree showing the relationships between *Schistosoma* species has the majority of species radiating from close to the origin (Kaukas et al., 1994), and consequently it would be unsafe to rely on RAPD for showing relationships at this level for this genus. There are also theoretical objections to the use of RAPD for classification. The origin of RAPD bands and hence their taxonomic significance is not known and nor is it known whether comigrating bands are of identical sequence nor are homologous regions necessarily amplified in all OTUs (Black, 1993). Despite these problems classifications based on RAPD have compared well with others based on isoenzyme electrophoresis when using small numbers of samples (Dias-Neto et al., 1993; Steindel et al., 1994). However in a study using 35 *Aedes* mosquitoes from five species it was found that RAPD correctly allocated all specimens to the expected species, but 23 *Aedes albopictus* from Beijing, Galveston, Singapore, and Zama did not cluster into geographical populations (Kambhampati et al., 1992).

1.12.2.4. Application of RAPD to the study of *Leishmania*

Old World *Leishmania* species have been identified by amplifying a monomorphic 461bp minicircle product using a pair of PCR primers followed by arbitrarily primed PCR on the fragment with the M13/pUC forward and reverse primers to produce species specific fingerprints (Bhattacharyya et al., 1993). Tibayrenc *et al.* (1993) compared classifications of some New World species of *Leishmania* generated by RAPD and isoenzyme electrophoresis and found good agreement between the two methods.

1.13. ANALYSIS OF DNA FINGERPRINT AND SEQUENCE DATA

Data derived from molecular biology must be used with some care if accurate phylogenies are to be produced. In comparative biology complex characters such as organs can be studied in great detail to determine if they are really homologous or if the apparent homology is due to parallel or convergent evolution - homoplasy. Fingerprint characters are either present or absent and DNA sequence data is comprised of the four bases ACGT, these characters cannot be subdivided in order to evaluate them.

Similarity between characters may be primary - due to common ancestry, or secondary - homoplasies. Primary similarity may further be subdivided into those similarities that define groups at the level of interest - synapomorphies, and those similarities that are derived from a common ancestor but are partially conserved across a number of groups and may confound the analysis - synplesiomorphies. Secondary similarities, homoplasies, may be due to reversal, parallelism, convergence and chance similarity (Williams, 1992b).

Homoplasies (false similarities) will usually be present in any set of DNA sequences and cannot be eliminated by inspection. Homoplasies accumulate in a gene in a probabilistic fashion at a rate that is loosely proportional to the rate of evolution of the gene. The more distantly related two taxa are the greater the number of homoplasies that will occur, until eventually the potential signal from the gene will be lost. The effect of homoplasies is that taxa appear to have diverged more recently than they actually have. Some of these problems can be partially overcome by outgroup rooting, ideally the outgroup should be closely related to the ingroup (Williams, 1992b). Phylogenies of the Trypanosomatidae indicate that *Endotrypanum* would be most suitable for rooting the *Leishmania* and that either *Crithidia* or *Leptomonas* would be suitable for rooting the *Endotrypanum* / *Leishmania* clade (Fernandes, et al., 1993; Marché et al., 1995; Maslov & Simpson, 1995).

There are two main approaches to analysis of sequence and fingerprint data - matrix methods and parsimony.

1.13.1. Matrix methods

In matrix methods the distance between each possible pair of taxa under study is calculated using an appropriate similarity or distance coefficient and the individual values are entered into a matrix. There are then a number of methods for compiling a tree from the matrix.

1.13.1.1. Similarity and distance coefficients

Priest and Austin (1993) list twelve different equations for calculating similarity coefficients which are widely used for comparing fingerprint data. Similarity coefficients usually give values of similarity from zero, for no similarity, to one for complete identity. Two coefficients are in widespread use, the simple matching coefficient and the Jaccard coefficient. Similarity coefficients compare two operational taxonomic units (OTU), for each pairwise comparison of a single character there are four possible outcomes (Table 1.2)

		OTU **j**	
		+	-
OTU **k**	+	*a*	*b*
	-	*c*	*d*

Table 1.2. The four possible outcomes for the comparison of a single binary character in OTU's **j** and **k** are represented by the letters *a, b, c* and *d*.

Each occurrence of the homologous band in both OTUs is represented by an *a*, each occurrence of a band in **k** but not **j** is represented by *b* and each occurrence of a band in **j** but not **k** is indicated by *c* (Table 1.2). If the number of times that a, b and c are found in a pair of OTUs is represented by A, B and C respectively then the equations for the similarity coefficients are as follows:-

Simple Matching Coefficient $S_{SM} = \frac{A + D}{A + B + C + D}$

Jaccard Coefficient:-

$$S_J = \frac{A}{A + B + C}$$

The difference between them is that the simple matching coefficient includes negative matches (*d*) whereas the Jaccard coefficient does not. When comparing only two OTUs by RAPD the problem of negative matches (the absence of a band from both members of the

pair) does not arise because the absent bands are not definable. However when comparing more than two OTUs a band may be present in one OTU that is not present in the two being compared, including the common absence of that band from the two OTUs under consideration in the equation would increase the similarity between the two. No publications have used the simple matching coefficient for comparing RAPD products. Sneath and Sokal (1973) suggest that it is appropriate to omit negative matches from the comparison of bacterial enzymes as a large group of negatives may be due to a single metabolic block which disables a particular pathway.

The Jaccard similarity coefficient has been widely used for comparing isoenzyme profiles of *Leishmania* and so it should be possible to compare results from isoenzymes with those from RAPD. However workers at Montpellier who have done extensive isoenzyme studies use the Jaccard distance coefficient (Thomaz Soccol, 1993; Tibayrenc et al., 1993). Distance coefficients are commonly equivalent to the similarity coefficient subtracted from one and can be compared by reversing the scale. However the workers at Montpellier use a different version of the equation which emphasises differences and is not monotonic with the Jaccard similarity coefficient.

Montpellier groups :-

Jaccard coef. $S_J = 1 - (A/(A + 2(B + C))$

Another version of the Jaccard similarity coefficient has also been described by Southwood (1978):-

$$D_J = A/(B + C)$$

Like Jaccard, Southwood was comparing the species found in two habitats and it may be that Southwood interpreted "Esp.[èces] distinctes sur [localités] 1 et 2" (Jaccard, 1908) to mean the number of species that were different in localities 1 and 2 rather than the number of different species in localities 1 and 2. Inspection of the data in Jaccard's appendices shows that the second interpretation was the one which Jaccard intended. The derivation of the Montpellier Jaccard distance equation is not clear. Southwood's equation has not been applied to *Leishmania*, however workers at Montpellier have used their form of the equation in a number of publications (Thomaz Soccol, 1993; Tibayrenc et al., 1993; Truc & Tibayrenc, 1993), whereas workers elsewhere have used the form originally described by

Jaccard although not always under that name (Cibulskis et al., 1986; Cupolillo et al., 1994). Care therefore needs to be exercised when comparing Jaccard coefficients.

All distance and similarity coefficients score all differences between two operational taxonomic units (OTU) equally. This has an important effect. The different migration of isoenzyme alleles, for example, may be caused by one or by several amino acid changes. Amino acid differences between two OTUs generally tend to accumulate over time and therefore scoring all differences equally tends to suggest that the OTUs are more closely related than they are in reality. The effect of this can be seen in many dendrograms where the distances between the nodes linking the most divergent taxa are small (Cibulskis et al., 1986; Cupolillo et al., 1994). This compression effect caused by scoring changes equally, irrespective of the number of individual changes of which the observed difference is composed, has a similar effect on all fingerprint data such as RFLPs and RAPD and is similar to the homoplasies described above for sequence data. The consequence of the compression effect is that the relationship between similarity and time as measured by any of these methods is not linear. To the extent that rates of change differ in individual lineages the compression effect will also distort the topology of a dendrogram.

1.13.1.2. Tree construction

The simplest approaches to tree construction are the phenetic clustering methods - single linkage, average linkage (UPGMA) and complete linkage that join successively more distantly related taxa to form the tree. The tree is constructed at a single pass and large data sets can be analyzed very quickly. Phenetic clustering methods will only generate a tree that indicates lines of descent if rates of change have been equal in all taxa, an assumption that becomes increasingly unreliable with increasing distance. Consequently although phenetic methods are still frequently used for population studies where unequal rates of change may not have an significant effect, they are unreliable for longer range studies (Hills et al., 1994)..

The alternatives to phenetic clustering methods are distance methods which were first described by Fitch and Margoliash in 1967 (Williams 1992a) and deal with rate heterogeneities by permitting unequal branch lengths and comparing the observed distances between taxa in the tree with the distances in the matrix. The tree can then be rearranged until the difference between observed and expected tree lengths is minimised. Initially a tree

is constructed in a similar manner to the phenetic clustering methods and the fit is assessed in the Fitch and Margoliash method by percentage standard deviation (SD) of the observed distances from the expected distances. If the tree perfectly fits the data the standard deviation will be zero. However there are a large number of trees to check and even using tree rearrangement techniques the tree with the lowest %SD might be missed (Williams, 1992a). The FITCH programme in the PHYLIP package used in the present study uses a least squares criterion in addition to % SD.

1.13.2. Parsimony methods

Parsimony methods examine all possible trees to find which tree requires the smallest number of changes to get from a hypothetical ancestral state to the observed OTUs. The numbers of trees to be examined grows rapidly with increasing numbers of OTUs. There are 945 possible trees for seven taxa but 2.2×10^{20} for twenty taxa. This problem can be overcome for groups of less than 25 taxa by using the branch and bound method in which the number of changes in an initial tree is compared with those required for succeeding trees. As soon as a set of trees starts to exceed the initial value the whole set can be discarded and if a tree is found with a lower number of changes that value is adopted as the new upper bound. Heuristic methods are used for data sets larger than 25 taxa, these cannot be guaranteed to yield the optimum tree (Williams, 1992a).

The mostly widely used method for evaluating the number of changes in trees derived from nucleotide sequences is that of Fitch. Fitch's method was an adaptation of that of Wagner and permits any nucleotide to change into any other and all such changes are equally weighted. This is the principle used in the DNAPARS programme in PHYLIP, and is applied to sequence data in this study. The alternative is to weight transversions between purines and pyrimidines more heavily than transitions amongst either pyrimidines or purines (Williams, 1992a). Transversions are assigned a weight of two in the distance matrix programmes used in the present study.

1.13.3. Evaluation of trees

The sensitivity of a tree to changes in the data set can be tested by constructing a new dataset by random sampling of the initial dataset. This will generate a new dataset containing only data from the original dataset but with some data duplicated and other data omitted. The "bootstrap" resampling can be repeated at least a hundred times and trees can then be

constructed from each dataset. Finally a consensus tree is constructed such that for each node the arrangement that is supported by the most trees is adopted. The proportion of trees in which a particular arrangement is found is then a measure of the robustness of that arrangement. These methods can require substantial computing time and memory capacity, as at least 100 datasets need to be accommodated.

There is a vigorous debate about the usefulness of various methods (Felsenstein, 1988; Hills et al., 1994; Sidow, 1994; Stewart, 1993). However all methods will fail to arrive at the true phylogeny when the true phylogeny contains a very short internal lineage separating two clusters, each cluster having one lineage that is quite long relative to its sister lineage (Sidow, 1994). These situations are frequently encountered, particularly when using high resolution techniques such as isoenzymes to determine long range relationships.

1.14. OBJECTIVES OF THE PRESENT STUDY

Central America has perhaps the highest density of named *Leishmania* species of any comparable sized region in the world, and therefore provides unique opportunities for the study of the diversity of these parasites. The work described below was undertaken as part of a collaborative evaluation of various methods of generating markers for *Leishmania* strains (see preface). The particular contribution of the British partners in the collaboration was to be the evaluation of PCR based techniques for the identification of *Leishmania* species and strains. In particular it was intended to identify putative hybrids between *Leishmania* species in Nicaragua (Para. 1.9), and to find markers for *L. chagasi* populations from Honduras and Costa Rica that correlated with particular disease states (Para. 1.8). The Central American partners in the collaboration supplied 84 strains of *Leishmania* that had previously been identified locally so that the local techniques could be compared with the PCR based techniques which were to be developed at Liverpool.

PCR using specific primers is well established for the identification of the *Leishmania* species complexes but primers suitable for higher resolution studies have not been found (Para. 1.12). RAPD was therefore tested to determine if this technique was suitable for identifying *Leishmania* species, *Leishmania* hybrids and virulence markers. The use of RAPD for the identification of *Leishmania* species and of putative hybrids between *L. braziliensis* and *L. panamensis* from Nicaragua is described in chapter three, and the use of RAPD for the identification of markers for different pathologies caused by *L. chagasi* is described in chapter five. Whilst it was found that RAPD was useful for the identification of *Leishmania* species it was not found to be useful for higher resolution population studies, therefore PCR based schizodemes were developed that might be suitable for the identification of individual strains of *Leishmania* (Chapter 4). The PCR based schizodemes were used to look for markers for the different pathologies caused by *L. chagasi* and compared with PFGE (Chapter 5).

31 of the 84 Central American strains studied could not be identified by comparison with reference strains using RAPD. The small subunit ribosomal RNA gene of eleven species of mammalian and lizard *Leishmania* was therefore investigated in a search for genus specific markers for the *Leishmania* (Chapter 6). The SSU rDNA based techniques were then applied to the identification of the parasites that could not be identified by RAPD (Chapter 7).

2. MATERIALS AND METHODS

2.1. PARASITE CULTURE

2.1.1. Parasites

Parasite reference strains are listed in table 2.1. Parasites received from Honduras are listed in table 2.3 and the parishes from which they were isolated are shown in figure 2.1. Parasites from Honduras were obtained from cutaneous lesions by aspiration, from cases of visceral disease by bone marrow aspiration and from sandflies by culture of flagellates detected by dissection. Parasites were isolated into Senekjie's medium with 10% rabbit blood overlaid with Locke's solution in which they generally grew well. Each isolate was typed by monoclonal antibodies at the Honduran Ministry of Health Central Laboratories in Tegucigalpa and the identification confirmed by isoenzyme electrophoresis in Dr R. Kreutzer's laboratory (Youngstown State University, Ohio, USA): all the *L. chagasi* strains were found to be identical to the WHO *L. chagasi* reference strain (MHOM/BR/74/M2682). Strains were deposited at the National Institutes of Health, (Dr F. Neva, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases) Maryland, USA, for cryopreservation. Parasites were not maintained in culture longer than necessary to identify them and consequently there was no opportunity for cross contamination between strains except for where more than one isolate was made at the same time. Parasites were received at the Liverpool School of Tropical Medicine in four batches and maintained in Schneider's insect medium (Sigma). The first batch received in August 1993 consisted of both visceral and cutaneous parasites. The second batch received in October 1994 consisted of nine strains each of *L. chagasi* and *L. mexicana* from cutaneous cases. The third batch received in May 1995 consisted of one visceral parasite and three cutaneous parasites, the parasites causing different pathologies were maintained in separate laboratories in Liverpool. The fourth batch received in November 1995 consisted of one cutaneous and one visceral isolate. DNA and parasites embedded in agarose blocks for PFGE were prepared when each batch were received and the parasites were then cryopreserved before further batches arrived.

Parasite isolates received from other colleagues in Latin America are listed in table 2.3. They had been typed by isoenzymes (Costa Rica, Honduras, Nicaragua and Guatemala) or schizodemes (Panama) before being sent to Liverpool but the identity of the parasites was not revealed until after they had been identified by RAPD.

2.1.2. Parasite maintenance

Parasites were routinely maintained in Schneider's insect medium (Sigma) pH6.8 containing heat inactivated foetal calf serum (10%v/v), penicillin (100Uml^{-1}) and streptomycin (100μgml^{-1}). The complete medium was sterilised by filtration (0.2μm). The Nicaraguan parasites were maintained in RPMI with 2% human urine (Howard et al., 1991) as recommended by their supplier. All cultures were incubated at 28°C unless otherwise stated.

Parasites were recovered from animals or cryopreservation into a modified Evans' semi-solid medium. 4g blood agar base (Oxoid No.2) made up with 350ml Locke's solution (65mM NaCl; 6mM KCl; 4mM $CaCl_2$; 2.7mM Na_2HCO_3; 5.5mM Glucose). The NaCl concentration is reduced to compensate for the NaCl in the blood agar base. The base was autoclaved and allowed to cool. The solidified agar was liquefied by shaking and filter sterilised $NaHCO_3$ was added to a final concentration of 2mM. 50ml of defibrinated sterile rabbit blood were added aseptically.

L. braziliensis parasites that failed to grow in monophasic liquid media were cultured on rat blood agar slopes (Bray & Munford, 1967). Blood agar base (Oxoid No.2) was prepared according to the manufacturer's directions and rat blood (Ludwig Wistar's) was added to a final concentration of 12.5%. The liquid phase was Schneider's insect medium.

Suspected *Crithidia* species were grown in the ATCC *Crithidia* selective medium:- 6.0g tryptone, 1.0g yeast extract, 0.1g liver concentrate, 15g sucrose, 5ml haemin solution (triethanolamine 2.5ml, haemin 25mg, distilled water 2.5ml), 1.0L distilled water pH7.8.

2.1.3. Parasite cloning

2% agar in Hank's solution (1.2mM $CaCl_2$; 5.4mM KCl; 44μM KH_2PO_4; 50μM MgCl; 40μM $MgSO_4$; 130mM NaCl; 4.1mM $NaHCO_3$; 3.3mM Na_2HPO_4; 5.5mM glucose; 10μM Phenol red, pH 7.0-7.2) was mixed with an equal volume of Schneider's insect medium with 10% foetal calf serum (FCS) at 40°C and poured into Petri dishes. 500 parasites were spread on each plate which was sealed with Parafilm and incubated at 28°C. After approximately ten days colonies were collected with a plastic needle or Pasteur pipette and transferred to 0.5mls of Schneider's insect medium.

2.1.4. Metacyclogenesis and *in vitro* generation of amastigotes.

Parasites were diluted to 10^6 promastigotes ml^{-1} in fresh Schneider's (10% FCS) pH5.6 and incubated at 28°C or 36°C.

2.1.5. Infection of sandflies with *Leishmania*

Female *Lu. longipalpis* were placed in individual vials with a nylon mesh cover and offered up to lesions on mice or hamsters. After feeding, vials were kept in sealed humidified containers. Cotton swabs soaked in saturated sucrose solution were placed on the mesh covers of the vials and changed daily. After five days the gut was extracted for examination or the whole fly was placed in Dubosq's Brazil (80% ethanol 150mls; formalin 60mls; glacial acetic acid 15mls; picric acid 1g) for sectioning for light microscopy as described below.

Reference strains:-		Tropism	Donor
L. amazonensis	MHOM/BR/73/LV78		Dr Chance
L. aristidesi	MORY/PA/69/1746;LV41		"
L. braziliensis	MHOM/BR/68/M2903		"
L. braziliensis	MHOM/BR/84/LTB300		A. Belli
L. chagasi	MHOM/BR/74/PP75	VL	Dr Chance
L. chagasi	MHOM/BR/76/150406;M4192	VL	"
L. chagasi	MHOM/HN/78/LV718	VL	"
L. deanei	MCOE/BR/XX/LV402	VL	"
L. donovani	MHOM/ET/67/LV9		"
L. guyanensis	MHOM/BR/75/M4147		"
L. herreri	MCHO/CR/74/016;Ch-97;LV344		"
L. herreri	ISHA/CR/74/010;Sh-1;LV341		"
L. herreri	IYLE/CR/74/012;Yleph;LV342		"
L. hertigi	MCOE/PA/65/C-8;LV42		"
L. hertigi	MCOE/PA/72/C119;LV43		"
L. infantum	MHOM/FR/78/LEM75	VL	Dr Evans
L. infantum	MHOM/FR/80/LEM188	CL	"
L. infantum	MHOM/TN/80/IPT1	VL	"
L. infantum	MHOM/FR/62/LV358	VL	Dr Chance
L. infantum	MHOM/FR/XX/LV405	CL	"
L. infantum	MHOM/BE/67/ITMAP263	VL	Dr Le Ray
L. major	MHOM/ET/XX/LV305		Dr Chance
L. major	MHOM/IR/XX/LV114		"
L. major	MHOM/IL/67/LRC-137;LV561/C22		"
L. mexicana	MNYC/BZ/62/M379		Dr Bates
L. panamensis	MHOM/PA/71/LS94		Dr Chance
L. peruviana	MHOM/PE/63/LV54		"
	MHOM/HN/78/LV639		"
	MHOM/HN/78/LV642		"
L. tropica	MHOM/IR/89/ARD22		Dr Motazedian
L. tropica	MHOM/IR/60/LV357		Dr Chance
L. tarentolae	RTAR/AL/39/LV414		"
L. tarentolae	RTAR/SE/XX/LV108		"
L. hoogstraali	RLIZ/SD/XX/LV31		"
L. gymnodactyli	RGEC/SU/XX/LV247		"
L. adleri	RLIZ/KE/XX/LV30		"
E. monterogeii	MCHO/CR/XX/LV88		"
E. schaudinni	MBRA/PA/XX/LV58		"
E. schaudinni	MCHO/PA/XX/LV59		"

Monoxenous parasites	Type host	
Crithidia luciliae (Strickland, 1911)	*Lucilia spp.*	Dr Chance
Crithidia fasciculata (Leger, 1902)	*Anopheles muculipennis*	"
Herpetomonas muscarum (Leidy 1956)	*Musca domestica*	Swiss Tropical Inst.
Leptomonas ctenophthalmi s.l. LV497	*Spilopsylus cuniculi*	Prof. Hommel

Table 2.1 Parasite reference strains.

Species	Strain No.	Tropism	Patient Sex/Age
L. chagasi	MHOM/HN/87/29	"	F 2y
"	ILUT/HN/87/92	Insect parasite	
"	MHOM/HN/88/115	Cutaneous	F 9y
"	MHOM/HN/88/116	"	F 14y
"	MHOM/HN/88/122	"	M 8y
"	ILUT/HN/88/125	Insect parasite	
"	MHOM/HN/89/167	Cutaneous	F 9y
"	MHOM/HN/89/168	Visceral	F 20m
"	MHOM/HN/93/310	Cutaneous	F 10y
"	MHOM/HN/93/336	"	F 9y
"	MHOM/HN/93/354	Visceral	F 2y
"	MHOM/HN/93/368	Cutaneous	F 8y
"	MHOM/HN/94/419	"	F 10y
"	MHOM/HN/94/420	"	F 12y
"	MHOM/HN/94/421	"	F 13y
"	MHOM/HN/94/462	"	M 12y
"	MHOM/HN/94/463	"	F 9y
"	MHOM/HN/94/504	"	M 6y
"	MHOM/HN/94/530	"	M 15y
"	MHOM/HN/95/552	"	M 11y
"	MHOM/HN/95/556	"	F 8y
N/D	MHOM/HN/95/560	"	F 13y
L. chagasi	MHOM/HN/95/564	Visceral	M 2y
N/D	MHOM/HN/95/572	Cutaneous	F 9y
"	MHOM/HN/95/586	Visceral	M 22m
L. braziliensis	MHOM/HN/90/294	Cutaneous	M 24y
L. mexicana	MHOM/HN/93/410	"	M 7y
"	MHOM/HN/93/413	"	F 10y
"	MHOM/HN/93/415	"	M 9y
"	MHOM/HN/93/416	"	F 12y
"	MHOM/HN/94/451	"	F 16y
"	MHOM/HN/94/453	"	M 6y
"	MHOM/HN/94/458	"	M 15y
"	MHOM/HN/94/470	"	F 8y
"	MHOM/HN/94/532	"	

Table 2.2 Parasite strains received from Honduras supplied by Dr C. Ponce and Dr E. Ponce, Ministry of Health, Tegucigalpa, Honduras. All Honduran strains except 560, 572 and 586 (N/D) were characterised by isoenzyme electrophoresis by Dr R. Kreutzer (Youngstown State University, Ohio). Age in years (y) or months (m).

Guatemala	**Nicaragua** Supplied by Mr A.Belli	
Supplied by Dr B. and Ms F. Arana	Strain	Species
MHOM/GT/XX/G338	MHOM/NI/87/ZF09	Not known
MHOM/GT/XX/G536	MHOM/NI/87/ZE09	*L. panamensis*
MHOM/GT/XX/G707	MHOM/NI/88/XD05	"
MHOM/GT/XX/U120	MHOM/NI/88/XD09	putative hybrid
MHOM/GT/XX/G408	MHOM/NI/88/XD13	" "
MHOM/GT/XX/G505	MHOM/NI/88/XD17	*L. braziliensis*
MHOM/GT/XX/G523	MHOM/NI/88/XD24	"
MHOM/GT/XX/G735	MHOM/NI/88/XD25	"
MHOM/GT/XX/G755	MHOM/NI/88/XD28	*L. braziliensis*
MHOM/GT/XX/U139	MHOM/NI/88/XD36	putative hybrid
MHOM/GT/XX/U154	MHOM/NI/88/XD42	" "
MHOM/GT/XX/G519	MHOM/NI/88/ZN05	*L. braziliensis*
MHOM/GT/XX/G753	MHOM/NI/91/WSL03	putative hybrid
MHOM/GT/XX/U265	MHOM/NI/91/WSL06	" "
MHOM/GT/XX/U266	MHOM/NI/91/ZF01	*L. panamensis*
MHOM/GT/XX/U267	MHOM/NI/91/ZF02	"
MHOM/GT/XX/U276	MHOM/NI/91/ZF03	"
MHOM/GT/XX/U277	MHOM/NI/91/ZF06	Not known
MHOM/GT/XX/U280	MHOM/NI/92/ZN01	" "
	MHOM/NI/92/ZN03	*L. panamensis*
	MHOM/NI/92/LV1	*L. chagasi*
	MHOM/NI/92/LV2	"
Costa Rica	**Panama**	**Venezuela**
Supplied by Dr R. Zeledon	Supplied by Dr P. de Carreira	Supplied by Dr Bonfante-Garrido
MHOM/CR/87/SIA127	CIDEP001	MHOM/VE/92/H-185
MHOM/CR/90/SIA209	CIDEP002	MHOM/VE/92/H-191
MHOM/CR/91/SIA263	CIDEP003	MHOM/VE/92/H-197
MHOM/CR/91/SIA264	CIDEP004	MHOM/VE/92/H-199
MHOM/CR/91/SIA266	CIDEP005	MHOM/VE/92/P-2
MHOM/CR/91/SIA283	CIDEP006	MHOM/VE/92/P-6
MHOM/CR/91/SIA296	CIDEP007	MHOM/VE/92/O-1
MHOM/CR/92/SIA343	CIDEP008	MHOM/VE/92/O-2
MHOM/CR/92/SIA426	CIDEP009	MHOM/VE/92/O-5
MHOM/CR/93/SIA433		MHOM/VE/92/O-9
MHOM/CR/93/SIA439		MHOM/VE/92/O-21
MHOM/CR/93/SIAEVER		MHOM/VE/XX/LbV

Table 2.3 Parasite strains received from colleagues in South and Central America for identification by RAPD. The identity of the strains from Guatemala and Panama was not reported at the time of supply. The identity of the strains from Nicaragua are indicated where known. The strains from Costa Rica were all reported to be *L. chagasi* from cutaneous lesions. The strains from Venezuela were reported to be *L. (Viannia)* species with the exception of MHOM/VE/XX/LbV which was reported to be *L. braziliensis*.

Figure 2.1. Map of Southern Honduras showing parishes from which strains used in the present study were isolated. Numbers after the names of parishes indicate the numbers of strains isolated there. ● Parishes in which cutaneous cases have been found, ■ parishes in which visceral cases have been found. Scale bar is equivalent to 50km.

2.2. MICROSCOPY

2.2.1. Light microscopy

500μl of parasite culture was centrifuged (10min, 1000g) and resuspended in 500μl Locke's solution (150mM NaCl; 6mM KCl; 4mM $CaCl_2$; 2mM $NaHCO_3$; 5mM glucose). 50μl was spread on a slide and air dried. The parasites were fixed with methanol, air dried, stained in 15:1 Giemsa : Giemsa buffer (7.6mM Na_2HPO_4; 2.9mM KH_2PO_4 pH7.2) (BDH) for 15 minutes and washed in tap water.

Sandflies in Dubosq's Brazil were dehydrated through a graded ethanol series then incubated in Supercedrol (BDH) at 60°C for 24 hours. The flies were then embedded in wax (Paramat, BDH) in a vacuum oven (60°C, 0.5bar) and sectioned at 5μm intervals. Sections were stained with Giemsa.

2.2.2. Electron microscopy

10^8 parasites were washed once in Locke's solution and fixed with 3% glutaraldehyde in 0.1M sodium cacodylate ($Na(CH_3)_2AsO_2$) buffer pH7.4 for 1 hour (RT). The pellet was passed to the EM unit for further processing where it was rinsed in 0.1M sodium cacodylate and post fixed in 1% OsO_4 in 0.1M sodium cacodylate (1 hour RT). The pellet was then washed with 0.1M sodium cacodylate, dehydrated through a graded ethanol series and embedded in Epon-araldite resin (8.5g araldite CY212; 15g Agar 100; 27g dodecenylsuccinicanhydride; 1.0g dibutylpthalate; 0.5g benzyldimethylamine). The resin blocks were sectioned at 130nm intervals and collected on 200 mesh hexagonal grids. Sections were stained with lead citrate and uranyl acetate (Reynolds, 1963) and examined in a Philips CM10 transmission electron microscope at 80kV.

2.3. DNA PREPARATION

2.3.1. Genomic DNA

2.3.1.1. Boiling

250μl of parasites (5x16^6 parasites ml^{-1}) in culture medium were incubated at 100°C for 5 minutes. 1μl was used in each PCR reaction.

2.3.1.2. Crude proteinase K lysates

$5x10^6$ parasites were washed once in Locke's solution by centrifugation (10min 1000g) in a microfuge. The pellet was resuspended in 100μl of Tris-HCl (150mM pH8.0), EDTA (1mM) to which were added 2.5μl Tween 20 and 2μl proteinase K (10mgml^{-1}). The solution was incubated at 55°C for one hour, then at 100°C for 5 minutes and centrifuged at 1000g for 15 seconds.

2.3.1.3. Lithium chloride preparation

$5x10^7$ parasites were harvested by centrifugation at 1000g for 10 minutes. The pellet was resuspended in 150μl of lysis buffer (50mM Tris-HCl, pH8.0; 62mM EDTA; 2.5M LiCl; 4%v/v Triton X-100) and incubated at room temperature for five minutes. 150μl of phenol chloroform 1:1 was added and the tube was shaken by hand for five minutes. The phases were separated by centrifugation (12,000g, 5min) and the DNA was precipitated from the aqueous phase by adding 300μl of absolute ethanol. The mixture was gently swirled for five seconds and incubated for five minutes at room temperature. The DNA was collected by centrifugation (12000g, 10min), washed once with 70% ethanol, dried under vacuum and resuspended in 100μl TrisHCl 10mM, EDTA 1mM (TE) pH8.0 (Medina-Acosta & Cross, 1993).

2.3.1.4. Phenol chloroform extraction

$5x10^8$ parasites were harvested by centrifugation (1000g, 4°C, 10min) washed once in Locke's solution, resuspended in 2ml lysis buffer (50mM NaCl; 50mM EDTA; 1% SDS; 50mM Tris-HCl, pH8.0; proteinase K 100μg/ml) and incubated for 16 hours at 37°C. The lysate was successively extracted with equal volumes of Tris-HCl pH7.6 equilibrated phenol; 1:1 phenol:chloroform and finally chloroform. The DNA was precipitated by addition of sodium acetate pH7.5 to a final concentration of 300mM and 2.5 volumes of absolute ethanol. The precipitate was removed with a plastic loop when practicable (Micheli et al., 1994), rinsed in 70% ethanol and resuspended in 500μl of TE. (Kelly, 1993)

2.3.1.5. DNA preparation for pulsed field gel electrophoresis

10^9 parasites were washed once in Locke's solution, resuspended in 1.5mls of L buffer (0.1M EDTA; 0.01M Tris-HCl pH7.6; 0.02M NaCl) and incubated at 40°C for 3minutes. The parasites were mixed with 1.5ml of 2% Seaplaque agarose (FMC) prepared with L buffer and cooled to 40°C. The warm parasite and agarose mixture was pipetted into moulds

placed on ice and allowed to cool for five minutes. The plugs were removed from the moulds into 10ml of lysis solution (L buffer containing 0.5% N-lauroylsarcosine and 0.5mg ml^{-1} of Proteinase K) and incubated at 55°C for 72 hours with a change of lysis solution after 36 hours. The plugs were transferred from the lysis solution into TE and stored at 4°C.

2.3.2. kDNA preparation

2.3.2.1. kDNA preparation by shearing genomic DNA

kDNA was prepared by the method of Gonçalves *et al.* (Gonçalves et al., 1984) with minor modifications. 10^9 parasites were washed once in Locke's solution and digested in 1ml lysis buffer (50mM NaCl; 50mM EDTA; 1% SDS; 50mM Tris-HCl, pH8.0; proteinase K 100μg ml^{-1}) at 55°C until the mixture cleared. The lysate was sheared by repeated passage through a 19g needle until the mixture was no longer viscous. The kDNA networks were collected by centrifugation at 100,000g, 1hr, 4°C in a Sorvall T60 rotor at 29,000 rpm. The supernatant was discarded and the pellet containing the kDNA networks was resuspended in TE, and recentrifuged under the same conditions. The pellet was resuspended in 500μl of TE, extracted twice with phenol:chloroform (1:1) and once with chloroform:isoamyl alcohol 12:1. The aqueous phase was precipitated with three volumes of absolute ethanol in the presence of 300mM sodium acetate. The networks were collected by centrifugation (12,000g 10min, 4°C).

2.3.2.2. kDNA preparation by centrifugation through sucrose

kDNA was prepared by a modification of the method of Chance *et al.* (1974). 10^9 parasites were washed once in Locke's solution and digested in 1ml lysis buffer (50mM NaCl; 50mM EDTA; 1% SDS; 50mM Tris-HCl, pH8.0; proteinase K 100μg ml^{-1}) at 55°C until the mixture cleared. The lysate was extracted once with an equal volume of phenol pH7.6, twice with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform:isoamyl alcohol (12:1). The aqueous phase was transferred to a 3ml ultracentrifuge tube, an equal volume of 40% sucrose in TE was added and the tube was filled to the brim with 20% sucrose in $T_{10}E_1$. The kDNA networks were collected by centrifugation at 100,000g, 1hr, 4°C in a Sorvall T60 rotor at 29,000 rpm. Alternatively the networks were collected by centrifugation in a 15ml tube at 15,000g, 1hr, 4°C in a Sorvall HB-4 rotor at 10,000 rpm. The supernatant was removed with a pasteur pipette and genomic DNA was precipitated from the supernatant with 3 volumes of absolute ethanol and 300mM sodium acetate. The tube containing the kDNA pellet was filled with 70% ethanol and

recentrifuged under the same conditions. The pellet was dried under vacuum and resuspended in 500μl of TE.

2.3.3. DNA extraction from gels

Bands excised from agarose gels were placed in a previously prepared 500μl Eppendorf tube. The bottom of the tube had been pierced with a 25swg needle and the hole overlaid with a plug of sterile glass wool. The 500μl Eppendorf containing the agarose plug was placed in a 1.5ml Eppendorf and spun (5sec) in a microfuge. The liquid collected from the 1.5ml tube was used directly in the PCR or labelled by random priming without further purification for use as a DNA probe.

2.3.4. RNA preparation

$5x10^8$ parasites were centrifuged (1000g, 10min) resuspended in 1ml Locke's solution, centrifuged (1000g, 10min) and the pellet was stored at -70°C until required. The tube containing the pellet was retrieved from the freezer into liquid nitrogen until use. The pellet was resuspended in 0.5ml of RNA extraction buffer (100mM NaCl; 50mM EDTA; 1%SDS) at 80°C. 0.5ml of acid phenol (pH4.3) (Sigma) at 80°C was added, the mixture was vortexed and centrifuged (3min, 12,000g, RT). The hot phenol extraction was repeated and followed by two phenol:chloroform extractions at room temperature. RNA was precipitated by incubation with 0.5ml *iso*-propanol for one hour at room temperature. The RNA was recovered by centrifugation (20min, 12,000g, RT), resuspended in 300μl RNAse free water (Sigma) and precipitated by incubation for 16 hours with lithium chloride (2.5M). The RNA was again recovered by centrifugation (20min, 12,000g, 4°C) and resuspended in 50μl RNAse free water with 50units of RNAsin (Promega). RNA was visualised by electrophoresis (1.5% agarose, 1xTAE (40mTris-acetate; 1mM EDTA; pH7.7), 0.1% SDS) and photographed with a Polaroid MP-4 camera and Polaroid P55 film.

2.4. POLYMERASE CHAIN REACTION

2.4.1. RAPD

A master mix was prepared (20mM $(NH_4)_2SO_4$; 75mM Tris-HCl pH9.0; 0.01% Tween; 2.0mM $MgCl_2$; 200μM dNTPs; 40Uml^{-1} *Taq* polymerase; 1μM primer) and vortexed (2sec). 24μl aliquots were pipetted into 500μl Eppendorf tubes and one drop of sterile mineral oil was added to each tube. 1μl of DNA solution diluted 1:10 in PCR water (ddH_2O, sterilised and 0.2μm filtered) was pipetted onto the side wall of each tube, the tubes were briefly centrifuged and placed in a thermal cycler (Perkin Elmer or Hybaid, Omnigene) and cycled through the programme indicated in table 2.4 according to the primers used. Primers used for RAPD are shown in table 2.4. Primers with the prefix AB1 are from the Advanced Biotechnologies (Leatherhead, UK) random primer kit 1. Primers with the prefix UBC are from the University of British Columbia random primer kit 3 (Nucleic Acid - Protein Service (NAPS) Unit, Biotechnology Laboratory, Room 237, Wesbrook Building, 6174 University Boulevard, University of British Columbia, Vancouver, V6T 1Z3, Canada).

2.4.2. PCR for specific products

PCR reactions for the amplification of specific sequences were prepared in the same way as RAPD reactions (Para. 2.4.1). Specific PCR primers and amplification conditions are shown in table 2.5. Primers were used at an individual concentration of 0.5μM unless otherwise indicated and *Taq* polymerase was used at a concentration of 20U ml^{-1} with primers 13Z and LiR.

2.4.3. PCR of kinetoplast DNA

Leishmania minicircles are composed of a conserved region approximately 120bp in length and a variable region 600-700bp in length. Part of the conserved region of *L. braziliensis* kinetoplast minicircles were amplified using primers MP1L and MP3H (Lopez *et al.* 1993) and the conserved region of the minicircles of all trypanosomatids was amplified using primers 13A and 13B (Rodgers *et al.* 1990). The whole minicircles of *L. braziliensis* species was amplified using primers LU and LB supplied by D. Barker. Whole minicircles of *L. braziliensis* and *L. mexicana* species were amplified using primers 13Y and 13Z (Rodgers *et al.* 1990). The sequence and amplification cycle of these primers is indicated in Table 2.5.

The minicircle variable region of a range of trypanosomatids were amplified with primer 13Z and primer LiR. Primer LiR was designed using *L. infantum* sequence data deposited

in Genbank. LiR is a 15mer, designed to match bases 111-125 of seven *L. infantum* minicircles sequences deposited by D. Barker at Genbank (accession numbers Z35501; Z35292; Z35269; Z35270; Z35272; Z35273; Z35274), the 10 bases at the 3' end were designed to match the conserved sequence block 1 (CSB1) (Ray, 1989). The primer 13Z was predicted to have an annealing temperature of 59.2°C by the program "Primer" (Lincoln, SE., Daly, MJ., Lander, ES. MIT Centre for Genome Research and Whitehead Institute for Biomedical Research, Nine Cambridge Centre, Cambridge, Mass. 02142 USA). The primer LiR was designed to have a similar annealing temperature by adjusting the length until the same program predicted that the annealing temperature would be 60.0°C.

2.4.4. PCR of the small subunit rRNA gene

As RAPD, isoenzymes and schizodemes are only suitable for examining the relationships within a genus, the SSU rRNA gene was examined for sequences that would be useful for identifying or classifying unknown trypanosomatids. The sequence data available on trypanosomatid SSU rRNA genes was reviewed and an 882bp sequence was identified for which sequence data was available for twenty two species of trypanosomatid, including fifteen species of *Leishmania*. This region covers the most heterologous region of the SSU RNA gene and includes part of the V4 and all of the V5 variable regions from helix E21-8 to helix 28 in the *Saccharomyces cerevisiae* secondary structure model described by De Rijk *et al.* (1992) (Fig 2.2). The PILEUP program in the GCG package (Genetics Computer Group, Wisconcin) was used to align the following sequences (Genbank accession numbers in brackets):- *Crithidia fasciculata* (X03450);*Leptomonas samuelpessoai* (X53914); *Leishmania donovani* (X07773); *Leishmania amazonensis* (X53912); *Endotrypanum monterogeii* (X53911); *Trypanosoma cruzi* (M31432); *Crithidia oncopelti* (L29264); *Trypanosoma brucei* (M12676); *Bodo caudatus* (X53910). In the PILEUP alignment of the 882bp *Leishmania* sequences with other trypanosomatid sequences, the 882bp region extended over 1006bp of the aligned sequences. Within this region three variable regions flanked by conserved regions were identified. The DNAPARS programme within the PHYLIP package (J. Felsenstein, Dept. of Genetics SK-50, University of Washington, Seattle, Washington 98195, USA.) was used to produce phylogenies of various subregions until a 677bp region was found which generated a dendrogram with a topology identical to one published for the whole gene (Marché et al., 1995). The average length of individual sequences within this region was 561bp with the exception of *T. cruzi* which had a 71bp insertion.

Primers for this region were designed using the PRIMER programme to meet the specifications of the University of Liverpool Department of Genetics sequencing service for automated sequencing, in particular an annealing temperature between 50-55°C is recommended. Primers SSUF and SSUR (Table 2.5), which have theoretical annealing temperatures of 55°C and 54.3°C respectively, were recommended by the programme.

Primers WSSUF and WSSUR for the complete SSU rRNA gene were also designed by identifying conserved regions at the 5' and 3' ends of the gene and using PRIMER to examine candidate primers. Primers WSSUF and WSSUR (Table 2.5) have theoretical annealing temperatures of 55.4°C and 54.8°C respectively.

The large subunit (LSU) rRNA gene was also examined as it was expected to show larger differences between genera and so provide more information about the relationship between closely related genera. However average distances computed with DNADIST in the PHYLIP package were smaller than those for the SSU gene. As there is also less sequence data available for the LSU gene it was decided not to pursue this avenue.

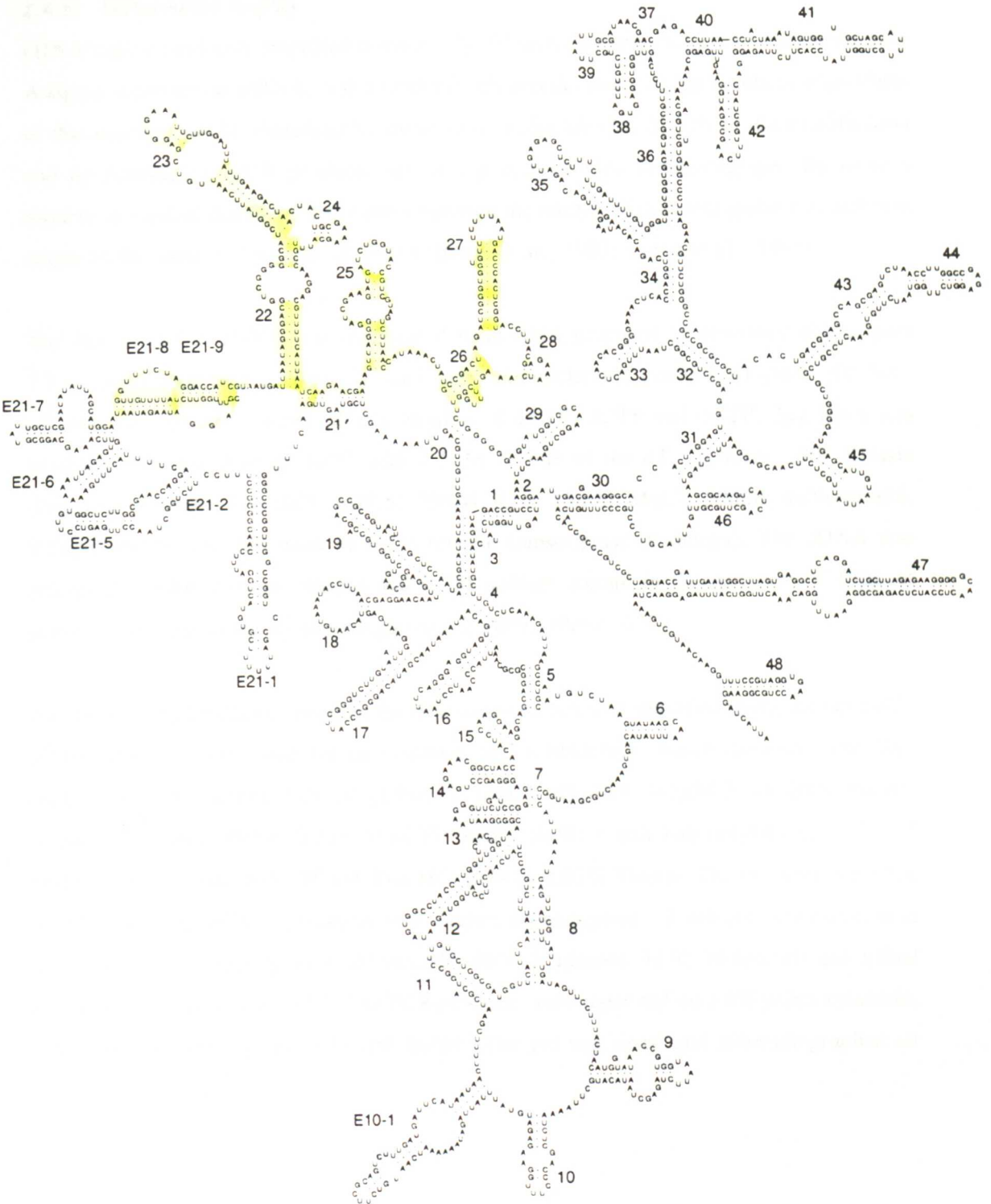


Figure 2.2 Model of the secondary structure of *Sacharomyces cerevisiae* SSU rRNA from de Rijk et al., (1992). The region amplified by primers SSUF and SSUR is highlighted.

2.4.5. Differential display

cDNA can be randomly amplified using a poly dT anchor primer, which anneals to the poly A signal sequence on mRNA, and a randomly chosen decamer. Large numbers of products of this reaction can be visualised by using isotopically labelled dNTPs in the amplification and by running the PCR products out on a polyacrylamide sequencing gel. By using a number of random decamers differences between the mRNA of different isolates or different stages of the same isolate may be found (Bauer et al., 1993; Liang et al., 1993).

The first strand of cDNA was transcribed from RNA prepared as described above (para 2.3.4) using four sets of poly dT anchored oligonucleotide primers $dT_{12}MA$, $dT_{12}MC$, $dT_{12}MG$ and $dT_{12}MT$, where M is a mixture of dATP, dCTP and dGTP. 2μg RNA was incubated for one hour at 40°C with 2.5μM of one of the dT_{12} primers; 20U RNasin (Promega), 50mM Tris-HCl, pH7.5; 75mM KCl; 15mM $MgCl_2$ 50mM dithiothreitol; 0.5mM dNTPs and 300 units M-MLV reverse transcriptase (Promega). The cDNA was precipitated with absolute ethanol and 0.3M sodium acetate in the presence of 10μg of glycogen and recovered by centrifugation (12,000g, 10min, 4°C).

For the differential display reaction the first strand cDNA was amplified using the same dT_{12} primer that had been used for its synthesis and a randomly chosen decamer. The 20μl reaction mixture consisted of 2μl cDNA; 2.5μM 3' $dT_{12}MV$; 0.5μM 5' random decamer primer; 2μM each dNTP; 0.2μl ^{32}P-dCTP 3000 Ci/μM; 1 unit *Taq* polymerase, 1.25mM $MgCl_2$; 20mM $(NH_4)_2SO_4$; 75mM Tris-HCl pH9.0; 0.01% Tween. The mixtures were run on the following PCR programme in a Techne thermocycler:- 3 minutes denaturation at 95°C then forty cycles of 94°C 30 seconds; 40°C 2 minutes; 72°C 30 seconds and a final extension of 5 minutes at 72°C. The PCR products were separated on a 6% polyacrylamide, 8.3M urea sequencing gel in 1xTBE buffer. The gel was dried and autoradiographed on Kodak XAR-5 film.

Primer	Sequence	Amplification*		Primer	Sequence	
M13	GTAAAACGACGGCCAGT	++	1	UBC-301	CGGTGGCGAA	2
AB1-01	**GTTTCGCTCC**	++	2	UBC-302	CGGCCCACGT	2
AB1-02	TGATCCCTGG	-	2	UBC-303	GCGGGAGACC	2
AB1-03	**CATCCCCCTG**	++	2	UBC-304	AGTCCTCGCC	2
AB1-04	**GGACTGGAGT**	++	2	UBC-305	GCTGGTACCC	2
AB1-05	TGCGCCCTTC	-	2	UBC-306	GTCCTCGTAG	2
AB1-06	**TGCTCTGCCC**	++	2	UBC-307	CGCATTTGCA	2
AB1-07	GGTGACGCAG	++	2	UBC-308	AGCGGCTAGG	2
AB1-08	GTCCACACGG	-	2	UBC-309	ACATCCTGCG	2
AB1-09	TGGGGGACTC	++	2	UBC-310	GAGCCAGAAG	2
AB1-10	CTGCTGGGAC	++	2	UBC-311	GGTAACCGTA	2
AB1-12	**CCTTGACGCA**	++	2	UBC-312	ACGGCGTCAC	2
AB1-13	**TTCCCCCGCT**	++	2	UBC-313	ACGGCAGTGG	2
AB1-14	TTCCCCCGCT	++	2	UBC-314	ACTTCCTCCA	2
AB1-15	**GGAGGGTGTT**	++	2	UBC-315	GGTCTCCTAG	2
AB1-16	TTTGCCCGGA	+	2	UBC-316	CCTCACCTGT	2
AB1-17	AGGGAACGAG	-	2	UBC-317	CTAGGGGCTG	2
AB1-18	**CCACAGCAGT**	++	2	UBC-318	CGGAGAGCGA	2
AB1-19	**ACCCCCGAAG**	++	2	UBC-319	GTGGCCGCGC	2
AB1-20	GGACCCTTAC	-	2	UBC-320	CCGGCATAGA	2
A1[a]	**CAGGCCCTTC**	++	2			
A4[a]	**AATCGGGCTG**	++	2			
A7[a]	**GAAACGGGTG**	++	2			
A8[a]	**GTGACGTAGG**	++	2			
3301[b]	TCGTAGCCAA	++	3			
ILO525[c]	GGACGTCGC	++	2			
UK1	GATATCAACCCC	++	2			
UK2	TGAGCTGGTGTA	++	2			
P1	TACAACGAGG					
P14	GATCAAGTCC					

Table 2.4 RAPD primers and amplification conditions

Thermal cycles: 1) 2 cycles of 94°C 2min; 40°C 5min; 72°C 5min; followed by 35 cycles of 94°C 30sec; 60°C 1min; 72°C 2min.
2) 35 cycles of 94°C 30 sec; 36°C 1min; 72°C 2min.
3) 30 cycles of 94°C 1min, 40°C 1.5min, 72°C 2min.

(* Quality of amplification: "-" none; "+" weak; "++" strong.)

Primers in bold were used to calculate Jaccard coefficients between four *L. (Viannia)* species' (Para 3.1.4).

References: [a] (Tibayrenc et al., 1993) [b](Neto et al., 1993) [c](Waitumbi & Murphy, 1993).

Primer	Sequence	Thermal cycles
13A[a]	GTGGGGGAGGGGCGTTCT	1
13B[a]	ATTTTACACCAACCCCCAGTT	1
13Y[a]	CGCCCCTCCCCCACGGG	1
13Z[a]	ACTGGGGGTTGGTGTAAAATAG	1
LiR	TCGCAGAACGCCCCT	1
MP3H[b]	GAACGGGGTTTCTGTATGC	2
MP1L[b]	TACTCGCCGCACTGCCTCTG	2
SSU561F	TGGGATAACAAAGGAGCA	3
SSU561R	CTGAGACTGTAACCTCAAAGC	3
WSSUF	GCTTGTTTCAAGGACTTAGCC	3
WSSUR	GAAATATCGGTGAACTTTCGG	3

Table 2.5 PCR primers and amplification conditions
Thermal cycles: **1)** 30 cycles of 94°C 30 sec; 54°C 90 sec.
2) 30 cycles of 94°C 30 sec; 50°C 90 sec.
3) 30 cycles of 94°C 30 sec; 54°C 1min; 72°C 2min.
References: [a] (Rodgers et al., 1990); [b] (Lopez et al., 1993).

2.5. ANALYSIS OF PCR PRODUCTS

2.5.1. Agarose gel electrophoresis

Loading buffer (20% w/v Ficoll; 100mM EDTA; Orange G) was added to the PCR products (20%v/v) and loaded onto agarose gels in 1xTAE with 200μ gl^{-1} of ethidium bromide. For analysis of RAPD products 15μl of PCR product was loaded onto a 1.5% agarose gel. For analysis of specific PCR products 5μl was loaded onto a 1% agarose gel. A mixture of *BglI* and *HinfII* digests of pBR328 (Boehringer Mannheim) were used as molecular weight markers for size determination (Molecular weights 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 234, 220, 154, 154bp). The gels were run at 4-8V cm^{-1}, visualised under UV light and photographed with Ilford FP4 film.

2.5.2. Polyacrylamide gel electrophoresis

5% polyacrylamide gels were prepared by diluting 30% 29:1 acrylamide: bisacrylamide stock solution (Severn biochemicals) with 0.5xTBE (0.045M Tris-borate; 0.001M EDTA pH8.2) and adding 10% ammonium persulphate (10μl ml^{-1}) and 1μl ml^{-1} of TEMED. PCR products were mixed 1:1 with loading buffer (20% Ficoll; 100mM EDTA; Orange G) and 5μl of the mixture was loaded onto the gel. Gels were run in 0.5xTBE under the conditions indicated in the figures and silver stained by the method of Bassam *et al.* (1991). Gels were fixed in 10% acetic acid for twenty minutes, washed 3x3 minutes in ddH_2O, incubated with shaking in 0.15% $AgNO_3$, 0.15% formalin for 30 minutes, rinsed 2x5 seconds in ddH_2O,

developed in 3% Na_2CO_3, 0.15% formalin, 2mg ml^{-1} $Na_2S_2O_3.5H_2O$ and fixed in 10% acetic acid using sufficient volumes to cover the gel.

2.5.3. Restriction endonuclease digestion of PCR products

For analysis on agarose gels, 10μl of PCR product was diluted 1:1 with 2x restriction enzyme buffer and digested for 16 hours with 1 unit of enzyme under the conditions recommended by the enzyme supplier (Boehringer). When products were to be analyzed on polyacrylamide gels 3-5μl of PCR product was first run on a 1% agarose gel and the concentration was estimated visually by comparison with size markers. 100ng of PCR product was made up to 20μl final volume with water, 2μl of 10X enzyme buffer and 1 unit of enzyme and incubated for 16 hours at 37°C.

2.5.4. Sequencing of PCR products

A 5μl aliquot of PCR product to be sequenced was checked for purity by agarose gel electrophoresis. An image of the gel was captured onto disk using a UVP Image Store 5000 version 7.12 and the DNA concentration was estimated by comparison with size markers using a BioImage Whole band Analyser version 3.2, running on a Sun SparcV workstation. Primers and buffer were removed from the remainder of the PCR product by passage through an S400 spin column (Pharmacia). The purified PCR product was ethanol precipitated and resuspended in water to a final concentration of 100ng μl^{-1}. The sequence of the Guatemalan leptomonad was determined by the University of Liverpool Department of Genetics using the PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit on a 373A DNA sequencing system (Applied Biosystems). The sequence of *L. hertigi* was determined at the Liverpool School of Tropical Medicine on a 377 DNA sequencing system (Applied Biosystems) using the same chemistry.

2.6. PULSED FIELD GEL ELECTROPHORESIS (PFGE)

Very large DNA molecules above 50kb will not enter or resolve on agarose gels under conventional conditions. Schwartz and Cantor (1984) used alternating electric fields to resolve entire yeast chromosomes. PFGE made it possible to examine the karyotype of *Leishmania* for the first time as the chromosomes of *Leishmania* do not condense at mitosis. The karyotype of *Leishmania* has been found to be very variable and PFGE provides the highest resolution method available for the study of *Leishmania* populations (Bastien et al., 1992b).

Blocks prepared as described in paragraph 2.3.1.5 were cut to size and inserted into wells in agarose gels and run in a Bio Rad contour clamped homogenous electric field (CHEF) apparatus in 0.5xTBE buffer under the conditions stated in the figures. Gels were stained with ethidium bromide (400μg l^{-1}) for 30 minutes and destained in water.

2.7. PREPARING AND PROBING SOUTHERN AND DOT BLOTS

2.7.1. Dot Blots

Samples were diluted to the appropriate concentration in 6xSSC (standard saline citrate) and applied to a Hybond N^+ (Amersham) nylon filter through a 'Minifold' (Schleicher & Schnell) apparatus. The filter was then transferred successively to Whatmans 3MM paper soaked with 1) denaturing solution 5min (0.5M NaOH, 1.5M NaCl); 2) neutralising solution 5min (3M NaCl, Tris-HCl pH5.6); 3) 2xSSC 5 min (1 x SSC is 0.15M NaCl, 0.015M trisodium citrate). The filter was air dried and baked at 80°C for 2 hours.

2.7.2. Southern Blotting

Gels for blotting were placed in denaturing solution (0.5M NaOH, 1.5M NaCl) for 1 hour with shaking and then changed to neutralising solution (NaCl 3M, Tris-HCl pH5.6) for 2 x 30 minutes. The blot consisted of a pad of paper towels overlaid with two sheets of Whatmans 3M and soaked with 20xSSC, the gel was placed on this and a nylon membrane (Amersham N+) soaked in 2xSSC was laid on top. The membrane was overlaid with two sheets of Whatmans 3M soaked in 2xSSC and one dry sheet, on top was placed a pad of dry paper towels and the whole stack was weighted with approximately 1kg distributed over a glass plate. The blot was left for 16 hours at room temperature. The membrane was removed from the blot and baked at 80°C for two hours.

2.7.3. Alkaline Southern Blotting

Pulsed field gels were placed on a transilluminator for 15 minutes to degrade the DNA and transferred into 0.25M HCl for 15 minutes. The gel was then washed 3x3 minutes in water and the DNA was denatured by incubation in alkali transfer solution (0.25M NaOH; 1.5M NaCl) 2x30 minutes. The gel was finally rinsed in water and placed in 20xSSC (0.3M sodium citrate; 3M NaCl) whilst the blot was prepared as described in paragraph 2.7.2.

2.7.4. Labelling probes

DNA probes were labelled with [α-^{32}P]dCTP to $\geq 10^9$cpm by the random priming method of Feinberg and Vogelstein (1983) using the Boehringer random priming kit. PCR products that were to be used as probes were separated from dNTPs by passage through a S400 spin column (Pharmacia), other probes were used as supplied. 20-50ng of DNA in a 11μl volume was boiled for ten minutes then cooled on ice. After a brief centrifugation 2μl of random hexanucleotide mix, 5mmoles each of dATP, dTTP and dGTP, 20μCi dCTP^{32}, and 2 units of Klenow enzyme were added. The mixture was briefly centrifuged and incubated for 30 minutes at 37°C.

Oligonucleotides were labelled using a terminal transferase kit (Boehringer) according to the directions of the manufacturer.

Labelled probes were separated from free nucleotides by passage through a sephadex G50 column. The activity of fractions collected from the column were measured in a scintillation counter (LKB). Fractions corresponding to the first peak of radioactivity were pooled, boiled for 5 minutes and cooled on ice.

2.7.5. Probing Southern Blots

Filters from Southern blots were soaked in 2xSSC for 10 minutes (RT) and prehybridised in BEPS hybridisation buffer (0.2ml/cm^2) (1%BSA, 1mM EDTA, 0.5M Na_2HPO_4 pH7.4, 7%w/v SDS) for 16 hours at the temperature indicated in the figures. The labelled probe was added and the filter incubated for at least four hours. The filter was then washed in 3xSSC, 0.1% SDS for 20 minutes at RT. The stringency of washes was increased by reducing the salt concentration and raising the temperature.

Radioactive filters were autoradiographed on pre flashed X-ray film (Fuji). The films were exposed for a suitable period at -70°C and developed for 2 minutes in Phenisol high contrast developer (Ilford), stopped for 1 minute in 5% acetic acid, fixed for 1 minute in fixer (Ilford), washed in water and air dried.

Filters that were to be reprobed were stripped in 0.1M NaOH (15min), rinsed briefly in water and neutralised in 0.5M Tris-HCl pH 7.0 (20min) and air dried.

2.8. ISOENZYME ELECTROPHORESIS

Starch gels were prepared with 11g of electra starch in 125ml of TBE. The mixture was heated until it became translucent, degassed before pouring and left for 16 hours at 4°C. 10^8 parasites were washed once in Locke's solution and the pellet was frozen at -70°C. The pellet was lysed by grinding in 100μl of 5% Triton X-100 in a ground glass homogenizer. 4mm squares of cellulose acetate membrane were soaked in the homogenate and inserted into slots in the gel. The gels were run at 160V, 90mA, at 4°C. Developing solutions were mixed with an equal volume of 2% agar and poured into a mould laid over the gel.

Cellulose acetate membranes were pre-soaked in running buffer and parasite homogenate, prepared as for starch gel electrophoresis, were applied to the membrane with an applicator.

Three enzyme systems were used, the developing solutions for which are as follows:-

1. Nucleoside hydrolase. (NH_i) E.C.2.4.2.-. (E.C.3.2.2.1. Le Blancq et al., 1987) also known as Nucleoside Purine Phosphorylase (NP) E.C.2.4.2.1. (Rioux et al., 1990).

Enzyme reaction:- NH_i

Inosine + orthophosphate → hypoxanthine + ribose-1-phosphate

Developer solution: 10ml 0.2M Tris-HCl pH 7.0; 10mg inosine; 0.13IU xanthine oxidase; 5mg MTT; 1mg PMS; 8ml H_2O; 10ml 2% agar.

2. Glucose-6-phosphate dehydrogenase. (G6PD) E.C.1.1.1.49.

Enzyme reaction:- G6PD

D-glucose-6-phosphate + NADP → 6-phosphogluconate + NADPH

Developer solution: 10ml 0.2M Tris-HCl pH8.0; 20mg glucose-6-phosphate; 10mg EDTA; 1ml 1%w/v NADP; 1ml 1%w/v NBT; 1ml 1%w/v PMS; 5ml 2%w/v agar.

3. Glucose phosphate-isomerase (GPI) E.C.5.3.1.9.

Enzyme reaction:-

$$\text{D-fructose-6-phosphate} \xrightarrow{\text{GPI}} \text{D-glucose-6-phosphate}$$

$$\text{D-glucose-6-phosphate} + \text{NAD} \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NADH}$$

$$\text{NADH} + \text{NBT} + \text{PMS} \rightarrow \text{formazan}$$

Developer solution: 10ml 0.2M Tris-HCl pH8.0; 10mg fructose-6-phosphate; 1ml 0.5M $MgCl_2$; 0.5ml 1%w/v NADP; 1ml 1%w/v NBT; 1ml 1%w/v NAD; 5ml 2%w/v agar; glucose-6-phosphate dehydrogenase 50μl; 0.5ml 1%w/v PMS; 5ml 2% agar.

2.9. ANALYSIS OF FINGERPRINT AND SEQUENCE DATA

2.9.1. Analysis of RAPD products

RAPD data was compared using similarity and distance matrices as described in paragraph 1.13.1.1 using the following similarity and distance coefficients.

The Jaccard Similarity Coefficient (Cibulskis et al., 1986; Cupolillo et al., 1994; Jaccard, 1908):-

$$S_J = \frac{A}{A + B + C}$$

The Jaccard Distance coefficient (Thomaz Soccol, 1993; Tibayrenc et al., 1993):-.

$$S_J = 1 - \frac{A}{A + 2(B + C)}$$

The Dice similarity coefficient also known as Nei and Li's coefficient (Beverley et al., 1987; Nei & Li, 1979; Sneath & Sokal, 1973):-

$$S_D = \frac{2A}{2A + B + C}$$

Matrices of the products of these equations were compiled for all pairs of OTUs. Phenograms were prepared from the matrices by inspection.

Figure 2.3 Dice Leraas diagram, the vertical line indicates the mean; the horizontal line joins the extreme values of the range; the black rectangles the standard deviation; and the white rectangles indicate twice the standard error (Fig. 2.3). Where the white rectangles do not overlap the means are significantly different ($P < 0.05$). In the present case n (number of primers) was less than 30 and so the white rectangles indicate the confidence limits ($\pm ts/\sqrt{n}$)($P < 0.05$) and not twice the standard error.

Dice Leraas diagrams (Gardener et al., 1976) were used to show the range, mean, standard deviation and confidence limits of the values of the similarity or distance coefficient generated by each primer used for each pair of OTUs under study (Fig. 2.3).

The means of the Jaccard coefficients for different GC content primers were compared using equations 22 and 21 (Bailey, 1981), which are as follows:

(22) $s^2 = (1/(n_1+n_2-2))(\Sigma_1x^2 - (\Sigma_1x)^2/n_1 + \Sigma_2x^2 - (\Sigma_2x)^2/n_2)$

(21) $t = (\bar{x}_1 - \bar{x}_2)(s\sqrt{(1/n_1 + 1/n_2)})$

2.9.2. Analysis of RFLPs of SSU rDNA

Restriction fragments of PCR amplified SSU rDNA were scored as present or absent. The presence/absence data was then analysed in two different ways. Firstly the presence/absence data was entered directly into the MIX programme in PHYLIP which uses Wagner parsimony. For data with two states Wagner parsimony is essentially the same as the method of Fitch and Margoliash (Para. 1.13.2). Secondly the similarity coefficient of Nei and Li was calculated for each pair and entered into a distance matrix. A dendrogram was then compiled from the distance matrix using the FITCH programme in PHYLIP which also uses the method of Fitch and Margoliash.

2.9.3. Analysis of sequence data.

DNA sequences were aligned using the PILEUP programme in GCG and the alignment was optimised by hand in the GCG LINEUP sequence editor. The aligned sequences were analyzed using the DNAPARS programme in PHYLIP to produce a phylogeny directly from the sequence data or DNADIST was used to produce a distance matrix from the aligned sequences which could then be converted to a phylogeny using FITCH.

3. RAPD FOR THE IDENTIFICATION AND CLASSIFICATION OF *LEISHMANIA*

3.1. RESULTS

3.1.1. DNA Preparation

DNA prepared by three different methods:- crude lysates, lithium chloride mini preparations and standard phenol chloroform preparations, were tested for differences in the RAPD fingerprints that they might generate. Crude lysates were proteinase K treated, Tween-20 digested washed parasites, incubated at 55°C for 1 hour (Para. 2.3.1.2). The lithium chloride mini-prep involved a five minute lysis of 10^7 parasites in 2.5M LiCl and Triton-X100 followed by phenol chloroform extraction (Para. 2.3.1.3). The standard phenol chloroform extraction was performed on parasites lysed in proteinase K and 1% SDS (Para. 2.3.1.4). Figure 3.1a and 3.1b show comparisons of phenol chloroform extracts with crude lysates and figure 3.1c shows fingerprints of DNA prepared by the lithium chloride mini-prep. Additionally two methods of recovery of the ethanol precipitated DNA were compared, the DNA was either looped out or recovered by centrifugation (Fig. 3.1a). In Figure 3.1a different preparations of the majority of parasite strains yielded the same fingerprints. The exception was M379 in which doublets at about 2200 and 2000 bp in the phenol chloroform prepared DNA appeared as singlets in the crude lysate and doublets at about 1200 and 1000bp in the crude lysate appeared as singlets in the phenol chloroform extract. The fingerprints produced from crude lysates stored at 4°C became weaker or disappeared within one week to three months.

3.1.2. Reproducibility

All DNA preparation methods gave sufficiently reproducible results for identification of parasite species, although occasionally aberrant patterns were seen such as U139 in figure 3.1b and HN458 in figure 3.1c. Anomalous fingerprints such as that of HN458 (Fig. 3.1c) were unlike all reference strains used in the same reaction. Retesting of a fresh DNA preparation of HN458 produced the expected *L. mexicana* fingerprint (Fig. 3.1a). Reactions sometimes failed to produce any products, such as G519 in figure 3.1b; HN410 in figure 3.1c; LV114 in figure 3.1d.

RAPD was also sufficiently reproducible for identification purposes when the reaction was conducted by different workers at different times. A RAPD on unknown parasites from Pakistan with *L. major*, *L. tropica* and *L. infantum* reference strains generated fingerprints

that were like published fingerprints of the same species (Fig. 3.1d & Motazedian *et al.*, In Press)

Although RAPD could reliably identify *Leishmania* species the fingerprint patterns could vary. This variation took two principal forms, either higher or lower molecular weight products could vary in intensity or disappear altogether, or the whole fingerprint pattern could vary in intensity, although the banding patterns were identical in so far as they were visible. This is illustrated by results shown in chapter 5, in figure 5.1c *L. infantum* LEM188 produced lower molecular weight products with primer AB3 and in figure 5.1b LV639 and LV640 produced fingerprints of different intensities. In cases of variable intensity the fingerprints had to be interpreted with care or the reaction repeated. Although the intensity might vary, all the products visible in the weaker reactions were also present in the stronger reaction but not *vice versa*. When repeating weak or failed reactions a new crude lysate was prepared or the lysate from the previous experiment was first passed through a Sephadex G-50 spin column prior to use.

Not all primers amplified template DNA even under identical conditions for example primer AB2 failed to amplify any of the samples tested (Fig. 5.1c), and some only produced very few (< 5) products. All primers that did amplify DNA produced different fingerprints as can be seen in figure 5.1c in which the same four strains were amplified with four different primers.

Bands were sometimes seen in the negative control (Fig. 3.1a & 3.1b), these were usually quite unlike the bands seen in the samples containing DNA and were disregarded.

3.1.3. RAPD For identification of *Leishmania* species

Intraspecific variation was slight and strains of the same species isolated at different places and times usually gave identical fingerprints (Fig. 3.1c & 5.1a). Although some variation was sometimes seen (Fig. 3.1d & 5.2a), this was not sufficient to hinder identification.

Fingerprints of members of species complexes varied from 0-100% homology (Appendix 3.1). Most of the variation was in the weaker bands and only a minority of primers tested would produce strong diagnostic bands in each of a given pair of species. Twenty eight primers were surveyed to find ones suitable for discriminating between four significant

human pathogens in the *L. (Viannia)* complex. No single primer was found that would generate a primary diagnostic band in all of these species but primers were found that were suitable for discriminating between all possible pairs of these species (Fig. 3.2a & Table 3.1).

Gross differences were found between members of different species complexes with all primers (Fig 3.2b & 3.1d).

L. br	-			
L. pa	AB104	-		
L. gu	AB118	AB109	-	
L. pe	A8	AB118	AB113	-
	L.br	L.pa	L.gu	L.pe

Table 3.1 RAPD-PCR primers that separate pairs of species of *L. Viannia*. The primers that are indicated against pairs of species generate at least one major unique product in each member of the pair. L.br *L. braziliensis*; L.pa *L. panamensis*; L.gu *L. guyanensis*; L.pe *L. peruviana*.

3.1.4. RAPD for the classification of *Leishmania (Viannia)* species

RAPD was used to prepare a classification of *L. braziliensis*, *L. guyanensis*, *L. panamensis* and *L. peruviana*. Fingerprints of the 13 primers shown in bold type in table 2.4 yielded reproducible multiple banding patterns with *L. (Viannia)* species and were used for numerical analysis. Data for each RAPD primer was pooled to give a single Jaccard distance for each pair of species. Figure 3.3a shows a dendrogram compiled from the pooled data by inspection. The four *L. (Viannia)* species tested form two pairs in the dendrogram. *L. braziliensis* and *L.peruviana* had a Jaccard distance of 0.50 and *L. guyanensis* and *L. panamensis* had a distance of 0.53. All other pairwise comparisons had Jaccard distances of between 0.79 and 0.74.

Results from each RAPD primer were also treated separately. Using the data in this way makes it possible to quantify the variability of the results. Jaccard distances were calculated for each pair of species for each primer. The range, means, standard deviation and

confidence limits of the Jaccard distances were found for each pair of species (Appendix 3.1) and displayed in a Dice Leraas diagram (Fig 3.3b). The means of the Jaccard distances calculated for each pair of primers were not significantly different from the distance calculated from the pooled data ($P<0.01$). The Dice Leraas diagram in figure 3.3b shows that members of the pairs of species *L. guyanensis* and *L. panamensis* on the one hand and *L. braziliensis* and *L.peruviana* are significantly more closely related to the other member of the pair than to any other species ($P<0.05$).

Jaccard similarity coefficients were also calculated from the same data and the same classification was obtained (Appendix 3.2).

3.1.5. The effect of primer GC content on RAPD fingerprints

The GC composition of the primers may have been significant for their discriminatory ability. Of the thirteen primers used to classify *L. (Viannia)* species (Para. 3.1.4), 5 were 70% GC and 8 were 60% GC. As the means of the Jaccard distance coefficient for the four pairs of species *L. guyanensis/L. peruviana*; *L. guyanensis/L. braziliensis*; *L. peruviana/L. panamensis*; *L. panamensis/L. braziliensis* were not significantly different (0.79-0.81 $\pm$ SE 0.09), the data for the four pairs of species was pooled and the mean and standard error ($P<0.10$) for the Jaccard distance coefficient was calculated for primers with 70% and 60% GC contents (Appendix 3.1). The mean Jaccard distance ($\pm$ the standard error) for these four pairs of species calculated from primers with 70% GC content was 0.834 ± 0.030 whilst that for 60% GC content primers was 0.785 ± 0.024. The *t*-test for the comparison of means of two small samples (unknown variances assumed equal) gives a value of t of 1.9873 which with 50 degrees of freedom on a two tailed test is significant at the 5.24% level.

3.1.6. RAPD for the identification of possible interspecific hybrids

Two isolates which had previously been typed as hybrids between *L. braziliensis* and *L. panamensis* by RFLP and isoenzymes by A. Belli, Ministry of Health, Nicaragua, (1994) were screened for hybrid RAPD fingerprints.

Primer 3301 produced a unique product at 1,500bp in *L. panamensis* and the hybrids (Fig 3.4a); the M13 primer produced a unique product in *L. braziliensis* and the hybrids at 2,550bp (Fig 3.4b). In order to generate a set of unique products for each of the parents

which would both appear in the hybrid under the same reaction conditions the two primers were combined. The banding pattern obtained was not a mixture of the patterns obtained with the individual primers, however two new products were present, one at 850bp in *L. braziliensis* and the hybrids and the other at 950bp in *L. panamensis* and the hybrids (Fig 3.4c).

Using primer AB104 the hybrid strains also showed products common to both supposed parent strains. A 460bp product was found in *L. panamensis* and the hybrids but not in *L. braziliensis*. A 1,400bp product was found in *L. braziliensis* and was also present in the hybrids though at lower intensity but was not present in *L. panamensis*. Strong bands at 870bp and 710bp in *L. panamensis* and *L. braziliensis* respectively were also present as strong bands in the hybrids but at much lower intensity in the other putative parent (Fig. 3.4d).

A mixture of *L. braziliensis* and *L. panamensis* DNA generated a set of products different from the two alone and different from the hybrids (Fig 3.4d).

A suspected *L. braziliensis/L. guyanensis* hybrid from Venezuela was screened with 25 primers (UBC1-UBC17, AB13, AB18, AB19, OPA1, OPA4, OPA7, OPA8, 3301) but no convincing hybrid patterns were detected (not shown).

3.2. DISCUSSION

3.2.1. Reproducibility

Although large numbers of caveats have been entered against RAPD, the technique was found to be a robust method for the identification of species, provided fingerprint patterns were interpreted with care (Black, 1993). None of the four methods tested for preparing template DNA was significantly more reliable or gave stronger fingerprints. Crude lysates were the simplest to prepare and were found to be very useful for screening large numbers of cloned parasites or identifying new isolates. However when more detailed or long term studies were planned parasites DNA was prepared by phenol chloroform extraction, as crude lysates tended to degrade over time. The lithium chloride miniprep was also found to be simple and satisfactory.

It has been proposed that recovering ethanol precipitated DNA by spooling gives stronger and more reproducible RAPD fingerprints than recovery by centrifugation (Micheli et al., 1994). This finding was not corroborated in the present study (Fig. 3.1a), however Micheli *et al.* proposed that spooled DNA performed better as spooling freed the DNA of ethanol precipitated contaminants such as low molecular weight DNA and RNA that would be collected by centrifugation. It may be true that the presence of low molecular weight contaminants are an important source of variability in RAPD. It was found in the present study that samples that gave weak fingerprints could sometimes be improved by passage through a Sephadex G-50 spin column. It was also found that excess template ($>$50ng) could lead to complete reaction failure whereas insufficient template gave rise to a weak or variable fingerprint, which is consistent with the concentration of inhibitory contaminants being significant for the outcome of the reaction.

The size range of products may vary with quantity and quality of the DNA and the absence of a particular product in a size range where no other products are visible should not be interpreted as evidence of the absence of the corresponding template DNA. For example in figure 3.1d the highest molecular weight bands in strain 02 are absent in strain 03, although in other respects these two strains appear identical. Nevertheless variation in the size range of products did not normally interfere with identifications, and fingerprint patterns were stable over DNA concentrations which varied by up to one order of magnitude. The size range of products may vary from batch to batch of DNA nevertheless we have produced identical banding patterns from both high quality DNA prepared for RFLPs and from crude

lysates indicating that RAPD is amenable to simple sample preparation. The results obtained for *Leishmania* from Central American foci are encouraging for the general applicability of the RAPD-PCR method to isolates from widely separated geographical regions since the patterns obtained from test strains were consistently similar to reference strains when compared in a single run.

The products occasionally seen in the negative controls were disregarded unless they showed some homology to the test reactions. Carlton *et al.* (1995) invariably found products in the negative controls and checked them for homology with test reactions by excising some of these products and using them as probes, no homology was found and the products were presumably due to the amplification of extraneous DNA. RAPD may be particularly sensitive to contamination of negative controls, as any contaminating DNA will produce a banding pattern under the low stringency reaction conditions. However, as RAPD is a competitive reaction, template DNA will be amplified preferentially unless gross contamination has occurred, particularly if the contaminating DNA is of lower complexity such as bacterial DNA which offers fewer potential binding sites (Williams, et al., 1993).

Although some run to run variation does occur particularly in the minor products and at the extremes of the product size range this is not a serious obstacle to the use of RAPD for the identification of *Leishmania* species as they can be identified using major products in the mid size range. However this variation may become an obstacle if RAPD is used for subtler purposes such as the detection of markers for particular traits or for linkage analysis.

3.2.2. RAPD for identification of *Leishmania* species

RAPD can identify parasites to the species or population level whereas PCR primers are only available for identification to the level of the species complex. However as RAPD can only be used on cultured parasites PCR is likely to be a more satisfactory method of diagnosis, as for this purpose it is usually sufficient to identify parasites to the species complex level. Consequently RAPD is unlikely to find extensive applications in diagnosis. However in epidemiology where an accurate determination of the *Leishmania* species may become increasingly important, RAPD has a number of advantages over competing methods. RAPD requires far fewer parasites and fewer reagents than isoenzymes, and it permits a much more positive identification than is possible with monoclonal antibodies which are only

available for the commoner species and may give anomalous results with unusual strains (Dr B. Arana, University del Valle, Guatemala, personal communication).

The high degree of intraspecific homology was unexpected as RAPD is generally described as a technique for population analysis of sexual species. It was found that the amount of variation detected was generally too low for RAPD to be used routinely for examining *Leishmania* populations, possibly because of the predominantly clonal structure of *Leishmania* populations. However others have used RAPD successfully for the study of *Leishmania* at various taxonomic levels.

Gomes *et al.* (1995) used RAPD to investigate intraspecific variation in *L. braziliensis* in Brazil and found that an average of 55% of bands were shared between strains, which is comparable to the amount of similarity we found between *L. braziliensis* and *L. peruviana* strains. However that group used a higher *Taq* concentration than used in the present study (80U ml^{-1} v 40U ml^{-1}), longer primers (17-36bp v 10-17bp), lower annealing temperatures (30° and 40° v 40° and 60° for equivalent primers) and analyzed their products on silver stained polyacrylamide gels, all of which would tend to increase the number of products, and particularly minor products, generated or detected. As the minor products tend to be the most polymorphic these differences in protocols would probably be sufficient to account for the greater number of polymorphisms detected in that study.

Tibayrenc *et al.* (1993) used RAPD to investigate the relationship between *Leishmania* complexes and found smaller distances between species than were found in the present study. They used lower *Taq* concentrations (20U ml^{-1} v 40U ml^{-1}) and lower primer concentrations (0.2μM v 1.0μM) than used in the present study, both of which would tend to reduce the number of products, and particularly minor products, detected.

It would appear that the number of polymorphisms detected by RAPD is dependant on the conditions used and consequently that these could be manipulated to suit the level of genetic distance that was to be investigated.

3.2.3. RAPD for *Leishmania* classification

The classification of four *L. (Viannia)* species based on the RAPD data was identical in topology to previous classifications based on isoenzymes (Cupolillo et al., 1994; Thomaz-

Soccol et al., 1993b). A comparison of published values of the Jaccard distance coefficient with values derived from the RAPD data showed some correlation with results of Thomaz-Soccol (1993) and Cupolillo *et al.* (1994), but there was no similarity to the values found by Tibayrenc *et al.* (1993) by RAPD and isoenzymes (Table 3.2). As noted above (Para. 3.2.2) Tibayrenc's group were using different conditions for RAPD which may account for the difference in the value of the Jaccard distance coefficient for the *L. braziliensis/L. guyanensis* pair, the particular isoenzymes used in their study were not described but the use of less polymorphic isoenzymes may have lead to the lower value of Jaccard distance coefficient which they found compared to those found by other workers.

Group	Method	L.pa/L.g	L.pe/L.br	L.b/L.g
Thomaz Soccol 1993	Isoenzymes	0.33	0.60	0.92
Cupolillo *et al.* 1994	Isoenzymes	0.67		0.79
Tibayrenc *et al.* 1993	RAPD			0.38
Tibayrenc *et al.* 1993	Isoenzymes			0.47
Present study	RAPD	0.46±0.13	0.50±0.12	0.83±0.05

Table 3.2 Jaccard distances found by different groups. L.pa *L. panamensis*; L.g *L. guyanensis*; L.pe *L. peruviana*; L.br *L. braziliensis*.

The similarity in the topology of classifications of *Leishmania* calculated from RAPD and isoenzyme data is encouraging for the general applicability of RAPD to the classification of a broader range of *Leishmania* strains. A number of novel *Leishmania* parasites which may be new species have been described (Lainson & Shaw, 1987) and simple methods for their classification would be a significant contribution to the determination of their status. However most of these undescribed strains are clearly very different from existing species and produce high values of the Jaccard distance coefficient when compared with named species using isoenzymes (Cupolillo et al., 1994). These high values of the Jaccard distance coefficient indicate that there is very little homology and so classifications in this region are based very largely on absence of shared characters which is an unreliable basis for determining relationships. The general similarity of the values of the Jaccard distance coefficient calculated from RAPD and isoenzyme data suggests that if RAPD is used to

describe these unclassified taxa then high values of the Jaccard coefficient will probably also be found by this method and so RAPD may not contribute much to their classification.

Uncertainty about the reliability of RAPD has been one of the chief drawbacks to the technique however it is possible to apply basic statistical techniques to RAPD data and hence to quantify the uncertainty of measurements of genetic distance.

The mean, standard deviation and confidence limits of the Jaccard distance and Jaccard similarity coefficients were calculated from the fingerprint patterns of individual primers. This data for the Jaccard distance coefficient is presented as a Dice Leraas diagram (Fig. 3.3b). The diagram suggests that the precision of the Jaccard distance coefficient is much worse for the two closely related pairs (standard deviation 0.21) than for the four other pairs (mean standard deviation 0.082). A large part of this difference is attributable to the lower resolution of the Jaccard distance for more remotely related species. The slope of the graph of the Jaccard distance when it is equal to 0.5 is twice that for when it is equal to 0.8.

3.2.4. The effect of primer GC content on RAPD fingerprints

Increasing the GC content of primers from 60% to 70% increased the values of the Jaccard distance coefficient indicating that less homology is found with higher GC content primers. This effect was significant at the $P < 0.0524$ level. In *Bulinus* spp. the same effect has been found (C. Jones, University of Aberdeen, formerly Oxford University, personal communication). This effect has not been formally described and needs to be tested with a larger number of primers, if it is a real effect, it offers another method for manipulating the amount of difference found between strains. Intra specific variation could be examined using high GC content primers and interspecific variation could be examined using lower GC content primers.

3.2.5. RAPD for the identification of possible interspecific hybrids

The RAPD results from the Nicaraguan hybrid strains supported results from RFLPs and isoenzymes (Belli et al., 1994). Both suspected hybrids did appear as such by RAPD with primers M13 and 3301 both individually and combined. *Leishmania* are generally considered to be diploid for at least some chromosomes (Bastien et al., 1992a) and consequently isolates that appear as heterozygotes by isoenzymes or RFLP between homozygote parents can reasonably be interpreted as being hybrids. However as the origin of the RAPD products are

not known they cannot be directly used as evidence for the existence of hybrids. Probing Southern blots of the RAPD fingerprints with the hybrid bands would be required to demonstrate that these products were homologous, but as the source of the DNA in these bands is unknown the significance of a small number of hybrid products would be difficult to determine. Nevertheless RAPD can provide a suitable method for quickly screening larger samples for the presence of possible hybrids, which can then be tested by a range of methods to determine their true status.

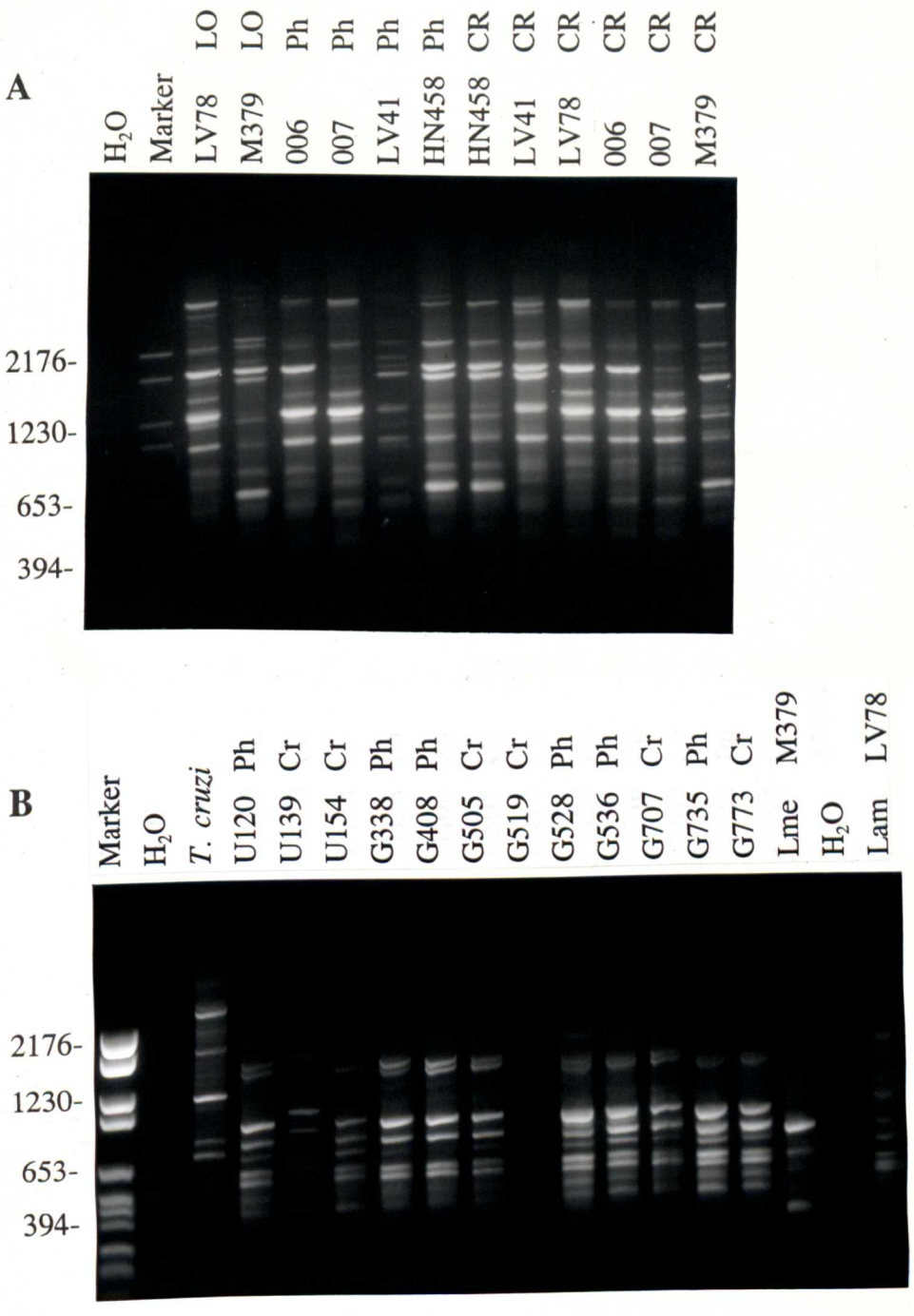


Figure 3.1A and 3.1B. **(A)** RAPD with primer UK1 on DNA prepared by different methods. Lanes 3 & 4 (LO) the DNA was prepared by standard phenol chloroform extraction and after ethanol precipitation the DNA was removed with a plastic loop. Lanes 5-8 (Ph) the DNA was prepared by a standard phenol chloroform extraction and after ethanol precipitation the DNA was retrieved by centrifugation. Lanes 9-14 (CR) a crude lysate was used in the RAPD. **(B)** Different preparations of unknown Guatemalan parasites (Chapter 7) amplified with primer M13. Note that U139 appears quite different from all other Guatemalan strains. (Ph) standard phenol chloroform extraction; (Cr) crude lysate.

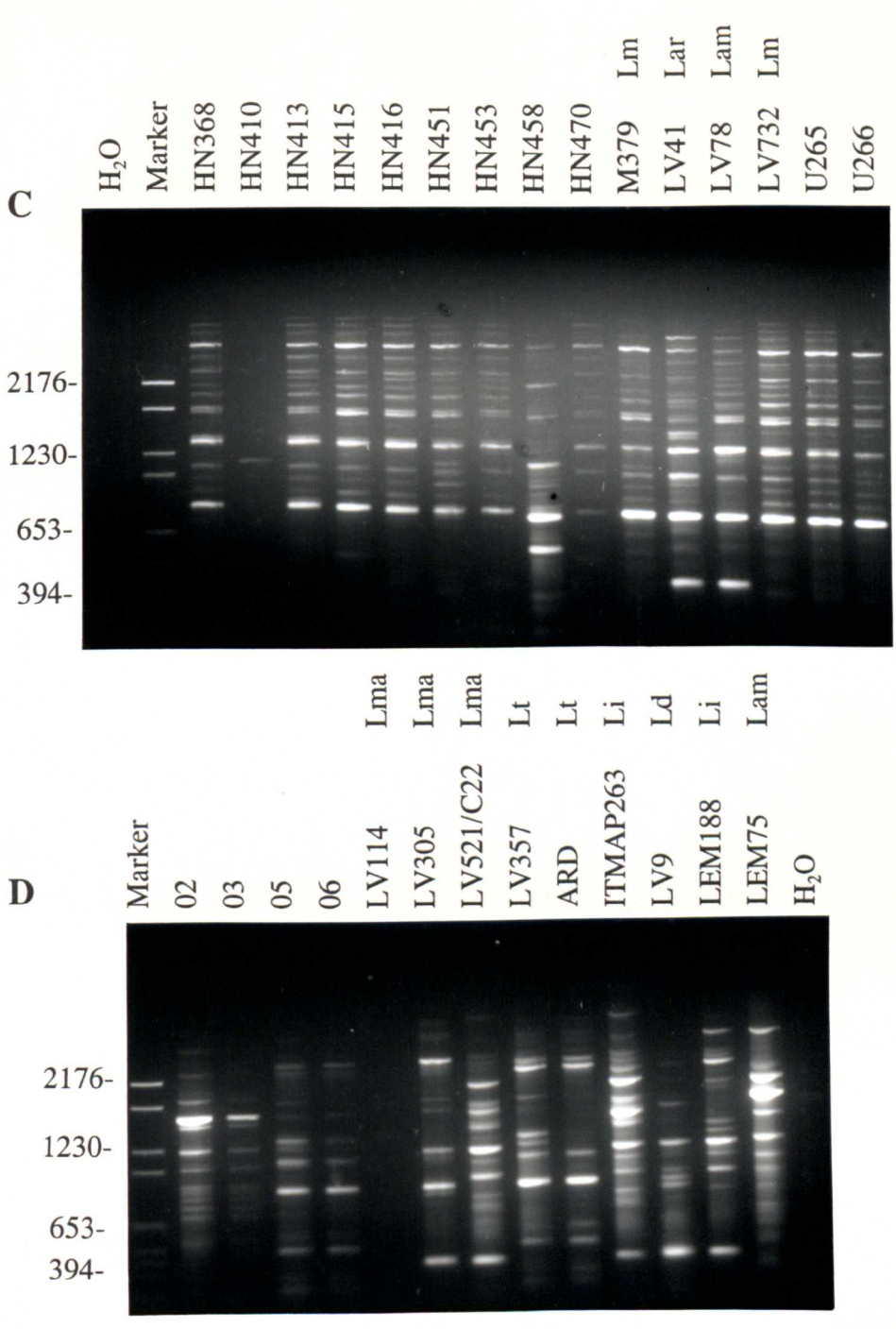


Figure 3.1C and 3.1D. **(C)** RAPD fingerprints of *L. mexicana* complex parasites from Honduras (HN prefix) and Guatemala (U prefix) and reference strains amplified with primer A1. (Lm *L. mexicana*; Lar *L. aristidesi*; Lam *L. amazonensis*). All DNA was prepared by the lithium chloride miniprep method. All members of the complex have very similar fingerprints with this primer. Note the anomalous fingerprint of HN458, whereas in figure 3.1a HN458 gives a typical *L. mexicana* fingerprint. **(D)** RAPD fingerprints of Old World parasites amplified with primer M13 comparing four unknown strains from Pakistan with various reference strains. (Lma *L. major*; Lt *L. tropica*; Li *L. infantum*; Ld *L. donovani*) The Pakistani test strains (02, 03, 05, 06) were amplified from crude lysates. The DNA used for the reference strains had either been prepared by Dr Moatazedian (ARD, LV114, LV305, LV357) a year before by the lithium chloride mini-prep method, or had been prepared by phenol chloroform extraction of crude lysates over three years previously (LV561/C22, ITMAP263, LV9), or by the author by phenol chloroform extraction (LEM188 & LEM75) one year previously. The fingerprints are very similar to published fingerprints of the same species with the same primer (Motazedian *et al.*, In Press).

Fig. 3.2

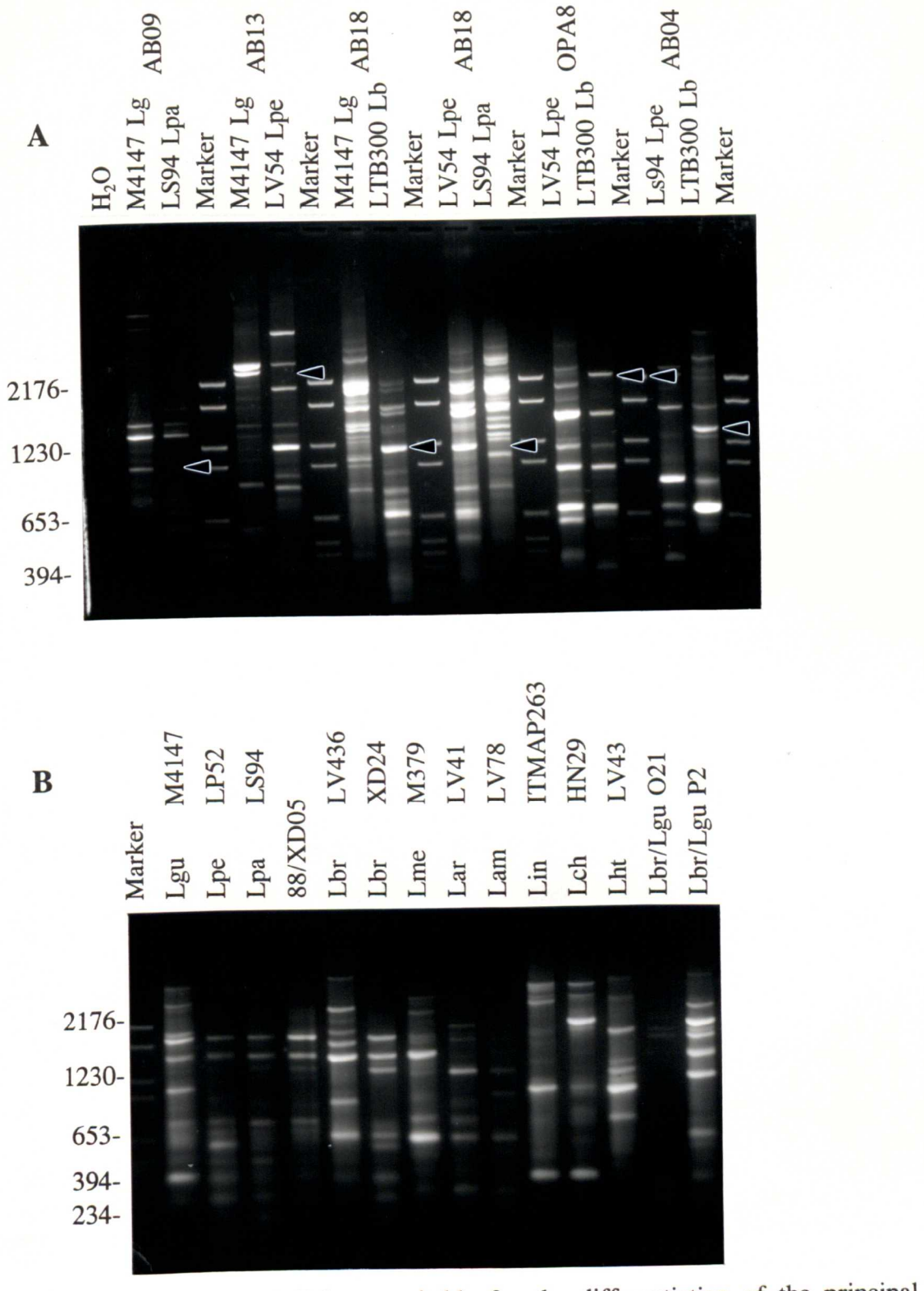
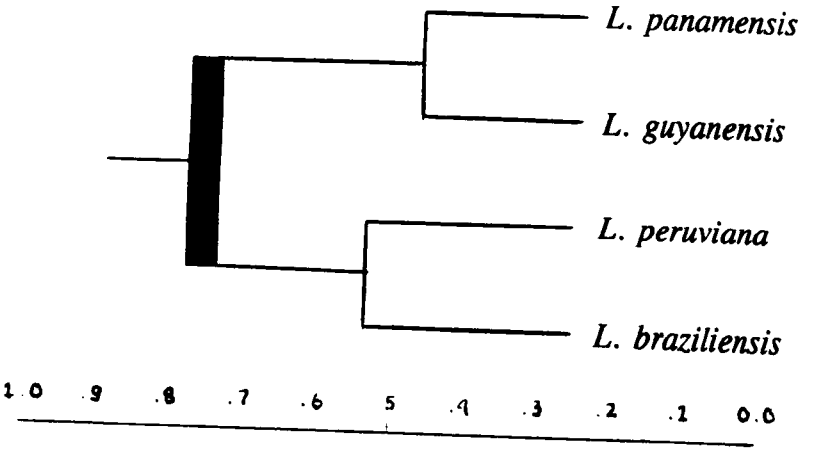


Figure 3.2A and 3.2B. **(A)** Primers suitable for the differentiation of the principal pathogenic *L. Viannia* species. (Lgu *L. guyanensis*; Lpa *L. panamensis*; Lpe *L. peruviana*; Lbr *L. braziliensis*). **(B)** RAPD fingerprints of strains from different complexes amplified with OPA8. (Lbr *L. braziliensis*; Lpa *L. panamensis*; Lpe *L. peruviana*; Lme *L. mexicana*; Lar *L. aristidesi*; Lam *L. amazonensis*; Lin *L. infantum*; Lch *L. chagasi*; Lht *L. hertigi*; Lbr/Lgu putative *L. braziliensis*/*L. guyanensis* hybrid).

A



B

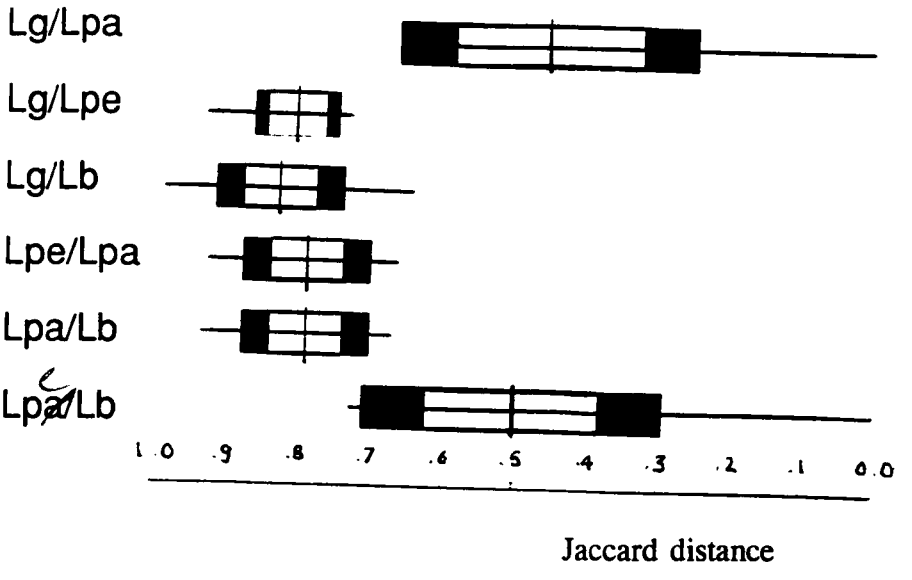


Figure 3.3A and 3.3B **(A)** Dendrogram of four *L. Viannia* species derived from Jaccard distances (Appendix 3.1) by inspection. The broad bar linking the two groups represents the range of values of the Jaccard distance between the four pairs of species *L. panamensis/L. peruviana*; *L. panamensis/L. braziliensis*; *L. guyanensis/L. peruviana*; *L. guyanensis/ L. braziliensis*.

(B) Dice Leraas diagram of the range, mean, standard deviation and standard error of the Jaccard distances for each primer for each pair of species. The vertical lines indicate the mean, the horizontal lines the range the white rectangles twice the confidence limits ($P < 0.05$) and the black rectangles the standard deviation. Where the white rectangles do not overlap the means are significantly different.

Fig. 3.4

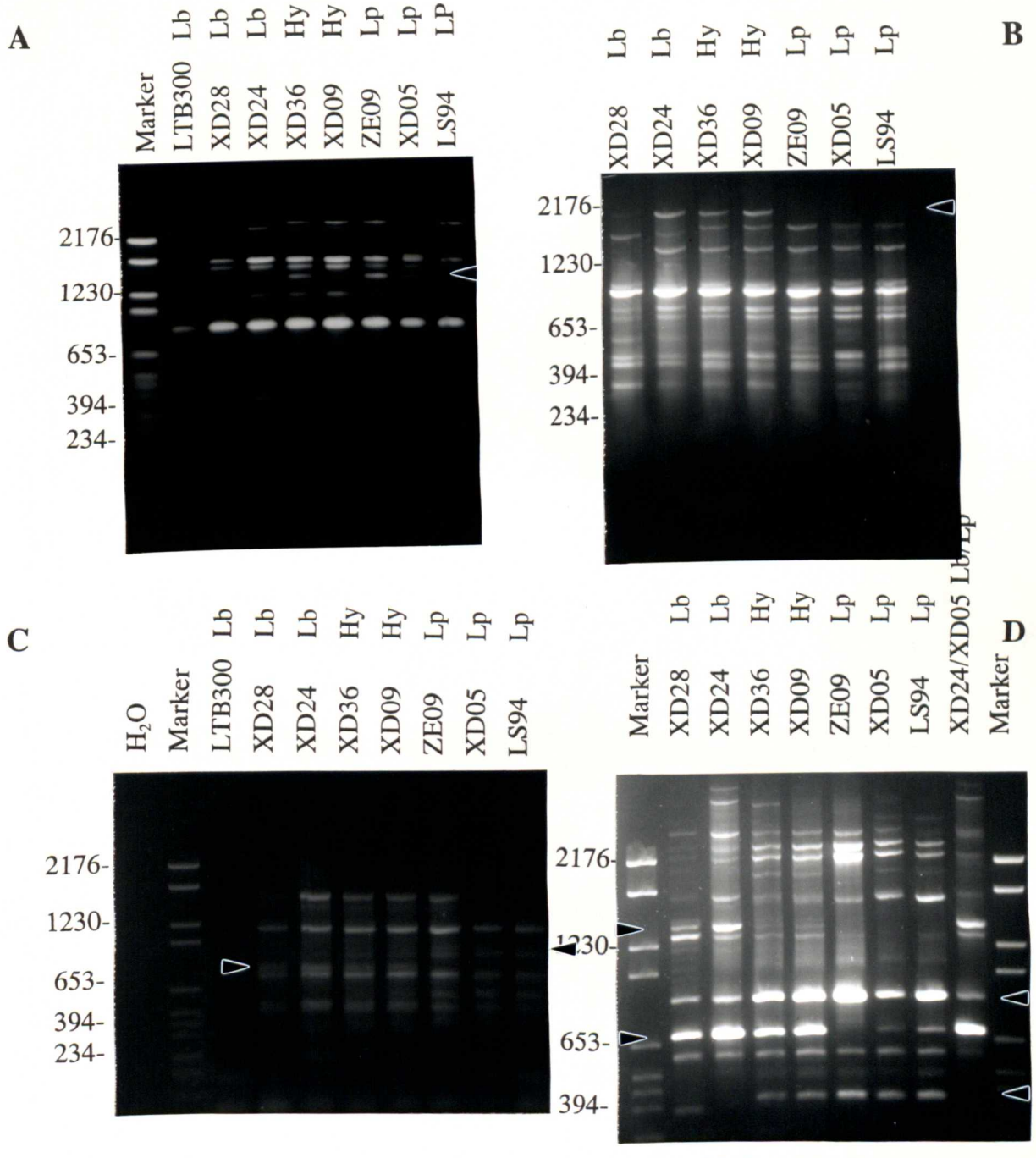


Figure 3.4A-D. RAPD for the identification of interspecific hybrids. Parent strains of *L. braziliensis* (Lb) and *L. panamensis* (Lp) and putative hybrids (Hy) amplified with: **(A)** primer 3301 which generates a product at 1,500bp in *L. panamensis* and the hybrids; **(B)** primer M13 which produced a band at 2,550bp in *L. braziliensis* and the hybrids; **(C)** a mixture of primers 3301 and M13 which produced a product at 850bp in *L. braziliensis* and the hybrids and a product at 950bp in *L. panamensis* and the hybrids; **(D)** primer AB104 produced a460bp product was found in *L. panamensis* and the hybrids, a 1,400bp product was found in *L. braziliensis* and the hybrids at lower intensity, strong bands at 870bp and 710bp in *L. panamensis* and *L. braziliensis* respectively were also present as strong bands in the hybrids but at much lower intensity in the other putative parent. A mixture of DNA from the two parent strains (Lb/Lp) produced a fingerprint unlike that of the hybrids.

4. ANALYSIS OF KINETOPLAST DNA

4.1. RESULTS

4.1.1. Isolation of kinetoplast DNA

Two methods for separating kinetoplast DNA from nuclear DNA were tested. Crude proteinase K parasite lysates were sheared by passage through a 19 gauge needle and kinetoplast DNA was collected by centrifugation (Para 2.3.6.1). Alternatively, phenol chloroform extracts were centrifuged (15,000g 60mins) in the presence of 20% sucrose, under these conditions the kinetoplast DNA formed a pellet and the nuclear DNA was retained in the supernatant (Para. 2.3.2.2). Both methods produced satisfactory yields of kinetoplast DNA, however the sucrose method also permitted recovery of intact nuclear DNA by ethanol precipitation from the supernatant. Figure 4.1a shows the kDNA pellet and supernatant fractions of four samples of *L. chagasi* prepared by centrifugation through sucrose. The kDNA fraction is contaminated with a trace of nuclear DNA and the nuclear DNA is contaminated with a significant amount of kDNA. The nuclear DNA fraction was suitable for enzyme digestion, southern blotting and probing. *L. chagasi* DNA prepared in this way was digested with *PstI* and *XhoI* and probed with a kinetoplast minicircle DNA fragment to find differences correlating with pathology (Fig. 5.2b & 5.2c). kDNA isolated from parasites in the experiments described below was prepared by the sucrose method.

An attempt was made to simplify the sucrose method by omission of the phenol chloroform extraction step before centrifugation. This protocol produced a large gelatinous pellet from which a mixture of nuclear and kinetoplast DNA was recovered after phenol chloroform extraction (not shown).

4.2. PCR AMPLIFICATION OF KINETOPLAST DNA

4.2.1. Evaluation of existing primers

Four pairs of primers that had been previously described were evaluated. Primers 13A and 13B amplify the conserved minicircle region of all trypanosomatids. MP1H and MP3L amplify part of the conserved region of *L. (Viannia)* species. 13Y and 13Z were designed to amplify the variable region of *L. mexicana*. Primers LU and LB amplify the whole of *L. (Viannia)* minicircles.

Primers 13A and 13B were confirmed to amplify a 120bp product from all *Leishmania* tested. During a study of monoxenous trypanosomatids it was found that these primers also

amplified kDNA from *C. fasciculata*, *C. luciliae* and *H. muscarum* (Fig. 7.1a). In addition to the 120bp product a number of higher molecular weight products were also observed in the monoxenous trypanosomatids.

Primers MP1H and MP3L were also confirmed to amplify a 70bp fragment from *L. braziliensis*, *L. panamensis*, *L. guyanensis* and *L. peruviana*, they also amplified the diagnostic fragment from *L. mexicana* BEL21 but not other *L. (Leishmania)* (Fig. 7.1b).

Primers 13Y and 13Z amplified a 740bp product in *L. mexicana* and a 980bp product in *L. braziliensis*, *L. guyanensis*, *L. panamensis* and *L. peruviana* (Fig. 4.1b). 1950bp products were sometimes seen in *L. (Viannia)* species as well as some lower molecular weight products (not shown). Primers 13Y and 13Z generated products of approximately 300bp and 360bp from *L. infantum* and *L. chagasi* DNA or failed to amplify DNA from these species (not shown).

Primers LU and LB generated products at 700bp and 800bp in *L. braziliensis* (Fig. 4.1c), a band at about 1200bp was produced in an *Endotrypanum* strain (Fig. 4.1c), but none in *L. mexicana* (not shown).

4.2.2. Testing primer LiR for the *L. infantum* variable region.

As none of the primers tested would amplify the *L. infantum* minicircle variable region a new primer LiR was designed to be used in combination with primer 13Z (Para. 2.4.3). LiR and 13Z generated a primary product at 680bp and a weaker product at 800bp in *L. infantum* (Fig. 4.2a), the concentration of the 800bp product significantly reduced by reducing the *Taq* concentration from 40U ml^{-1} to 20U ml^{-1} (Fig. 4.2b).

Concentrations of template DNA and primers 13Z and LiR were titrated against each other to determine their relative effects on amplification efficiency. The concentration of product was directly correlated with the primer concentration. 4ng μl^{-1} of primer generating significantly more product than 1ng μl^{-1} which produced more than 0.4ng μl^{-1}. The concentration of product was not significantly effected by template concentrations from 400 to 4pg μl^{-1} but the concentration of product was stronger when 0.4pg μl^{-1} of template was used with primer concentrations of 1 and 0.4ng μl^{-1} (Table 4.1 & Fig. 4.2a).

	Primer concentration ngμl^{-1}			
DNA concentration pgμl^{-1}		4	1	0.4
	400	17.45	4.30	1.50
	40	14.95	3.55	1.65
	4	12.50	4.50	3.50
	0.4	16.15	8.00	9.00

Table 4.1 Concentration of PCR products (ng μl^{-1}) in figure 4.2a. Different concentrations of template DNA (*L. infantum* LEM188) and primers 13Z and LiR were compared. Whole band intensity was determined by densitometry and concentration calculated by comparison with known concentrations of molecular weight markers.

Primers 13Z and LiR also amplified kDNA from all other kinetoplastids tested (Fig. 4.1d). The size of the diagnostic band in various species is shown in table 4.2. Some species could be readily distinguished from each other by the size of the principal product.

In the Old World *L. donovani* complex species were readily distinguished from *L. major* but not from *L. tropica* or lizard *Leishmania*. *L. amazonensis* had a significantly smaller kDNA minicircle than all other New World species, but *L. mexicana*, *L. panamensis* and *L. chagasi* all had very similar sized minicircles. *L. hertigi*, *L. herreri* and *Endotrypanum* species all had large minicircles of approximately 850bp.

Primers 13Z and LiR would amplify various different DNA template preparations. Phenol/chloroform extracted DNA (Fig. 4.1b-d), crude proteinase K lysates (not shown), dilutions of parasites embedded in agarose PFGE blocks (Fig. 5.4) and boiled parasites (Fig. 4.2b) all amplified satisfactorily.

New World strains	Size	Old World strains	Size
L. amazonensis	570bp	*L. donovani*	780bp
L. mexicana	630bp	*L. infantum*	680bp
L. braziliensis	650bp	*L. tropica*	750bp
L. guyanensis	650bp	*L. major*	570bp
L. peruviana	650bp	*L. tarentolae*	720bp
L. panamensis	650bp	*L. hoogstraali*	720bp
L. herreri	850bp	*L. gymnodactyli*	720bp
L. hertigi	850bp	*L. adleri*	720bp
E. monterogeii	850bp		
E. schaudinni	850bp		

Table 4.2 Sizes of principal product of PCR primers LiR and 13Z with different species of *Leishmania* and *Endotrypanum*. Sizes were determined by measurement of migration from the loading well on negatives of agarose gels.

4.3. PCR BASED SCHIZODEMES FOR THE IDENTIFICATION OF *LEISHMANIA* POPULATIONS

Kinetoplast DNA amplified by PCR was readily digested by restriction enzymes without purification. The fingerprints produced by digestion of PCR products had fewer fragments than those produced by kDNA produced by conventional methods, however *L. chagasi* strains from Honduras (HN) have fragments that appear to be homologous in both preparations (Fig. 4.3a and 4.3b). Nevertheless both methods could readily discriminate between different strains of *L. chagasi* and *L. infantum* (Fig. 4.3a, 4.3b and 5.4).

kDNA from *L. infantum* strains LEM188 and IPT1, prepared both by PCR and by isolation from parasites, appeared to generate more fragments when digested with *HaeIII* than kDNA from *L. chagasi* from Honduras (Figs. 4.3a, 4.3b and 5.4). The sum of the sizes of fragments smaller than 801bp in LEM188 in figure 4.3b is 8932bp, equivalent to 11.16 complete 800bp sized minicircles, whereas the sum of the sizes of HN336 fragments is 7428bp equivalent to 9.28 complete minicircles. This suggested that the *L. infantum* strains LEM188 and IPT1 might be polyclonal or that they might contain more minicircle classes than the *L. chagasi* from Honduras. IPT1 was cloned to test whether it was polyclonal, kDNA from individual clones was PCR amplified and digested with *HaeIII*, but no differences were detected between the clones (Fig. 4.4a and 4.4b).

Strain							
LEM188		LV358		HN556		HN420	
Size	Int(%)	Size	Int(%)	Size	Int(%)	Size	Int (%)
				850	11	850	7
760	5	760	12	760	30	760	18
700	3	700	6	700	26	700	12
590	4	590	2	550	9	550	2
		490	2				
						480	1
420	8			380	1	380	3
330	4			340	2	340	5
300	18	300	9	280	2	280	6
220	2	220	10	230	2	230	4
165	5	165	1				
135	7						
115	17	115	8	115	13	115	16
85	4						
57	24	57	50	57	3	57	28

Table 4.3 Percentage of total lane intensity in individual products in figure 4.5b of an autoradiograph of Southern blot of *HaeIII* digests of kDNA.

Estimates of the number of minicircle classes in individual strains was also made by probing Southern blots of *HaeIII* digests of kDNA isolated from eight strains of *L. infantum* and *L. chagasi* with the 13Z PCR primer which binds to the minicircle origin of replication. The 13Z probe bound to 12 fragments in *L. infantum* LEM188 and 9 fragments in LV358. The *L. chagasi* strains were not sufficiently well resolved to determine the number of fragments to which the probe bound with confidence (Fig. 4.5). On visual inspection the radioactivity in the Honduran strains appeared to be concentrated in three bands of approximately 850, 760 and 660bp, whilst in the *L. infantum* strains radioactivity appeared to be concentrated in a single band at about 57bp but with a number of bands of intermediate strength. The proportion of radioactivity detected in different sized fragments was determined by densitometry over the whole band areas and is shown in Table 4.3.

Minicircle fragments at about 760, 700 and 115bp were detected by the 13Z probe in all *L. infantum* and *L. chagasi* strains and may represent conserved minicircle sequence classes.

A *HaeIII, MspI* and *DraI* mixed digest of *L. panamensis*, *L. braziliensis* and the *L. braziliensis/L. guyanensis* putative hybrid (P2) were also probed with the 13Z probe. No hybrid restriction pattern was observed (Fig. 4.5b).

4.4. kDNA PROBES FOR THE IDENTIFICATION OF *LEISHMANIA* SPECIES

A dot blot of 10ng and 1ng of kinetoplast variable region kDNA was prepared using kDNA amplified with primers LiR and 13Z. 33 *Leishmania* strains from 16 species (Table 4.4) were hybridised with isotopically labelled kDNA variable region probes from seven strains of *Leishmania*. The blot was first probed with the 13Z primer to test the loading and then probed with kDNA from the following species: *L. braziliensis* (LbV); *L. mexicana*, (M379); *L. guyanensis* (M4147); *L. chagasi* (HN29); putative *L. braziliensis/L. guyanensis* hybrid from Venezuela (P2); *L. major* (LV305); a putative *L. infantum* (LEM75).

Row	Column A	Column B	Column C
1	*L. mexicana* M379	*L. tropica* 05	
2	*L. chagasi* HN354	*L. tarentolae* 03	
3	*L. chagasi* HN168	*L. tarentolae* 02	
4	*L. chagasi* HN462	*L. peruviana* LV54	*L. amazonensis* 006
5	*L. chagasi* HN536	*L. braziliensis* LbV	*L. amazonensis* 007
6	*L. chagasi* HN29	*L. braziliensis/L. guyanensis* P2	*L. tarentolae* LV414
7	*L. chagasi* LV718	*L. guyanensis* M4147	*L. tarentolae* LV108
8	*L. chagasi* M4192	*L. panamensis* LS94	*L. gymnodactyli* LV247
9	*L. infantum* IPT1	*L. tropica* ARD	*L. hoogstraali* LV31
10	*L. infantum* LV405	*L. major* LV305	*L. adleri* LV30
11	*L. infantum* LV358	*L. aristidesi* LV41	*L. donovani* LV9
12	*L. infantum* LEM188	*L. amazonensis* LV78	*L. tropica* 06

Table 4.4 *Leishmania* strains on dot blots of kDNA (Figs 4.6, 4.7, 4.8). kDNA was prepared by PCR using primer 13Z and LiR. 10ng and 1ng of each PCR product was applied to the dot blot.

The 13Z probe indicated considerable variation in loading (Fig. 4.6a). The *L. (Viannia)* probes all cross hybridised with each other even at high stringency (0.1% SDS, 55°C) but only very faint traces of cross hybridisation were detected with *L. (Leishmania)*. The *L. guyanensis* (M4147) probe (Fig. 4.6b) hybridised with *L. panamensis* (LS94) at the same intensity as the homologous kDNA but at lower intensity with *L. braziliensis* (LbV) and with the putative *L. braziliensis* / *L. guyanensis* hybrid (P2). The *L. braziliensis* (LbV) probe (Fig. 4.6c) hybridised most strongly to itself and equally with the putative hybrid, *L. panamensis* and *L. guyanensis*. The putative hybrid probe (P2) (Fig. 4.6d) bound only

slightly more strongly to itself than to the other *L. (Viannia)* strains with which it bound equally. The signal from *L. (Leishmania)* species in figure 4.6d is residual radiation after stripping the previous probe (*L. chagasi* HN29), however only a very weak signal was detected from *L. (Viannia)* species after stripping the *L. chagasi* probe (Fig.4.7b). The signal from *L. peruviana* was weak in all cases due to lower loading (Fig. 4.6a).

The *L. chagasi* (HN29) probe cross hybridised with all other *Leishmania* at low stringency (0.1xSSC, 42°C) (Fig. 4.7a), under these conditions hybridisation was proportional to loading for all *L. donovani* complex some *L. tropica* and some lizard *Leishmania*, all other strains and particularly *L. (Viannia)* had relatively weaker signals. After stripping with NaOH at 65°C for 30 minutes the signal was weaker (Fig. 4.7b). There was still extensive cross hybridisation with *L. (Leishmania)* and lizard *Leishmania* species, particularly *L. tropica* (06) and *L. adleri* (LV30), but the *L. (Viannia)* signal was reduced to the residual signal which had remained after previously being probed with *L. guyanensis* (M4147) (not shown).

The *L. mexicana* (M379) probe bound proportionately to loading, as measured by the 13Z loading probe, with all *Leishmania* at low stringency (0.1xSSC, 42°C) (Fig. 4.7c). At higher stringency (0.1%SDS, 55°C) (Fig. 4.7d) hybridisation to the 1ng dot was only detectable in the homologous kDNA after 4 hours (not shown), although after 22 hours weak hybridisation to the 1ng dot was detectable in *L. (Leishmania)* species and lizard *Leishmania* (Fig. 4.7d).

An *L. infantum* (LEM75) strain that appeared to be contaminated with *L. mexicana* complex parasites by RAPD, cross hybridised at high stringency (0.1%SDS, 55°C) to 10ng of kDNA from all *Leishmania* though only weakly to *L. (Viannia)*. It hybridised more strongly to 1ng of kDNA from the Panamanian *L. amazonensis* (CIDEP006 and CIDEP007) than to 1ng of kDNA from the Brazilian *L. amazonensis* LV78. It also hybridised strongly to 1ng of kDNA from three *L. infantum* and *L. chagasi* strains (M4192, IPT1 and LV405) (Fig. 4.8a).

The *L. major* (LV305) probe was washed at higher stringency (0.1%SDS, 42°C) directly without an intervening lower stringency wash. The probe bound strongly to 1ng of

homologous kDNA and also 10ng of *L. infantum* and *L. chagasi* kDNA. The probe bound weakly to 1ng of all *L. (Leishmania)* and to lizard *Leishmania* strains (Fig. 4.8b).

4.5. DISCUSSION

4.5.1. kDNA preparation

The separation of kDNA from nuclear DNA by centrifugation through 20% sucrose proved to be as satisfactory as the conventional method of shearing the nuclear DNA through a syringe for the preparation of kDNA (Fig. 4.1). The sucrose method had the advantage of yielding intact nuclear DNA that could be used for RFLPs (Fig. 5.2). The principal modification of the method of Chance *et al.* (1974) was the omission of the ethanol precipitation step before centrifugation through sucrose. Consequently parasites could be washed, lysed, phenol extracted, centrifuged through sucrose and the nDNA and kDNA fractions collected and ethanol precipitated all in one day. Although high centrifuge speeds are normally recommended for collecting kDNA networks (Chance et al., 1974; Gonçalves et al., 1984), kDNA is reported to sediment at 1g and it was found that kDNA of adequate purity for schizodeme analysis could be prepared by both methods at 15,000g eliminating the need for an ultracentrifuge.

4.5.2. PCR primers

The published primers for *Leishmania* generally performed as described. The 13A and 13B primers (Rodgers *et al.* 1990), amplified a 120bp kDNA product from *Leishmania*, *Endotrypanum*, *Crithidia* and *Herpetomonas*. These primers were designed against the conserved sequence blocks 1 and 3 of *L. mexicana* minicircles. Since these same sequences are found in all trypanosomatids it was not surprising that amplification should not be genus specific. In the monoxenous trypanosomatids a number of higher molecular weight products were usually also amplified (Fig. 7.1). The precise origin of these products is not clear, *C. fasciculata* has two conserved regions at 180° to each other in a 2.5kb minicircle (Ryan, et al., 1988), so the additional products are too small to contain the variable region between two conserved regions. The higher molecular weight products were moderately reproducible so amplification would have to occur in a partially specific manner. They may represent the products of hybridisation between heterologous minicircles at the runs of oligo (dA) in the bend region. These oligo (dA) runs are particularly abundant in *C. fasciculata* in which 16 successive oligo (dA) runs form a 200-300bp loop (Simpson, 1987). Alternatively as single

stranded guide RNAs coded for by kDNA minicircles adopt a hairpin loop conformation (Piller et al., 1995) they may be capable of selfpriming the PCR reaction in one direction.

Primers MP1H and MP3L performed as described generating a diagnostic product at 70bp in *L. braziliensis* species (Fig. 7.1b) (Lopes *et al.* 1993). The supposed *L. mexicana* strain (BEL21) which also generated a 70bp product was later confirmed to be contaminated with *L. braziliensis* (not shown). The higher molecular weight bands in figure 7.1a may be read through products.

Primers 13Y and 13Z are complementary to 13A and 13B and amplify the variable region rather the conserved region, but, despite being complementary to the conserved sequence blocks, these primers did not amplify all *Leishmania*. 13Y and 13Z amplified *L. mexicana* and *L. braziliensis* but only unexpectedly small fragments at 300 and 360bp were generated in *L. infantum*. Rodgers *et al.* (1990) reported an 800bp product for *L. chagasi* with these primers which was not found in the present study and products of 350 and 200bp for *L. donovani* which are similar to the ones found at 360 and 300bp in *L. infantum* and *L. chagasi* (Table 4.5). When published minicircle sequences of *L. infantum* were compared with those of the primers (Para 2.4.2), it was seen that the homology of the 13Y primer to *L. infantum* was restricted to the 5' end of the primer as the 3' end of 13Y extended beyond the end of the conserved sequence block (Appendix 4.1). The primer LiR was designed to anneal to the full CSB at its 3' end and amplified all trypanosomatids tested.

The 13Y and 13Z amplification products were up to 300bp larger than expected whilst those of LU/LB and LiR/13Z were within 50bp of the expected size (Table 4.5). Size determinations from agarose gels should be treated with caution. The resolution of the negatives from which determinations were made were approximately 50bp mm^{-1}. As the mean band width was about 1mm, small errors in measurement could significantly alter the observed size. Furthermore the observed sizes are very sensitive to any skewing of the gel. The slight difference from the expected sizes of the LiR/13Z and LU/LB PCR products is therefore probably due to errors in measurement or calculation. However the large differences from expected values of *L. braziliensis* and *L. mexicana* minicircle sizes with primers 13Y and 13Z suggest that the actual product is different from the expected. The nature of the difference could be determined by sequencing and until this is done it is clearly necessary to be cautious in interpreting any data generated by primers 13Y and 13Z.

The size difference between *L. infantum* PCR products and those generated by *L. mexicana* or *L. braziliensis* templates was sufficient to differentiate between *L. infantum* and those species when they were run together on the same gel.

The only primer pair to consistently generate a single product was LiR & 13Z, after the *Taq* concentration had been reduced from 40U ml^{-1} to 20U ml^{-1}. Additional higher molecular weight bands have been previously reported to be generated by LU and LB (de-Bruijn & Barker, 1992). No attempt was made to eliminate additional products amplified with primers LU/LB by *Taq* titration, but it was found that if the diagnostic band was excised from the gel and reamplified a single product could be generated (not shown).

	L. mexicana	*L. braziliensis*	*L. infantum*
Minicircle length	710	750	800
13Y & 13Z Observed	740	980	300/360
13Y & 13Z Reported	750	-	800
13Y & 13Z Expected	630	670	720
LU & LB Observed		700	
LU & LB Reported		750	
LU & LB Expected		750	
LiR and 13Z Observed	630	650	750
LiR and 13Z Expected	630	670	720

Table 4.5 Size of minicircle amplified by different primer combinations. The minicircle size is from the respective sequences (de Bruijn & Barker, 1992). The observed minicircle length was calculated from 1% agarose gels, the reported minicircle lengths are from de Bruijn & Barker, (1992) (LU and LB) and Rodgers et al., (1990) (13Y and 13Z). The expected PCR product size is calculated from the length of the published sequences of the minicircles less 80bp of conserved region which is not amplified by primers LiR/13Z or 13Y/13Z.

4.5.3. PCR based schizodemes for the identification of *Leishmania* populations

As primers LiR and 13Z produced a single PCR product it was practical to prepare digests directly from the PCR product without further purification. As expected, schizodemes produced by digestions of PCR products were different from those generated by digests of

intact minicircles isolated from parasites. However *HaeIII* schizodemes of Honduran strains were all very similar to each other by both methods whilst *L. infantum* reference strains were more variable (Fig. 4.3a and 4.3b). It would be expected that schizodemes based on intact minicircles would show greater resolution as there would be a greater number of products in the size range detectable on agarose gels (> 200bp depending on conditions), but as schizodemes are such a high resolution technique this may not be a serious problem. The application of this method for the detection of differences between very similar *L. chagasi* strains is described in paragraph 5.1.3.

The sum of band sizes of *L. infantum* LEM188 (8932bp) and *L. chagasi* HN336 (7482bp) in figure 4.3b suggested that *L. infantum* LEM188 contained 11.16 minicircle classes whilst *L. chagasi* HN336 contained 9.28. Probing *HaeIII* digests of kDNA isolated from parasites with the 13Z primer for the minicircle origin of replication indicated that the LEM188 strain had twelve minicircle classes but did not resolve the Honduran *L. chagasi* strains sufficiently well to determine the number of minicircle classes of these strains (Fig. 4.5b). Other workers have observed approximately ten minicircle classes in *Leishmania* species (Barker, 1987). This is consistent with the values obtained in the present study, given the potential sources of error inherent in both methods of calculating the number of minicircle classes. There are two principal sources of error when calculating the number of minicircle classes by summing the fragments in a *HaeIII* restriction digest and by probing Southern blots with a probe for the minicircle origin of replication. Firstly partial digestions will increase the observed number of minicircle classes and secondly if two minicircle classes have *HaeIII* restriction sites equidistant from the origin of replication they will appear as a single class thereby reducing the observed number of minicircle classes. Consequently the number of minicircle classes detected by both methods should be treated with caution and the observed differences in the number of minicircle classes in different strains may not represent actual differences.

However the 13Z probe for the minicircle origin of replication indicated that the Honduran *L. chagasi* strains have a different distribution of minicircles between classes from the *L. infantum* strains. The majority of minicircles of the Honduran strains were in four minicircle classes, with *HaeIII* fragments of 850, 760,700 and 115, and the remaining minicircle classes containing relatively few minicircles. The minicircles of the *L. infantum* strains had a more variable distribution of minicircles with the largest class producing fragments of

about 57bp, but the remaining minicircles being rather more evenly distributed. The *C. fasciculata* Cf-C1 strain is reported to have 90% of minicircles in a single class which codes for a guide RNA of unknown function whilst five guide RNAs which are involved in editing the MURF4 and RPS12 genes are coded for by minicircles that comprise 2% of the minicircle population (Yasuhira & Simpson, 1995). Other *C. fasciculata* strains have very different distributions of minicircle classes (Simpson, 1987) so the considerable differences in the apparent distribution of minicircle classes in *L. chagasi* and *L. infantum* strains examined may not reflect differences in the activity of mature guide RNAs.

The numbers of minicircle classes estimated was consistent with an estimate from the sum of the minicircle restriction digest fragment lengths but did not give reliable results from all strains tested. A refinement of this approach would be to detect fragments bearing the origin of replication in digests of whole kDNA minicircles with the isotopically labelled 13Z primer, but instead of using the 13Z oligonucleotide as a probe it could be used as a primer in a Klenow reaction, the fragments could then be run out on a sequencing gel. This should produce both greater sensitivity and higher resolution.

4.5.4. kDNA variable region probes

The dot blots of PCR amplified minicircle regions probed with PCR amplified minicircle variable regions partially confirmed previous reports that this method can discriminate Old World species (Bozza et al., 1995). The *L. tropica* and *L. major* probes bound strongly to homologous kDNA and only weakly to kDNA of other species. However whilst Bozza *et al.* found that a *L. infantum* probe only bound weakly to *L. chagasi* and hardly at all to *L. donovani*, the *L. chagasi* (HN29) probe used in the present study bound equally to all *L. donovani* complex. This was observed both at relatively low stringency (0.1xSSC, 42°C) and after stripping with NaOH, it therefore seems likely that some permanent bonding of the probe to the substrate had occurred and that if the blot had been washed at higher stringency directly after hybridisation larger differentials might have been seen.

Bozza *et al.* hybridised their blots at 65°C in formamide and washed them in 0.1xSSC, 0.1% SDS at 65°C. In the present study blots were hybridised at 42°C and were initially washed in 0.1xSSC, 0.1% SDS at 42°C to examine patterns of cross hybridisation by autoradiography before continuing to a second wash in 0.1% SDS at 55°C. This difference in conditions may be sufficient to account for the greater cross hybridisation observed in the

present study. As a residual signal was invariably detected after stripping the blot in NaOH, this not would appear to be an effective method for obtaining maximum specificity. Therefore separate blots should ideally be prepared for studying specificity at different stringencies.

kDNA variable region probes prepared from New World species have not previously been described. The *L. mexicana* and *L. amazonensis* probes bound much more strongly to the homologous DNA than to the other members of the *L. mexicana* complex. The low levels of cross hybridisation between *L. amazonensis* and *L. mexicana* strains was consistent with the 60bp difference in minicircle size (Table 4.1). The *L. (Viannia)* LiR/13Z products were all approximately 650bp (Table 4.1) and probes prepared from them showed substantial cross hybridisation. The *L. guyanensis* probe bound more strongly to *L. panamensis* than to *L. braziliensis* which is consistent with the *L. (Viannia)* classification (Para 3.4). Very little cross hybridisation was observed between *L. (Viannia)* and *L. (Leishmania)* strains. High levels of cross hybridisation amongst *L. (Viannia)* strains but no cross hybridisation with *L. (Leishmania)* has been observed with a probe prepared from complete *L. braziliensis* kDNA minicircles, however a *L. (V) lainsoni* kDNA probe was found to hybridise weakly with *L. mexicana* complex strains (Eresh et al., 1995). An *E. monterogeii* probe cross hybridised weakly with *Leishmania* when washed in 0.1%SDS at 42°C (not shown) but after stripping in 0.1M NaOH the residual signal was only detectable from *Endotrypanum* strains after 20 hours exposure (Fig. 6.8b). Cross hybridisation between *E. schaudinni* and *L. braziliensis* whole kDNA has been reported (Pacheco et al., 1990b). The variable region probes do appear to be more specific than whole minicircle probes, they can therefore be used to detect to the genus level with confidence provided protocols are carefully followed and to the species level in some cases if caution is exercised in the interpretation of results.

As *T. brucei* hybrids have been found to have kDNA minicircles derived from each parent (Gibson, 1995), the *L. braziliensis/L. guyanensis* (P2) putative hybrid was tested to determine if it might have minicircle classes derived from each parent. The P2 kDNA variable region probe gave a stronger signal from the homologous kDNA than from either parent which is consistent with a mixture of DNA from both sources, however the results from the *L. braziliensis* and *L. guyanensis* probes gave no indication that P2 had greater homology with the probe than the heterologous species.

The 13Z probe did not detect any hybrid pattern in the digest of *L. braziliensis/L. guyanensis* kDNA (Para 4.3 & Fig. 4.5b). No *L. guyanensis* parasites were available from which to prepare kDNA and *L. panamensis* kDNA, being the closest relative, was used instead, consequently it is not surprising that no hybrid pattern was found. *L. guyanensis* is not found in the region of Venezuela from which the putative hybrid was isolated and there are not any potential parent strains with which to compare this possible hybrid. The identity of P2 therefore remains problematic.

DNA probes have not been widely used for the diagnosis of *Leishmania* due to insufficient sensitivity to detect the small numbers of parasites in clinical samples. However probes do offer an alternative to gel electrophoresis for the detection of PCR products and have recently been used for this purpose (Ashford, et al., 1995). However these probes must clearly be used under well defined conditions as cross hybridisation was much more extensive at 10ng than at 1ng. PCR yield from prepared parasite DNA frequently exceeds 20ng μl^{-1}, if 10μl of PCR product were applied to the filter a significant level of non-specific hybridisation could occur. PCR on clinical samples is likely to have substantially lower yields but nevertheless specificity may well remain a concern. However for many diagnostic purposes the infecting species is not important, consequently these probes could provide the basis of useful diagnostic systems such as the PCR-SHELA based on a specific sequence of *L. infantum* nuclear DNA described by Qiao *et al.* (1995).

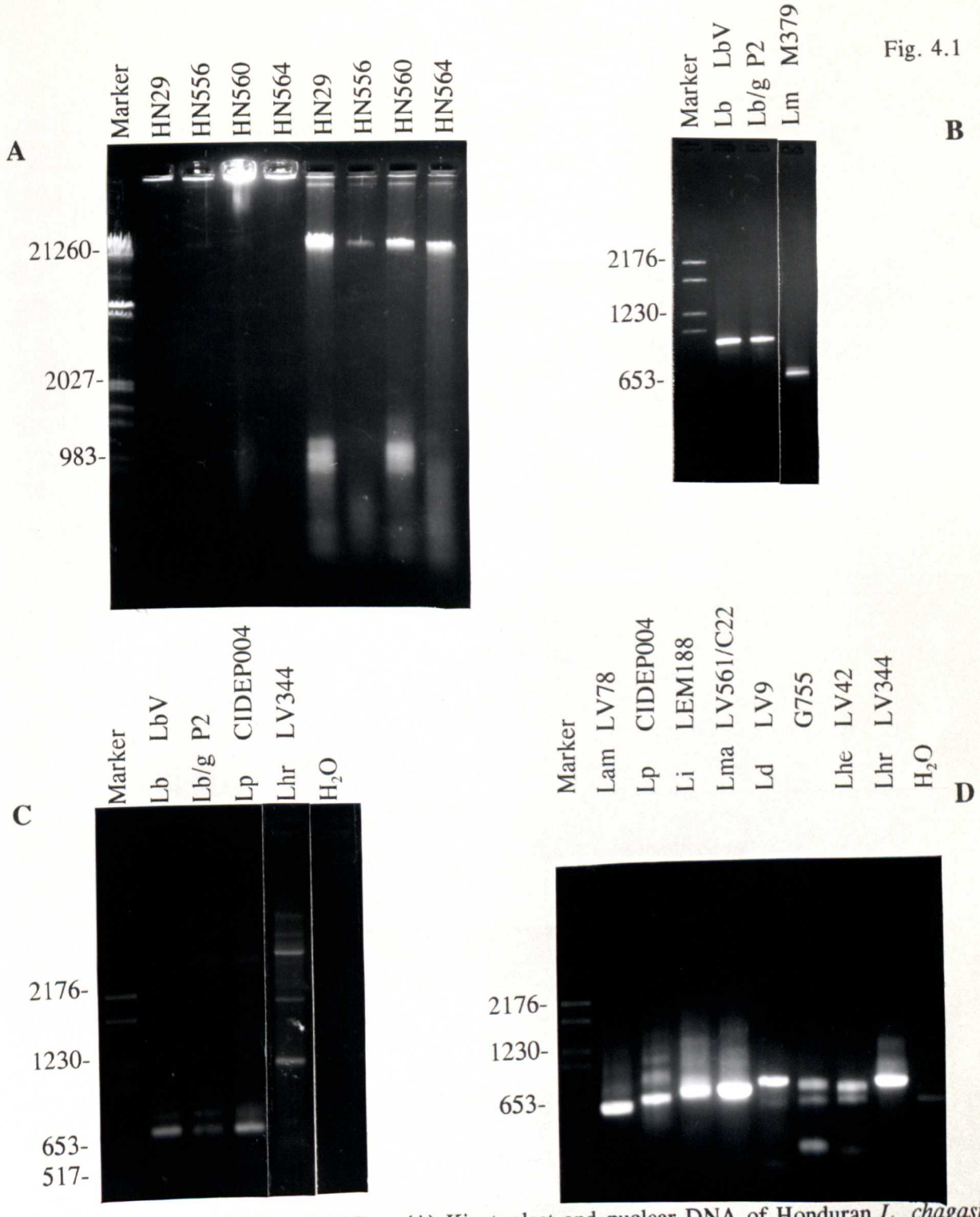


Figure 4.1A, 4.1B, 4.1C and 4.1D **(A)** Kinetoplast and nuclear DNA of Honduran *L. chagasi* strains prepared by centrifugation through 20% sucrose. The kinetoplast DNA networks (lanes 2-5) have not migrated out of the sample well. kDNA is also visible in the sample wells of the lanes containing nuclear DNA. The lower molecular weight bands in the nDNA lanes are presumed to contain kDNA minicircles that have been linearised during processing. **(B)** PCR products generated using primers 13Y and 13Z. **(C)** PCR products generated using primers LB and LU. **(D)** PCR products using primers 13Z and LiR. Abbreviations: *L. braziliensis* (LbV) (Lb); putative *L. braziliensis* / *L. guyanensis* hybrid (P2) from Venezuela (Lb/g); *L. mexicana* (M379) (Lm); *L. panamensis* (Lp); putative *L. herreri* (Lhr) *L. amazonensis* (Lam); *L. panamensis* (Lp); *L. infantum* (Li); *L. major* (Lma); *L. donovani* (Ld); Guatemalan leptomonad G755; *L. hertigi* (Lhe); *L. herreri* (Lhr).

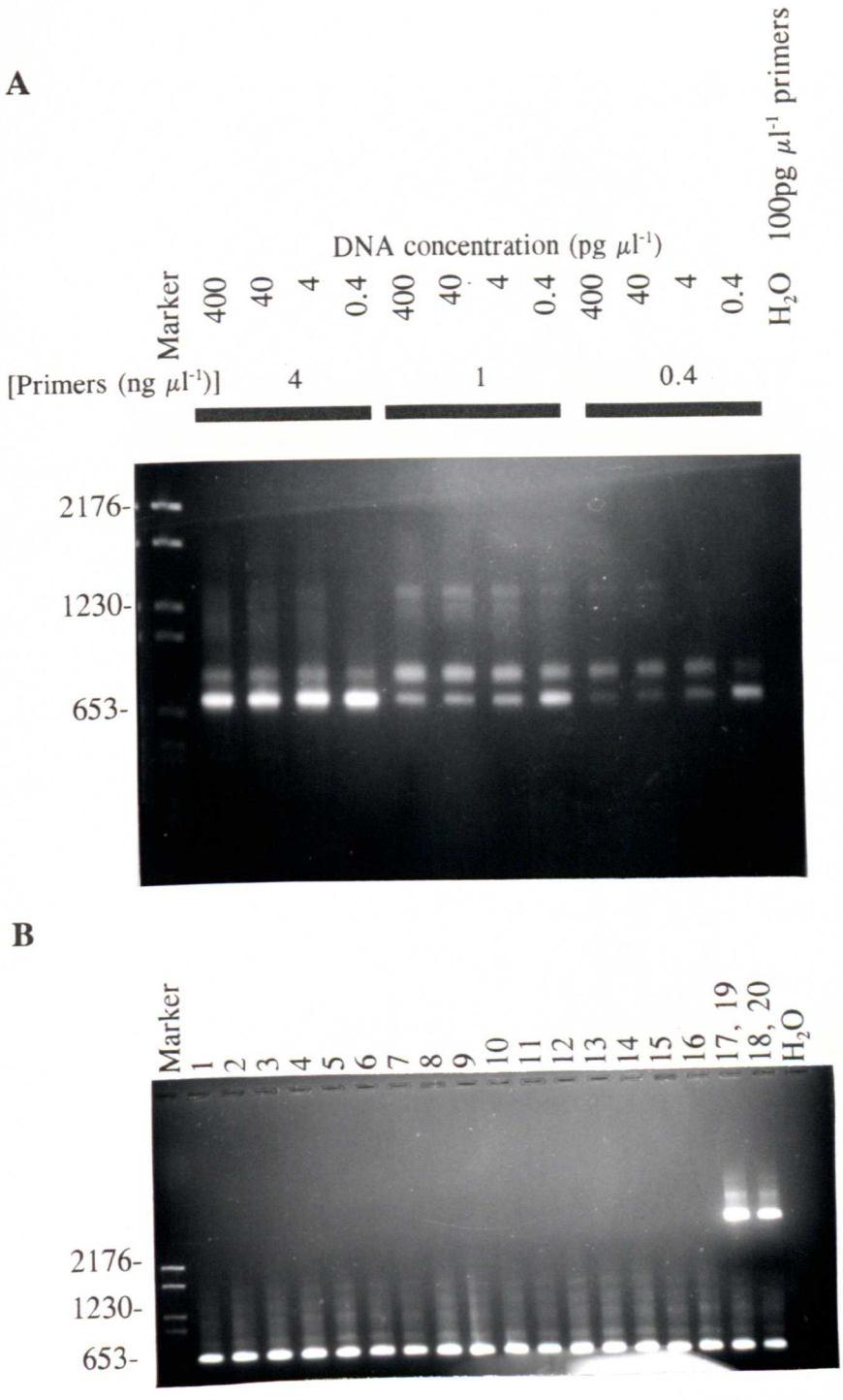


Figure 4.2A and 4.2B. **(A)** Titrations of *L. infantum* LEM188 DNA and 13Z and LiR primers to determine optimum concentrations (Table 4.1). **(B)** Clones 1-20 of *L. chagasi* strain HN29 amplified with primers 13Z and LiR. Clones 19 and 20 were loaded after the other samples had run into the gel. DNA was prepared by incubating 250µl of culture media containing approximately 10^6 parasites per ml at 95°C for 5 minutes. 1µl of the medium was then used in the PCR reaction.

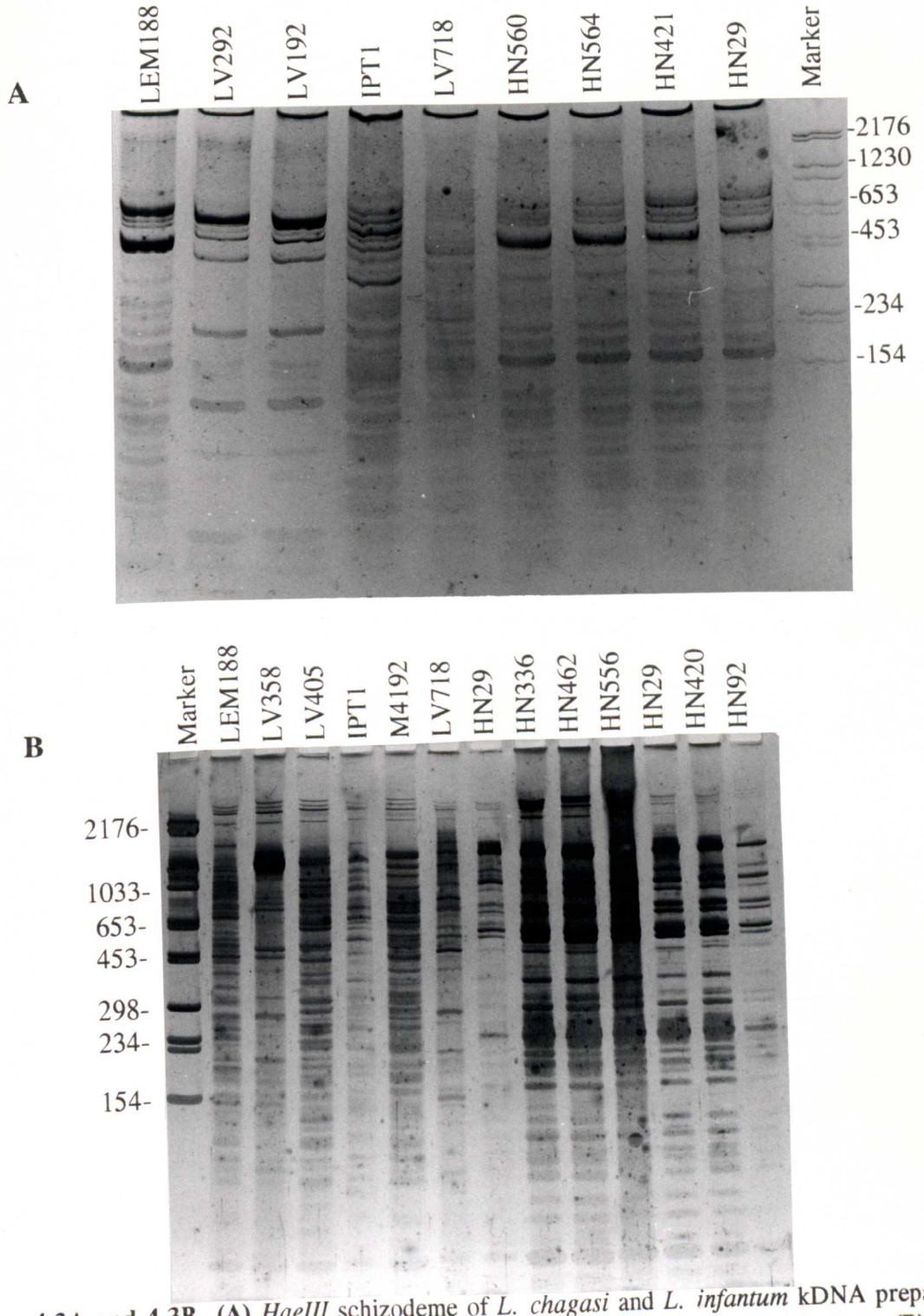


Figure 4.3A and 4.3B. **(A)** *HaeIII* schizodeme of *L. chagasi* and *L. infantum* kDNA prepared by PCR using primers 13Z and LiR run on a 6% polyacrylamide gel and stained with silver. **(B)** *HaeIII* schizodeme of *L. chagasi* and *L. infantum* kDNA prepared by centrifugation of total parasite DNA through 20% succrose. Strong bands in HN strains at approximately 653bp and 200bp are visible in both preparations.

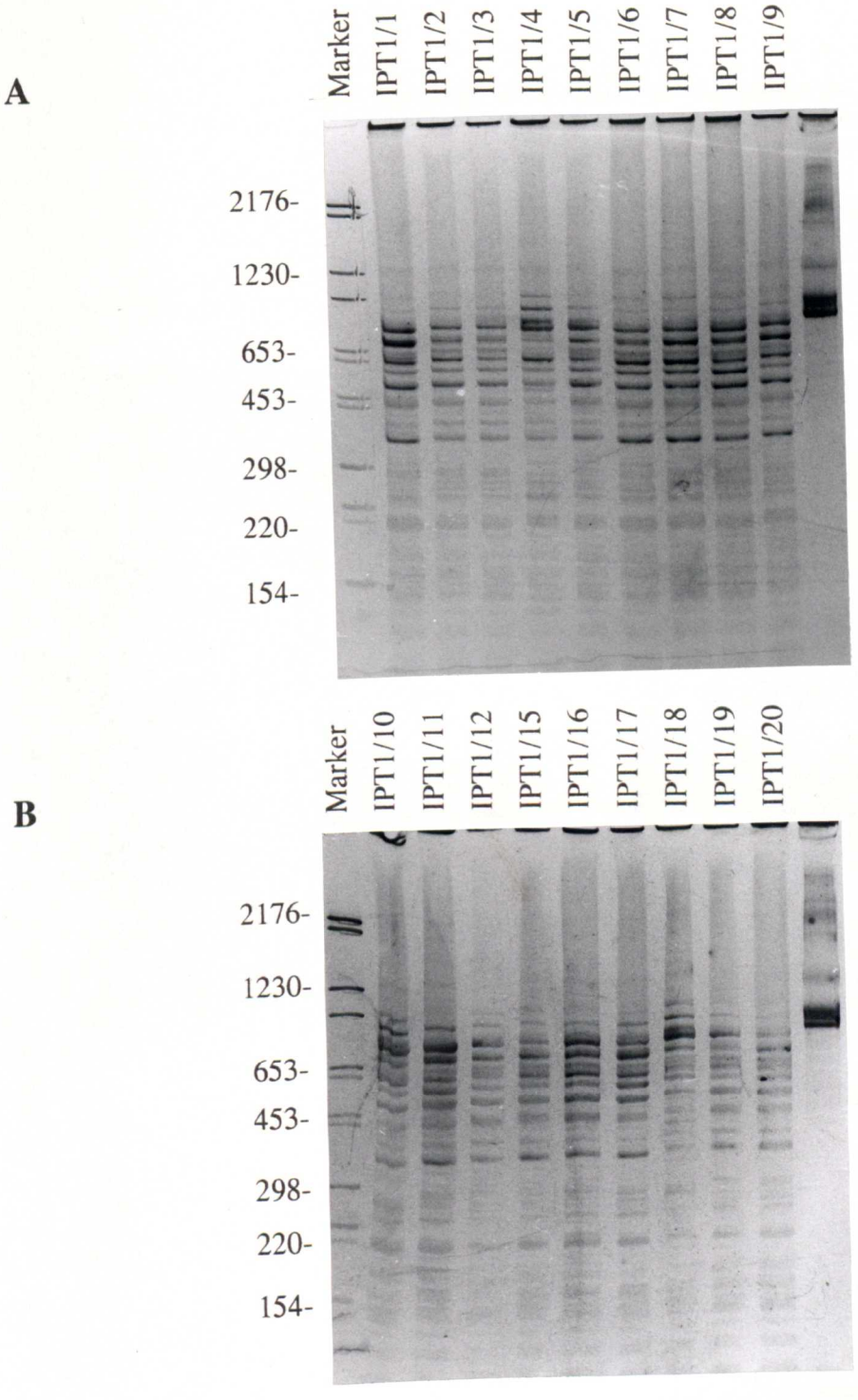


Figure 4.4A and 4.4B. **(A & B)** *HaeIII* schizodemes of kDNA of clones of *L. infantum* strain IPT1. kDNA was prepared by PCR using primers 13Z and LiR. The right hand lane in each panel contains undigested PCR product.

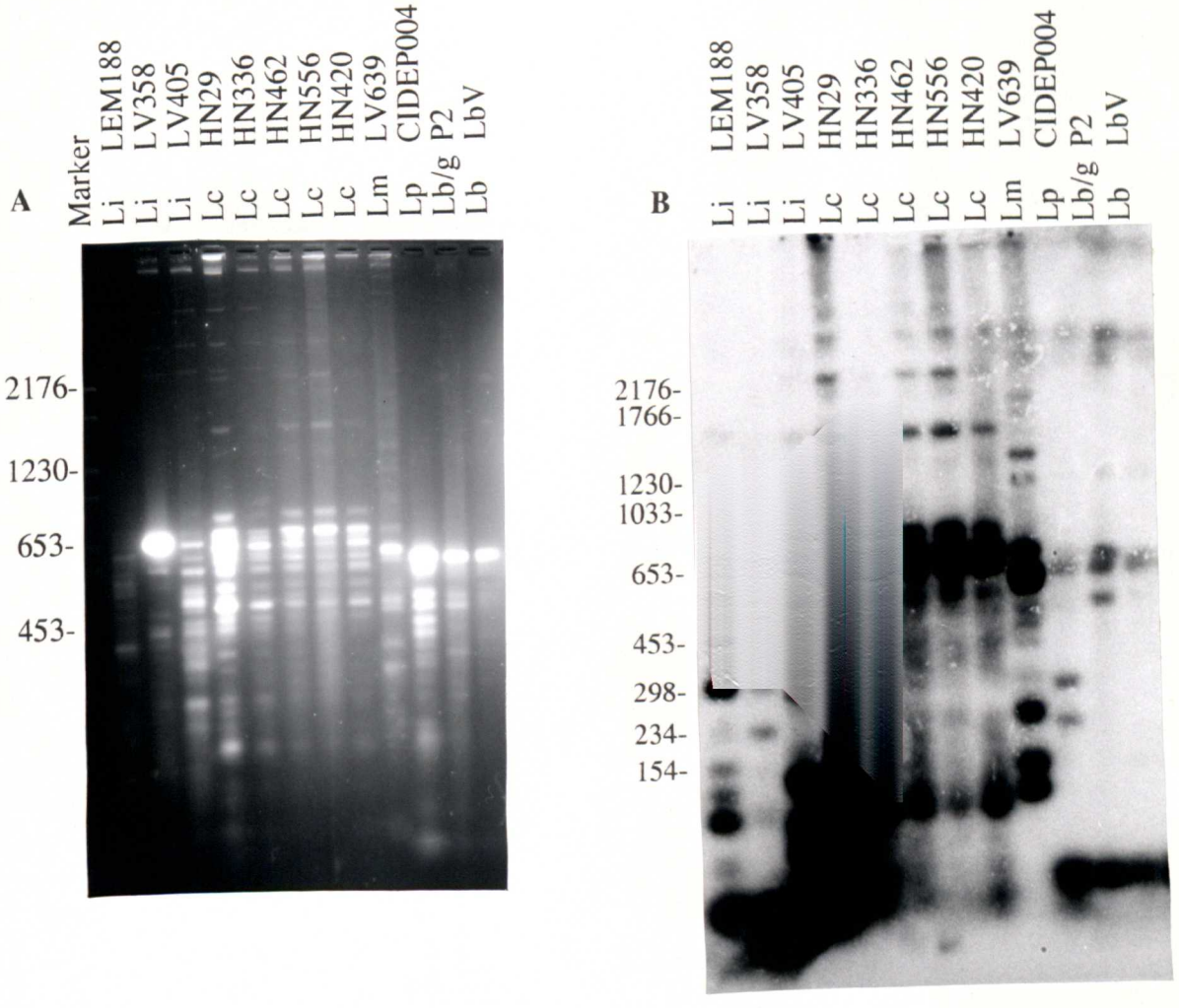


Figure 4.5A and 4.5B **(A)** Ethidium bromide stained 1.5% Nusieve agarose gel of kDNA digested with various restriction enzymes. Strains of *L. infantum*, *L. chagasi* and *L. mexicana* were digested with *HaeIII*. *L. braziliensis*, *L. panamensis* and a putative *L. braziliensis*/*L. guyanensis* hybrid were digested with a mixture of *HaeIII*, *MspI* and *DraI*. kDNA was prepared by centrifugation through 20% sucrose. *L. infantum* (Li); *L. chagasi* (Lc); *L. mexicana* (Lm); *L. braziliensis* (Lb); putative *L. braziliensis*/*L. guyanensis* hybrid (Lb/g); *L. panamensis* (Lp). **(B)** Autoradiograph of a southern blot of the agarose gel in (A) probed with the ^{32}P labelled 13Z PCR primer for the minicircle universal origin of replication. The blot was hybridised at 37°C and washed to a final stringency of 1xSSC, 0.1%SDS at 42°C.

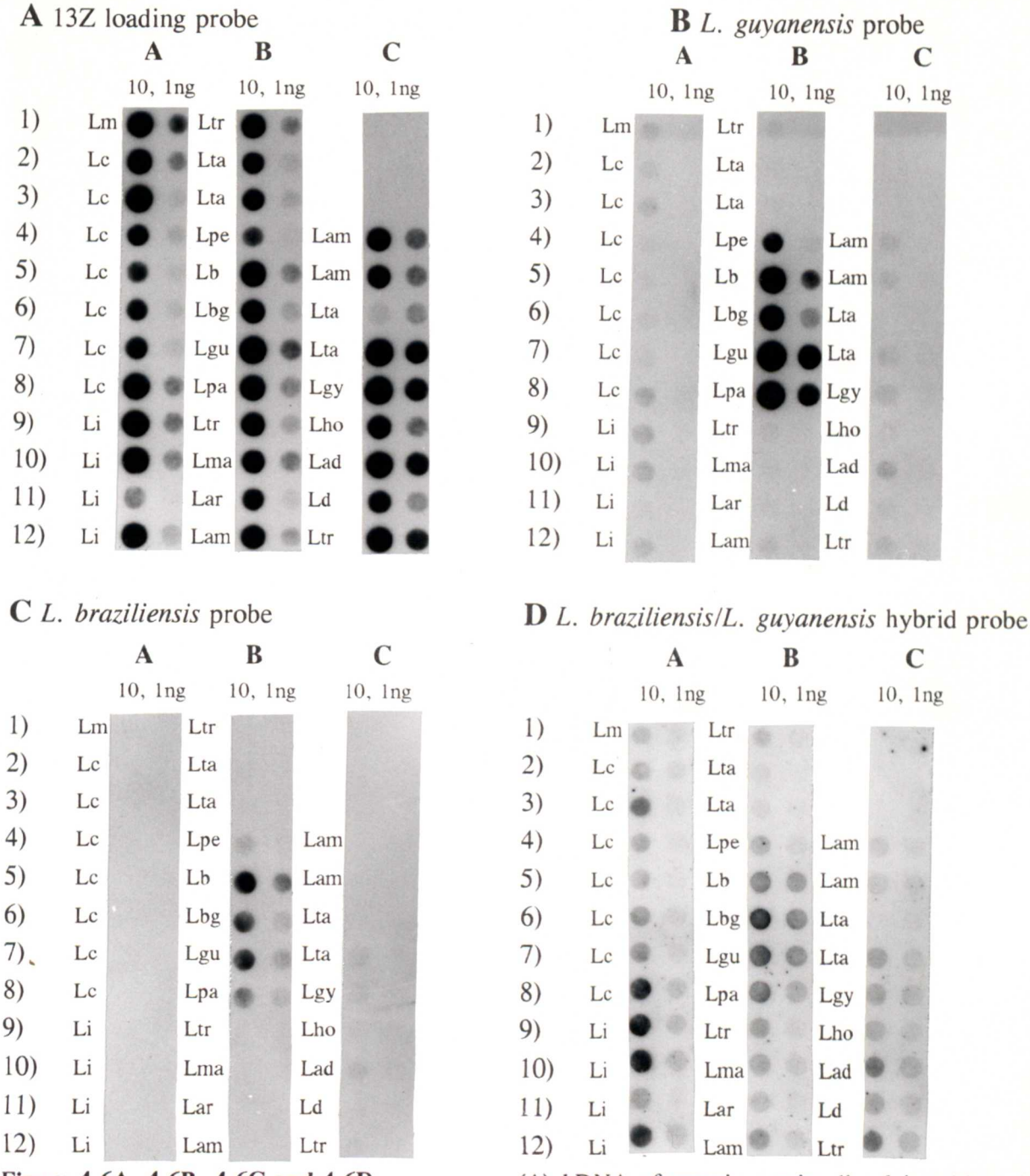


Figure 4.6A, 4.6B, 4.6C and 4.6D. **(A)** kDNA of parasite strains listed in table 4.4 amplified with primers 13Z and LiR dotted on to an Amersham N^+ membrane and probed with PCR primer 13Z which hybridises to the universal minicircle origin of replication to show kDNA loading. The blot was washed in 1xSSC, 0.1%SDS at 37°C and exposed for 24 hours. **(B)** The same dot blot as in (A) probed with kDNA PCR amplified from *L. guyanensis* (M4147) using primers 13Z and LiR. The blot was washed in 0.1% SDS at 55°C and exposed for 4.5 hours. **(C)** The same dot blot as in (A) probed with kDNA PCR amplified from *L. braziliensis* (LbV) using primers 13Z and LiR. The blot was washed in 0.1% SDS at 56°C and exposed for 22 hours. **(D)** The same dot blot as in (A) probed with kDNA PCR amplified from the putative *L. braziliensis/L. guyanensis* hybrid (P2) using primers 13Z and LiR. The blot was washed directly in water with 0.1% SDS at 55°C and exposed for 48 hours. See legend of figure 4.8 for abbreviations.

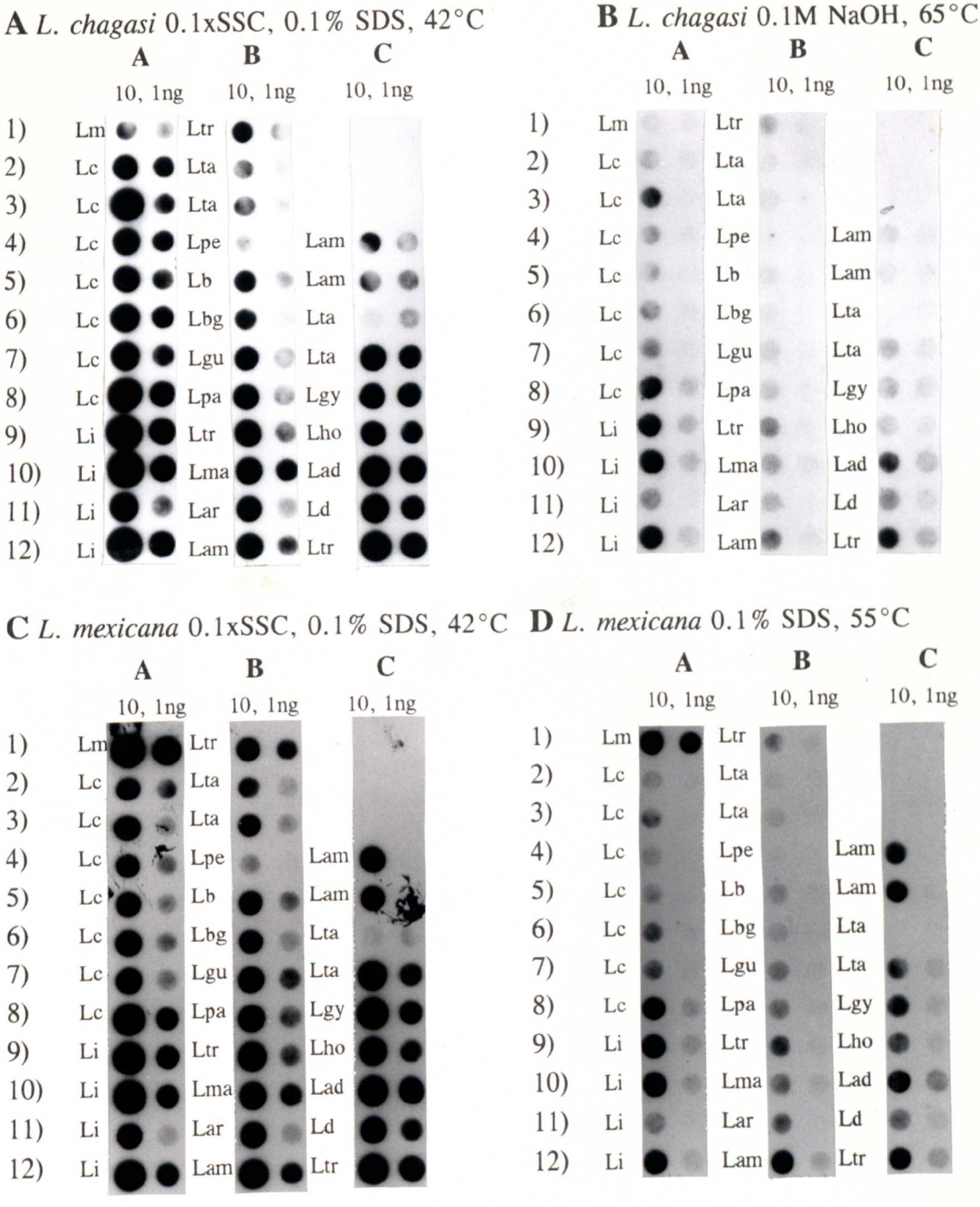


Figure 4.7A, 4.7B, 4.7C and 4.7D. **(A)** kDNA of parasite strains listed in table 4.4 amplified with primers 13Z and LiR dotted on to an Amersham N^+ membrane and probed with *L. chagasi* kDNA which was PCR amplified with primers 13Z and LiR. The blot was washed in 0.1xSSC 0.1% SDS at 42°C and exposed for 2.5 hours. **(B)** The same dot blot as in (A) after stripping in 0.1M NaOH at 65°C for 30 minutes and exposed for 4 hours. **(C)** The same dot blot as in (A) probed with kDNA PCR amplified from *L. mexicana* (M379) using primers 13Z and LiR. The blot was washed in 0.1xSSC, 0.1% SDS at 42°C and exposed for 6 hours. **(D)** The same dot blot as in (C) but washed in 0.1% SDS at 55°C and exposed for 22 hours. See legend of figure 4.8 for abbreviations.

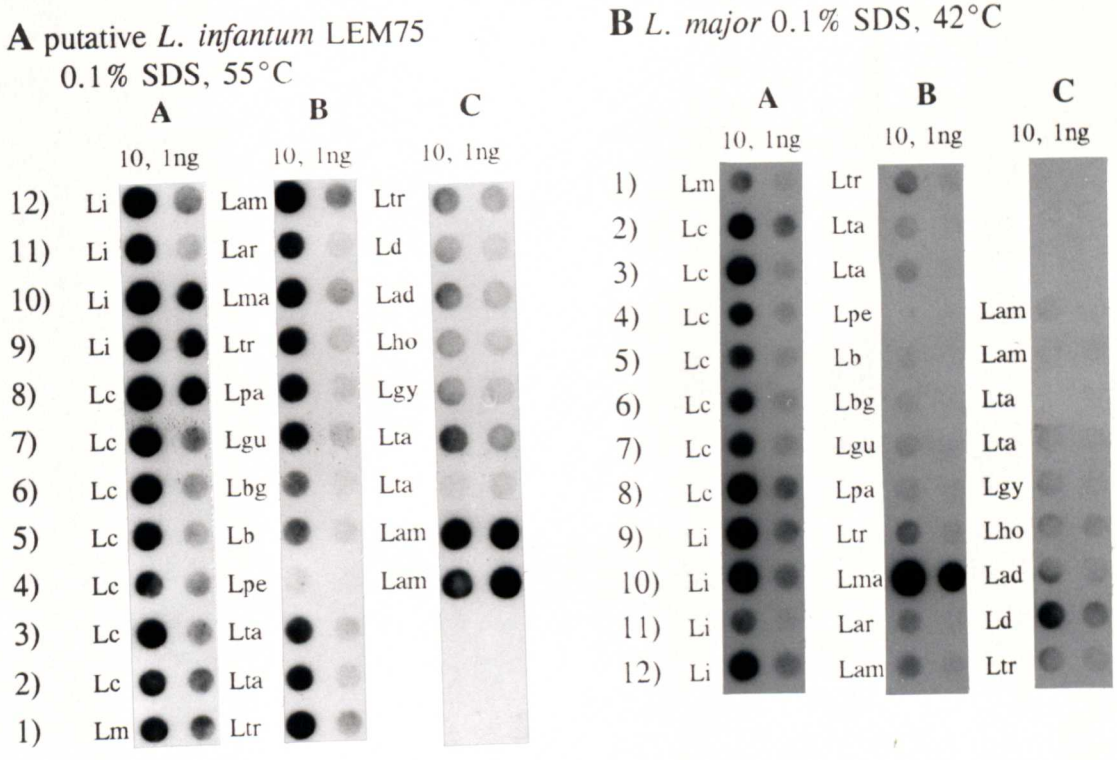


Figure 4.8A, 4.8B. **(A)** kDNA of parasite strains listed in table 4.4 amplified with primers 13Z and LiR dotted on to an Amersham N^+ membrane and probed with LEM75 kDNA which was PCR amplified with primers 13Z and LiR. The blot was washed in 0.1% SDS at 55°C, and exposed for 2.5 hours. **(B)** The same dot blot as in (A) probed with kDNA from *L. major* (LV305) amplified by PCR with primers 13Z and LIR. The blot was washed in 0.1% SDS at 42°C, and exposed for 1.5 hours. Abbreviations: Lad *L. adleri*; Lam *L. amazonensis*; Lar *L. aristidesi*; Lb *L. braziliensis*; Lbg *L. braziliensis*/*L. guyanensis* putative hybrid; Lc *L. chagasi*; Ld *L. donovani*; Lgu *L. guyanensis*; Lgy *L. gymnodactyli*; Lho *L. hoogstraali*; Li *L. infantum*; Lm *L. mexicana*; Lpa *L. panamensis*; Lpe *L. peruviana*; Lta *L. tarentolae*; Ltr *L. tropica*. For strain numbers see table 4.4.

5. SEARCH FOR GENETIC MARKERS FOR *L. CHAGASI* TROPISMS

5.1. RESULTS

5.1.1. RAPD

35 strains of *Leishmania* were received from Honduras which had all been previously identified by isoenzymes by Dr Kreutzer or monoclonal antibodies by Dr C. Ponce and Dr E Ponce or both (Para. 2.1.1). One was *L. braziliensis*, nine were *L. mexicana* and 24 were *L. chagasi*. All these identifications were confirmed by RAPD with the exception of HN294 which had been identified as *L. braziliensis* by isoenzymes by Dr Kreutzer but was found to be *L. chagasi* by RAPD when characterised in Liverpool. Nine strains were found to be *L. mexicana* (Fig. 3.1c) and 26 were *L. chagasi*.

Three of the twelve reference strains of *L. chagasi* and *L. infantum* were unlike *L. chagasi* by RAPD. Two strains of *L. chagasi* (LV639 and LV640) which had been isolated in Honduras from cases of visceral leishmaniasis in 1978 and sent to Liverpool shortly after proved to be *L. mexicana* (Fig. 5.1b). The standard reference strain for zymodeme MON1 used for electrophoresis at Montpelier (LEM75) was unlike *L. infantum* by RAPD (Fig. 5.1b). LEM75 had a RAPD fingerprint similar to *L. mexicana* complex species (not shown), LEM75 kDNA minicircle variable region hybridised strongly to two *L. amazonensis* strains from Panama and to two *L. infantum* strains and an *L. chagasi* strain from Brazil on a dot blot of 16 *Leishmania* species (Fig. 4.8a). A duplicate of LEM75 was obtained and was identical to the first (not shown).

DNA from four *L. chagasi* strains (HN167; HN115; HN29; HN92) was amplified with random primers M13, AB1-AB20, A1, A4, A7, A8, 3301,IL0525, UK1 and UK2 (Table 2.2) and with the following twenty two primer combinations:- AB1-2/AB1-4; AB1-2/AB1-5; AB1-2;AB1-6; AB1-2/AB1-7; AB1-2/AB1-8; AB1-3/AB1-6; AB1-3/AB1-13; AB1-3/AB1-15; AB1-4/AB1-6; AB1-4/AB1-12; AB1-4/AB1-15; AB1-4/AB1-18; AB1-6/AB1-12; AB1-6/AB1-13; AB1-6/AB1-15; AB1-6/AB1-18; AB1-12/AB1-13; AB1-12/AB1-15; AB1-12/AB1-18; AB1-13/AB1-15; AB1-13/AB1-18; AB1-15/AB1-18.

Six *L. chagasi* strains (HN29; HN92; HN421; HN 463; HN 510) and *L. infantum* IPT1 and LEM188 reference strains were compared using primers M13, UK1, UK2, AB1-18, AB1-19, UBC302, UBC303, and AB1-12/AB1-18. None of the primers or primer combinations produced reproducible polymorphisms that distinguished between any of the *L. chagasi*

strains. Figure 5.1a shows the fingerprints produced by primer M13 with six reference strains and three Honduran *L. chagasi* strains, all are indistinguishable.

Primer UK1 generated a polymorphism in *L. infantum* strain LEM188 that distinguished it from the *L. chagasi* stocks (Fig. 5.2a). The polymorphic band was excised from the gel, reamplified with the same primer, and used to probe Southern blots of *XhoI* and *PstI* digests of the same strains. This probe bound to a ladder of fragments with the following approximate molecular weights:- 850, 1450, 2000, 2500bp (Fig. 5.2). There is a band at 850bp visible in the ethidium bromide stained gel in some of the strains which may be the DNA to which the probe binds (not shown). The Southern blot was stripped and reprobed with a *L. chagasi* (HN29) kDNA variable region probe. This probe hybridised to all the bands to which the LEM188/UK1 RAPD product bound and five additional bands as well (not shown), indicating that the band at about 400bp in the UK1 RAPD was derived from kinetoplast DNA.

5.1.2. Isoenzymes

Representatives of reference strains and Honduran test strains were compared by isoenzyme electrophoresis using GPI, G6PD and NH. No differences were detected between any of the test strains and any of the reference strains by this method (Fig. 5.3).

5.1.3. Schizodemes

There was almost complete homogeneity in the PCR based *HaeIII* schizodemes of all of the Honduran *L. chagasi* strains in this study although substantial differences were seen between test strains and reference strains including LV718 from Honduras (Fig. 5.4). Two strains of *L. chagasi* isolated from cases of visceral leishmaniasis in Nicaragua, close to the frontier with Honduras, were identical to each other but quite distinct from the Honduran strains (represented by 92/LV1 in Fig. 5.4). In six Honduran strains (HN420, HN421, HN552, HN556, HN572, HN586) a band at about 400bp was absent that was present in all other strains (arrow in Fig. 5.4). This polymorphism was represented in three out of the four batches received and was identified in isolates from four different departments of Honduras from both visceral and cutaneous cases.

5.1.4. Differential display

cDNA from a sandfly isolate, a visceral isolate and two cutaneous strains of *L. infantum* and *L. chagasi* was amplified with the four polydT_{12} anchored primers dT_{12}NA, dT_{12}NC, dT_{12}NG and dT_{12}NT paired with the following random primers:- P1, P14, AB1-15, UK1, AB1-6, A7, UBC302.

No polymorphisms were found which were markers for the two types of disease caused by *L. chagasi* although a few polymorphisms were identified in individual stocks (Fig. 5.5a). A number of polymorphisms were observed between the *L. infantum* reference strains for cutaneous and visceral disease. The *L. infantum* IPT1 visceral leishmaniasis reference strain fingerprint shared more bands with all the *L. chagasi* stocks than it did with the *L. infantum* LEM188 cutaneous leishmaniasis reference strain, consistent with *L. chagasi* and IPT1 all being from zymodeme MON1.

5.1.5. Pulsed Field Gel Electrophoresis

Karyotypes of 22 Honduran *L. chagasi* isolates were obtained by pulsed field gel electrophoresis together with the *L. infantum* and *L. chagasi* reference strains (Fig. 5.6a). All Honduran *L. chagasi* included in the study appeared to be identical with the exception of HN336 which had a duplicated small chromosome at 300kb (Fig. 5.6a). A gp63 probe bound to two chromosomes at 770 and 750kb in HN462, whereas the gp63 probe bound to a single band at 770kb in all other Honduran test strains (Fig. 5.6b & 5.6c). A telomeric probe failed to reveal any differences between Honduran test strains (Fig. 5.5c). All the reference strains including LV718 from Honduras were different from each other and from the Honduran test strains by all these methods (Fig.5.5 and 5.6). *L. chagasi* M4192 from Brazil had a gp63 gene at about 740kb similar to the Honduran strains in the study, whereas LV718 isolated in Honduras in 1978 had a gp63 gene at 625kb which is similar to the *L. infantum* strains which had genes at 625kb (LEM188), 645kb (LV358), 630/640kb (LV405 and IPT1) (Fig. 8b). A gp46 probe failed to reveal any differences between any of the Honduran strains (not shown).

5.2. DISCUSSION

None of the techniques used to examine *L. chagasi* stocks from Honduras have produced any markers for the two types of disease found there, although PFGE and schizodemes detected slight differences between some of the Honduran test strains.

RAPD was useful for identifying species but detected very few differences between *L. infantum* and Honduran *L. chagasi*. The polymorphic band generated in LEM188 with primer UK1 proved to contain substantial homology to a 850bp fragment in *XhoI* and *PstI* digests of all strains tested and was found to be kinetoplast minicircle DNA (Fig. 5.2). As the RAPD derived probe bound to fewer bands than the probe made with conserved PCR primers which amplify all or most minicircle classes, the RAPD probe may have been from a single minicircle class.

PCR based schizodemes proved to be a simple and specific method for identifying parasite strains. The homogeneity of the Honduran test strains was quite remarkable in comparison with the heterogeneity of the reference strains. Only one polymorphic band was identified which was found in six out of twenty five strains on agarose gels (Fig. 5.4). Other workers have also detected schizodeme differences in parasites with apparently identical karyotypes (Pacheco et al., 1995). There may be corresponding differences in the nuclear DNA between strains that can be distinguished by schizodemes. However pulsed field gels cannot detect size differences of less than a thousand base pairs and are incapable of detecting a single base substitution such as may have given rise to the difference in the schizodemes.

Differential display was also demonstrated to be a powerful method for discriminating between strains. Although much more laborious than PCR based schizodemes it is capable of higher resolution as many more products are detectable on the sequencing gels used to visualise the reactions. Also differential display may provide leads to the identification of virulence genes. The differential display experiment conducted here suffered from two flaws. Firstly promastigotes were used for mRNA preparation and not metacyclics or amastigotes and secondly a sandfly isolate (HN92) was used inadvertently instead of one of the two planned visceral isolates. An attempt was made to prepare axenic metacyclics for this experiment but was unsuccessful. Although axenic *L. chagasi* metacyclics are routinely produced within the Liverpool School, it has been found that *L. chagasi* loses its capacity

to differentiate into metacyclics after no more than five subpassages (H. Zakai, LSTM, personal communication). The Honduran *L. chagasi* had been in culture for some time even before they were sent to Liverpool, so it is unlikely that it would be possible to generate metacyclics from these strains. Consequently it may well be worth repeating the differential display experiment using fresh isolates, maintained in rodents, to produce axenic metacyclics and amastigotes.

The distribution of apparent *L. chagasi* clones in Honduras over time and space is quite remarkable. Twenty five strains collected over nine years from 1987 to 1995 in Southern Honduras and analyzed in four separate batches over two years are all virtually indistinguishable. A single strain LV718, isolated in 1978 and supplied by a separate source is quite distinct, two strains 92/LV1 and 92/LV2 isolated in 1992 in the adjoining part of Nicaragua, were identical to each other and distinct from the Honduran strains by schizodemes (Fig. 5.4). In contrast, Blaineau *et al.* (1992) examined the karyotype of 22 *L. infantum* strains from three closely related zymodemes, collected from sandflies and human cases in a restricted focus of cutaneous leishmaniasis in the Pyrenees. Even though they described the homogeneity amongst these strains as remarkable each strain had a unique karyotype, with the small chromosomes (< 350kb) being particulary variable.

The unexpected homogeneity of the Honduran *L. chagasi* strains compared with European *L. infantum* strains raises two questions:-

- What mechanisms could account for such extensive homogeneity?
- Can the differences in pathology be accounted for in the absence of differences in the pathogen?

5.2.1. Mechanisms that could generate or maintain high levels of homogeneity

It is generally accepted that reproduction in *Leishmania* is clonal, however there is uncertainty about the extent and significance of variation between *Leishmania* clones. No previous study using high resolution techniques such as PFGE have found such extensive homogeneity in the stocks being studied. However a study of the karyotypes of a range of *L. donovani*, *L. infantum* and *L. chagasi* strains found no obvious karyotype differences between four out of five *L. donovani* strains from India, but the fifth strain showed significant differences in the small chromosomes (Bishop & Akinsehinwa, 1989). A study

of *L. peruviana* karyotypes in Peru found substantial though not complete homogeneity within each of three biogeographical areas each bounded by barriers to parasite movement (Dujardin et al., 1993), however a subsequent study found remarkable heterogeneity in RFLPs of the gp63 locus within villages (Espinoza et al., 1995).

Momen and Grimaldi (1987) surveyed over 100 *L. chagasi* strains using isoenzymes and reviewed published reports of a further 38 strains and found that all these strains were identical by at least eleven enzyme systems with the exception of one strain which differed at one locus. In Bangladesh it has recently been found that 15 *L. donovani* parasites from cases of visceral disease and post kala azar dermal leishmaniasis are all identical by enzyme electrophoresis (Elmasum & Evans, 1995).

L. chagasi is believed to have been introduced into South America within the last five hundred years (Momen & Grimaldi, 1987). The lack of diversity of these parasites may be a consequence of the introduction of only one or a few clones which have had insufficient time to diversify. *L. chagasi* was first detected in Central America in 1949 (Zeledon, 1985), although it may have been present for much longer. The *L. chagasi* endemic zone in Honduras was very sparsely populated until the present century. In 1822 the population of Honduras was about 100,000 in a country of 112,000 Km^2, and was concentrated in the mining districts and the wetter central mountains where *L. chagasi* is unknown (Barahona, 1991). So although *L. chagasi* may have been present for several hundred years the current distribution and transmission pattern must have become established in the twentieth century during which the population of the whole country has grown to 4.8 million. The much greater diversity of *L. infantum* in the Mediterranean is consistent with this parasite being endemic in this area for much longer.

mRNAs analyzed by differential display showed greater similarity between the WHO *L. infantum* reference strain IPT1 (zymodeme MON1) from Tunisia and the Honduran test strains than between IPT1 and LEM188 from France (Fig. 5.5a), this is consistent with the recent introduction of MON1 parasites into Honduras from the Mediterranean.

An alternative source of homogeneity is the cross contamination of strains which is probably more common than is generally admitted (Chapter 7, Shaw & Camargo, 1995) and is difficult to disprove. When dealing with closely related strains, which in this case were

indistinguishable by isoenzyme electrophoresis when first characterised by Dr Kreutzer, it is now impossible to demonstrate a change in type, although this might have been revealed if the stocks had been initially characterised by a higher resolution method such as PFGE.

One of the strains supplied however did change in type, HN294 had been characterised by a range of methods as *L. braziliensis* before arriving in Liverpool. This strain died out during early culture rounds, a stabilate was recovered into liquid culture but was found to be *L. chagasi* by RAPD, so at least some cross contamination has occurred. However there are a number of reasons for supposing that cross contamination is not responsible for the similarity of all the Honduran strains. Firstly, small differences were found in the karyotypes of HN336 and HN462 (Fig. 5.6) and in the schizodemes of HN420, HN421, HN552, HN556, HN572 and HN586. Although similar minor karyotype differences have been found between strains on subcloning they have not been reported in uncloned strains (Navarro et al., 1992). Secondly four successive batches of parasites received at Liverpool over a period of 21 months and maintained separately were all substantially identical, so the opportunities for cross contamination were limited to cross contamination within batches.

LV718 which was isolated in Honduras in 1978 was very different from all the Honduran test strains. It is possible that LV718 has been contaminated. Three strains of *L. chagasi* isolated in Honduras in the 1970's were held in the cryobanks at Liverpool, of these, two (LV639 and LV640) were found to be *L. mexicana* on recovery from liquid nitrogen (Fig. 5.1b). Although the third strain (LV718) was identified as *L. infantum/L. chagasi* by RAPD, it is impossible to be confident that it is the same as the original isolate from Honduras. If it is assumed that neither LV718 nor the Honduran test strains have been contaminated, then it is necessary to propose that there has been a dramatic change in the dominant strain of *L. chagasi* in Southern Honduras in less than twenty years. A literature search did not reveal any report of such a change in dominant *Leishmania* type. Consequently it is more likely that cross contamination has occurred than that a change in type has occurred. Given that four separate batches of test strains were all almost indistinguishable, it seems most likely that the cross contamination has occurred in LV718. Honduran *L. chagasi* isolates have been characterised at Montpellier (Moreno et al., 1986), and if they are still available they may be suitable for resolving this problem.

The *L. infantum* reference strain LEM75 was also contaminated (Para. 5.1.1 & 4.4). It is more difficult to dismiss the Nicaraguan strains of *L. chagasi*, these were isolated in Nicaragua in 1992 (Duarte et al., 1994) and DNA prepared by A. Belli at the London School of Hygiene and Tropical Medicine was sent to Liverpool. These strains had a quite distinct schizodeme from the Honduran strains indicating that quite different parasites are circulating in Nicaragua less than 50km from the places where *L. chagasi* parasites were isolated in Honduras and in very similar ecological conditions.

Consequently cross contamination has occurred in one instance in the Honduran test strains (HN294) and at least three instances in the reference strains (LV639, LV640 and LEM75). However, as three different karyotypes and two different schizodemes have been found in the Honduran test strains, cross contamination cannot account for the similarity between all the strains

5.2.2. Possible reasons for different responses to the same pathogen

Although none of the methods used in the present study detected differences between cutaneous and visceral *L. chagasi* isolates from Honduras, it is nevertheless possible that undetected differences in genotype could be responsible for the differences in pathology. However it is also possible that different environments in the vector or the host could elicit different responses from the same parasite.

The critical difference between mice that are resistant or susceptible to acute infection with *L. donovani* appears to be a glycine to aspartate substitution at nucleotide position 596 of *Nramp1*, (Vidal et al., 1995). If a single base difference can control susceptibility in the host, a single base difference could conceivably control virulence in the parasites. The differential display method could in principle detect the deletion of a single amino acid but it would not detect a single base change. Furthermore differential display products range in size from 20-400bp, and as the 3' untranslated region may be several hundred base pairs in length differential display may only detect size differences in a relatively limited region of a gene. RAPD is capable of detecting a single base change but only if the primer binds to the polymorphic site. It would be necessary to screen a very large number of primers to be confident of finding one that would bind to the polymorphic region. Consequently none of the methods we have used would necessarily detect a virulence polymorphism analogous to the polymorphism in the *Lsh* gene.

It is notable that in Southern Honduras *L. mexicana* also causes papular lesions indistinguishable from those caused by *L. chagasi* and does not cause ulcerating lesions. Consequently very different parasites can cause identical pathology and almost identical parasites can cause very different pathologies. This suggests that non parasite factors may be the most important for determining pathology.

A number of host and vector mechanisms have been described which can account for the differences in pathology. Resistance to *Leishmania* has a genetic component (Cabello et al., 1995; Liew & O'Donnel, 1993; Vidal et al., 1995), and nutritional status may also be important (Dye & Williams, 1993). Age at onset seems to be a significant factor for susceptibility to the two types of disease. The median age of patients with cutaneous disease in the present study was 9.5 years (n=17, range 6-15 years). The median age of patients with visceral disease was 2 years (n=5, range 20 months to 2 years) (Table 2.1). 210 children with confirmed visceral leishmaniasis were registered with the Ministry of Health in Honduras between 1974 and 1990. 96% of these 210 cases were under six years old and 85% were under two years old. In contrast all patients with cutaneous leishmaniasis due to *L. chagasi* were over six years old and 90% were between 8 and 16 years old (Drs C. & E. Ponce, Ministry of Health, Honduras, personal communication). In a previous study of the epidemiology of visceral leishmaniasis in Honduras it was found that the median age at onset of visceral disease was 17 months, with 98% being under 43 months (n=64) (Navin et al., 1985).

There is also a significant sex difference in susceptibility, 70% of the 210 children with VL were girls ($P<0.001$). Navin *et al.*. (1985) also found that girls were at higher risk but were unable to identify any activity of young girls that placed them at higher risk of acquiring VL, nor were girls more frequently admitted to hospital.

Vector related mechanisms may also be involved. It has been shown that components of sandfly saliva inhibits IFN-γ mediated macrophage activation to kill parasites (Hall & Titus, 1995). Warburg *et al.* (1994) proposed that concentrations of a vasodilator and of an erythema inducing peptide (maxadilan) in the saliva of *Lu. longipalpis* from Costa Rica caused *L. chagasi* infections to remain local and not become systemic. In contrast, the saliva of Colombian strains of *Lu. longipalpis* is reported to promote early dissemination of the parasite which may enhance the risk of visceral disease. Furthermore the same authors found

that the concentrations of these compounds correlated with putative *Lu. longipalpis* sibling species and with visceral disease in Columbia and Brazil and cutaneous disease in Costa Rica. An examination of the sex pheromones of sandflies found that in Costa Rica there are at least two *Lu. longipalpis* populations separated by sex pheromone mating barriers. In contrast in Honduras, sandflies captured in foci of the two types of disease have the same sex pheromone type suggesting that the same sandfly population is capable of transmitting parasites that cause both pathologies (Hamilton et al., In Press). However sex pheromone mating barriers do not necessarily correlate with maxidilan or vasodilator distribution which are still to be determined for the Honduran sandflies. Alternatively the virulence of an infection may be contingent upon the source and number of sandfly blood and sugar meals prior to the infective bite, as these have all been found to effect transmission in experimental systems (Elnaiem et al., 1994; Schlein & Jacobson, 1994; Warburg & Schlein, 1986) and may effect the outcome of infection in nature.

Different pathologies caused by *L. infantum* in the Mediterranean are correlated with different parasite zymodemes, 90% of strains from 138 VL cases were MON1 whilst only 20% of 92 strains from CL cases were MON1 (Jimenez et al., 1995). This kind of population difference between visceral and cutaneous parasites does not appear to occur in Honduras, even using techniques with a much higher resolution than isoenzymes. However direct comparison is difficult as no isoenzyme differences were found between any of the Mediterranean reference strains from different zymodemes and different pathologies. Only three enzyme systems were tested, GPI, G6PD and NH_1, but in previous studies these have been found to be the most sensitive for distinguishing between *L. infantum* strains (Martin Sanchez et al., 1995; Rioux et al., 1990). The implications of this are not clear as we have found significant differences between these strains by differential display, schizodemes and PFGE. As it has not been possible to reproduce the isoenzyme markers used to distinguish between visceral and cutaneous strains in the Mediterranean it is not possible to say to which group the Honduran parasites are most closely related. Some of the strains used in the present study have been compared with the WHO *L. chagasi* reference strain and found to be identical by 14 enzymes, however NH_1 was not used (Ponce et al., 1991). MON1 parasites have previously been reported from Honduras (Moreno et al., 1986) and the differential display results suggest that the Honduran strains are more like MON1 than MON11. As MON1 is the only *L. infantum*/*L. chagasi* zymodeme found in the New World

(Momen & Grimaldi, 1987; Moreno et al., 1986), it seems likely that the strains used in the present study are also MON1.

5.2.3. Conclusions

In the Mediterranean different pathologies caused by *L. infantum* are correlated with different parasite strains. In contrast there is a unique degree of homology amongst *L. chagasi* strains from Honduras and a clear age difference in susceptibility to the two forms of disease. Consequently, although it should be emphasised that a parasite virulence factor cannot be ruled out, the data presented here suggest that the host and vector are likely to be the major determinants of pathology.

PCR based schizodemes should provide an excellent method for testing this conclusion. It has been demonstrated here that PCR based schizodemes are as sensitive as PFGE for detecting polymorphisms in strains in this study. It should therefore be possible to examine the heterogeneity of parasites directly from clinical samples without an intervening culture step and all the artifacts that that may introduce.

With the increasingly detailed knowledge of the immune response to *Leishmania* and other intracellular parasites it should also be possible to devise studies of cases of cutaneous and visceral leishmaniasis caused by *L. chagasi* that might reveal a differential response to infection with identical parasites.

Southern Honduras may provide an ideal site for both parasite and host studies and the present investigation provides valuable baseline data for future work. As there are few population differences between parasites it may be possible to detect small virulence polymorphisms without the grosser differences between populations introducing excessive noise. A repeat of the differential display experiment using metacyclics and amastigotes may reveal differences that were not detected between promastigotes. It should also be possible to investigate different host responses to essentially the same population of parasites. The Class II MHC is known to control the course of late disease so a study of MHC haplotypes might find correlations between different types and different pathologies. Further studies on vector saliva will also shed light on the contribution of maxadilan and other components to pathology.

Fig. 5.1

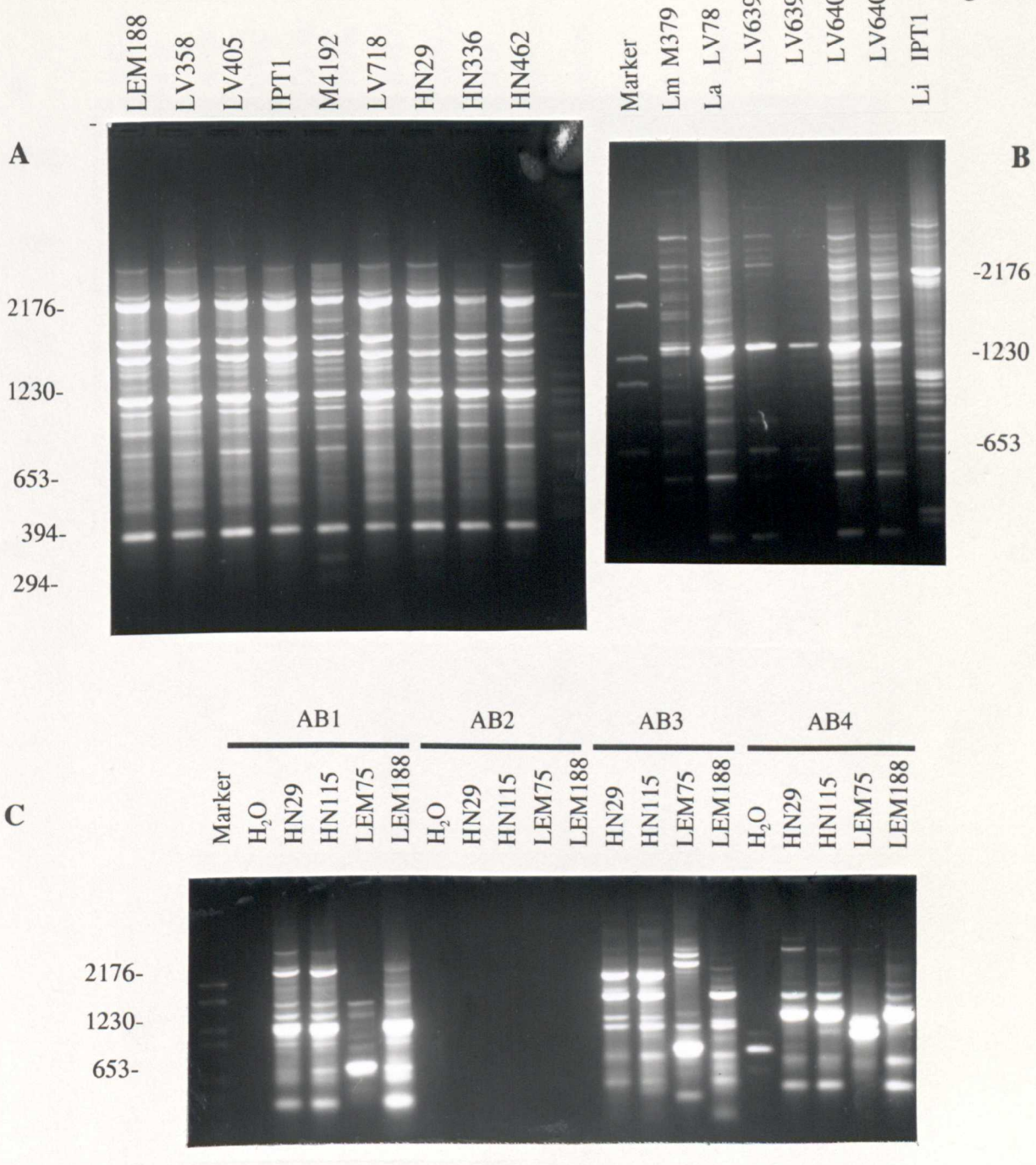


Figure 5.1A, 5.1B and 5.1C. **A** RAPD on six *L. infantum* and *L. chagasi* references strains together with three *L. chagasi* test strains (HN strains) amplified with primer M13, showing monomorphic fingerprints generated by most primers. The same strains were analysed by PFGE and all were distinguishable (Fig 5.5b & c). **B** RAPD on LV639 and LV640 with primer OPA8 and *L. mexicana* and *L. chagasi* reference strains. LV638 and LV639 were expected to be *L. chagasi* but are shown to be *L. mexicana*. **C** RAPD primer screen with primers AB1, AB2, AB3, AB4 using two Honduran *L. chagasi* strains (HN) and two *L. infantum* strains. LEM75 is the MON1 standard reference strain used by Montpellier but showed no similarity to other *L. infantum* in this or any other test. LEM188 from a cutaneous case also shows some differences by these primers but this is probably an artifact as it consistently takes the form of weaker higher molecular weight bands. No differences are observed between the two Honduran strains test strains.

Fig. 5.2

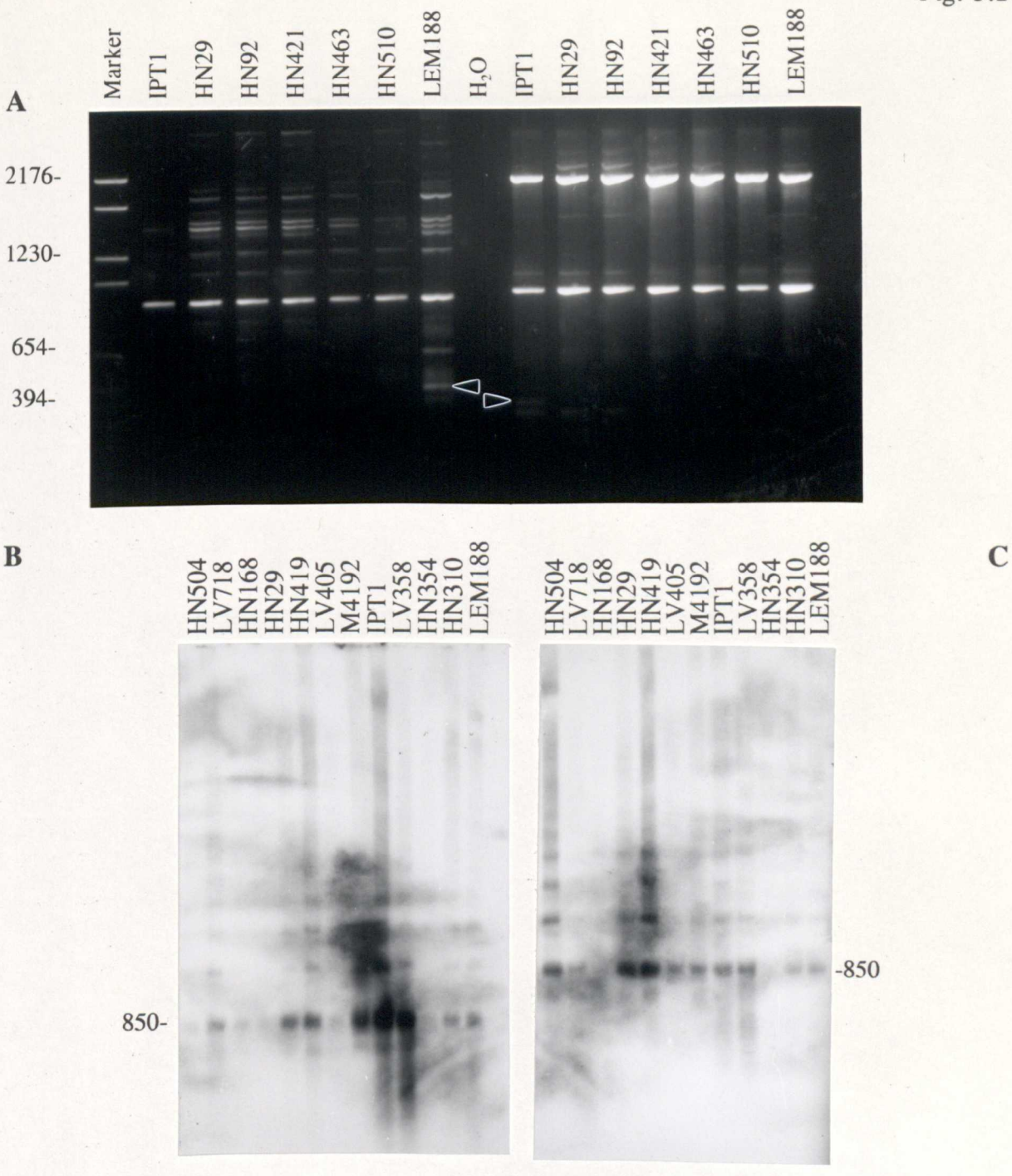


Figure 5.2A, 5.2B and 5.2C **A** RAPD of Honduran *L. chagasi* test strains (HN) and two *L. infantum* reference strains using primers UK1 and AB19. Two products unique to the *L. infantum* strains are visible (Arrows). The LEM188 product at about 400bp amplified by UK1 was excised from the gel, labelled and used to probe southern blots of **B** *XhoI* and **C** *PstI* digests of a number of Honduran *L. chagasi* strains (HN) with *L. chagasi* and *L. infantum* reference strains. The blot was washed to a final stringency of 0.1xSSC at 42°C and exposed for 2.5 hours. Although the RAPD product appeared to be unique to LEM188 it bound to all the strains tested. The differences in intensity correspond to DNA loading differences on the gel. The ladder pattern particularly visible in HN504 and HN419 in both digests is regularly spaced on a logarithmic scale indicating that it is a repeat unit.

Fig. 5.3

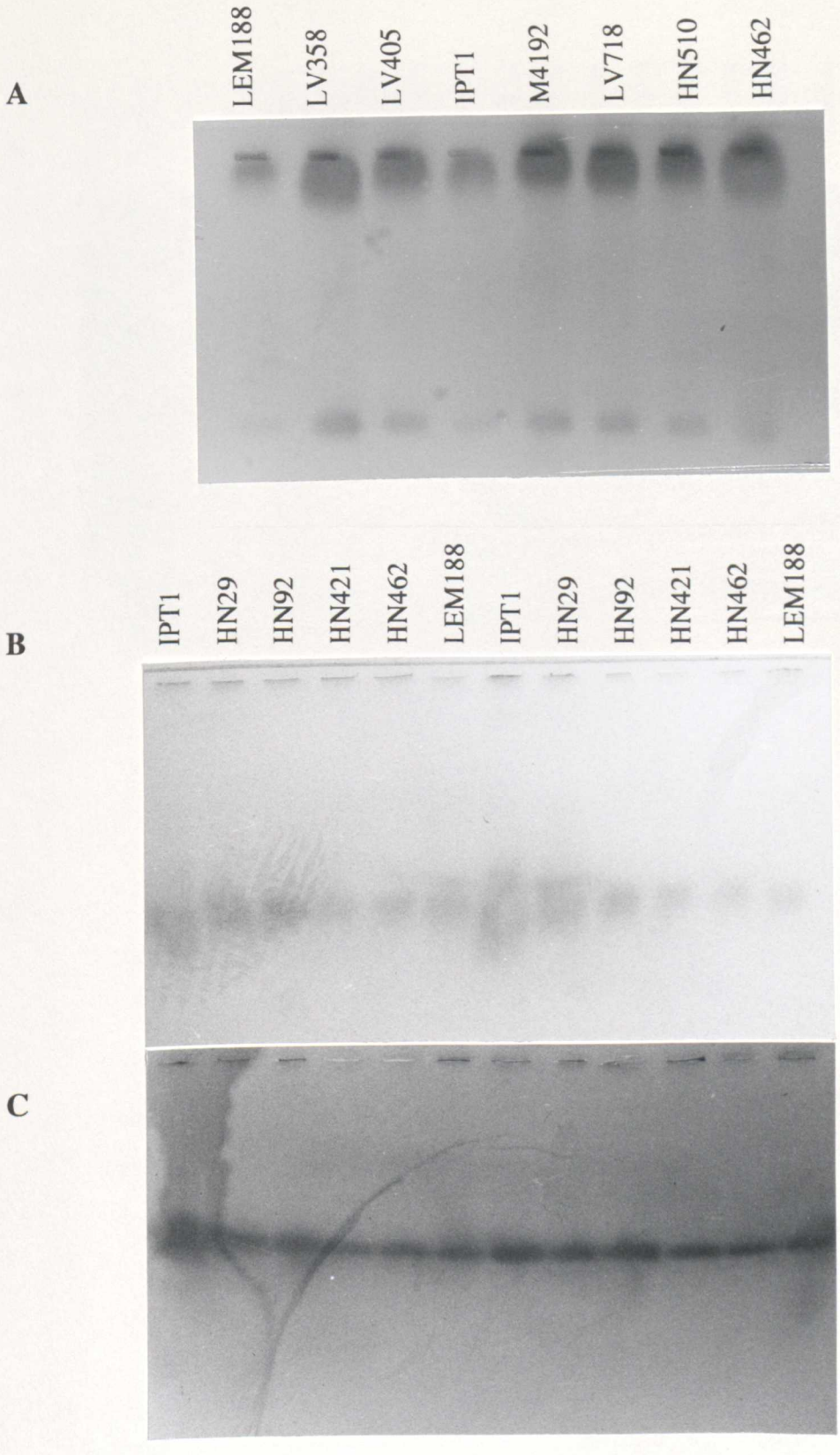


Figure 5.3A 5.3B and 5.3C Isoenzymes of Honduran *L. chagasi* test strains (HN) and *L. chagasi* and *L. infantum* reference strains. **A** NH, **B** GPI, **C** G6PD.

Fig. 5.4

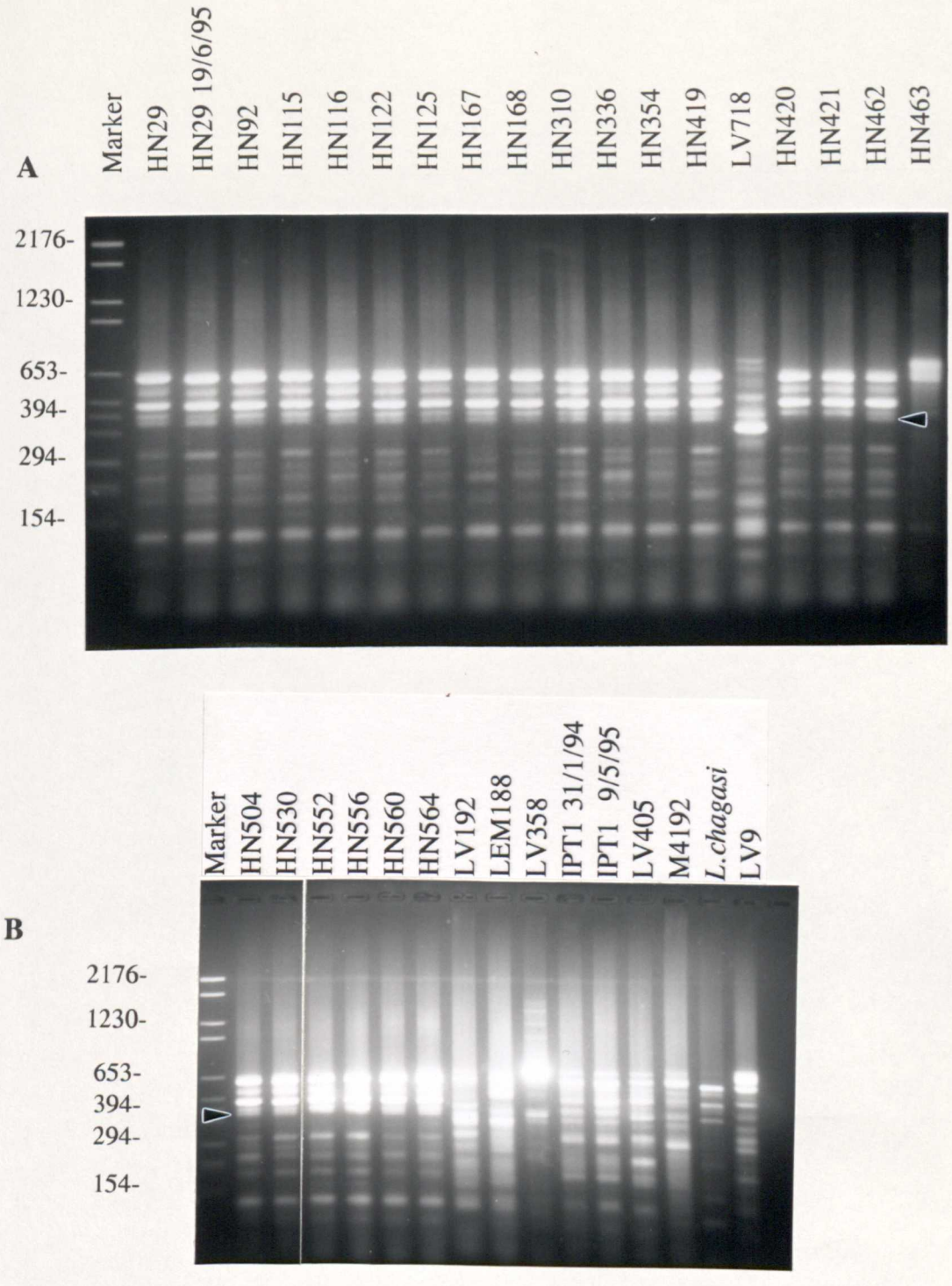


Figure 5.4 Schizodemes of *L. infantum* and *L. chagasi* strains. kDNA was amplified with primers LiR and 13Z and digested with *HaeIII*. DNA for amplification was prepared by melting an agarose block of parasites prepared for PFGE in 0.5ml of ddH_2O and diluting the solution 10:1 with ddH_2O. 1μl of the diluted solution was used in the PCR with the exceptions of HN168, HN564 and LV192 for which PFGE blocks were not available. When two lots of PFGE blocks of a given strain prepared at different times were available they were used and the date of preparation of the blocks is indicated above the strain number. Strains HN463 and LV358 do not appear to have digested fully.

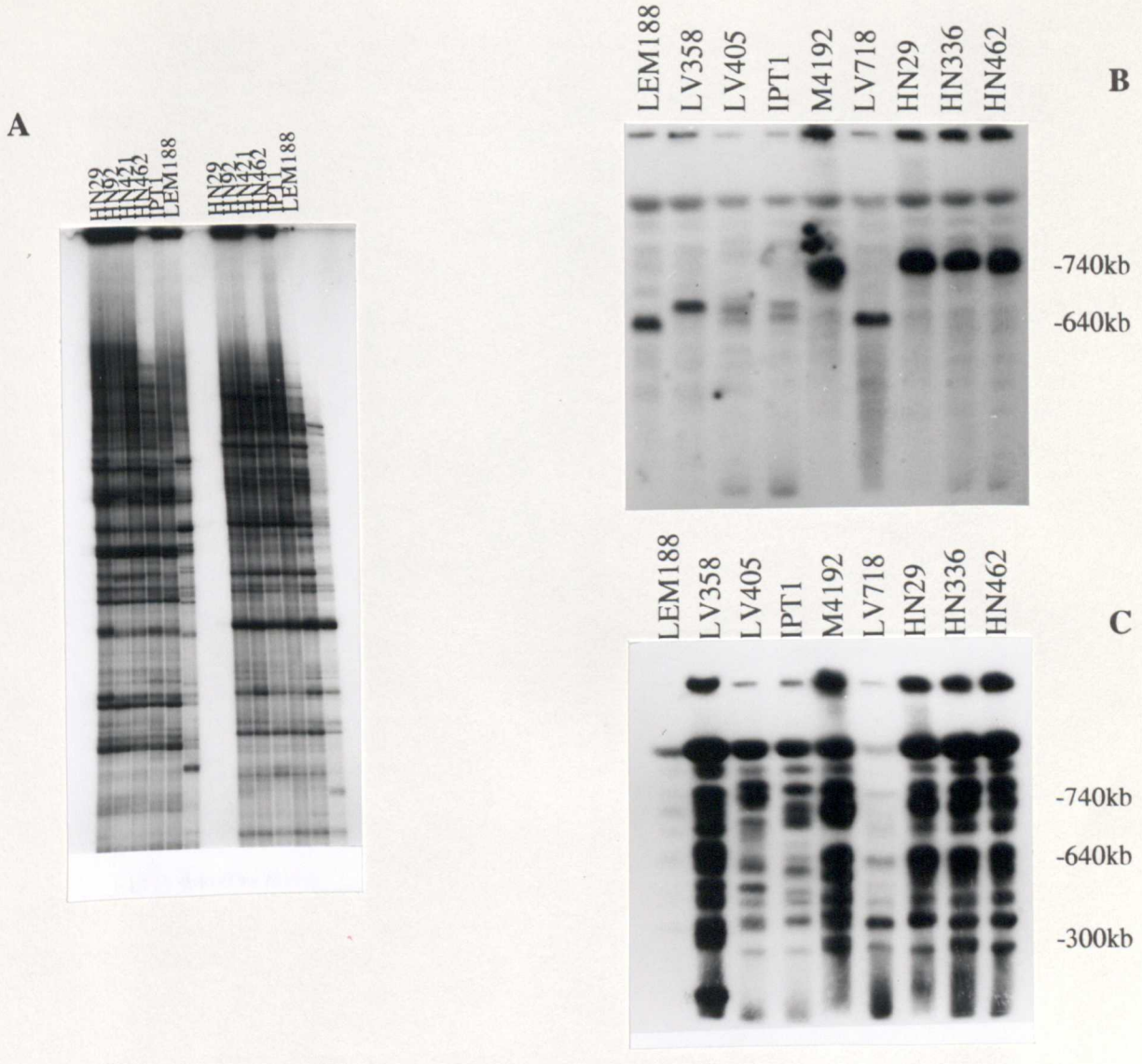


Figure 5.5A, 5.5B and 5.5C **A** Differential display of randomly amplified cDNA from promastigotes of Honduran *L. chagasi* test strains (HN) and *L. infantum* reference strains. A number of differences between LEM188 and the remaining strains are detectable. cDNA was amplified with primer P14 and $dT_{12}NC$ (left hand group) and P14 and $dT_{12}NG$ (right hand group)
B Southern blot of PFGE gel of *L. chagasi* and *L. infantum* reference strains and Honduran *L. chagasi* test strains (HN) probed with a gp63 probe, washed 0.0xSSC 42°C exposed 5 days. LV405 and IPT1 produce doublets with this probe, HN462 is also a doublet (Fig. 5.6b) but is not clearly resolved in this gel.
C The same gel as in B probed with a telomeric probe,. All the reference strains are readily distinguishable but the Honduran *L. chagasi* test strains are almost indistinguishable except a broad band at 300kb in HN336 which is resolved as a doublet in figure 5.6a.

Fig. 5.6

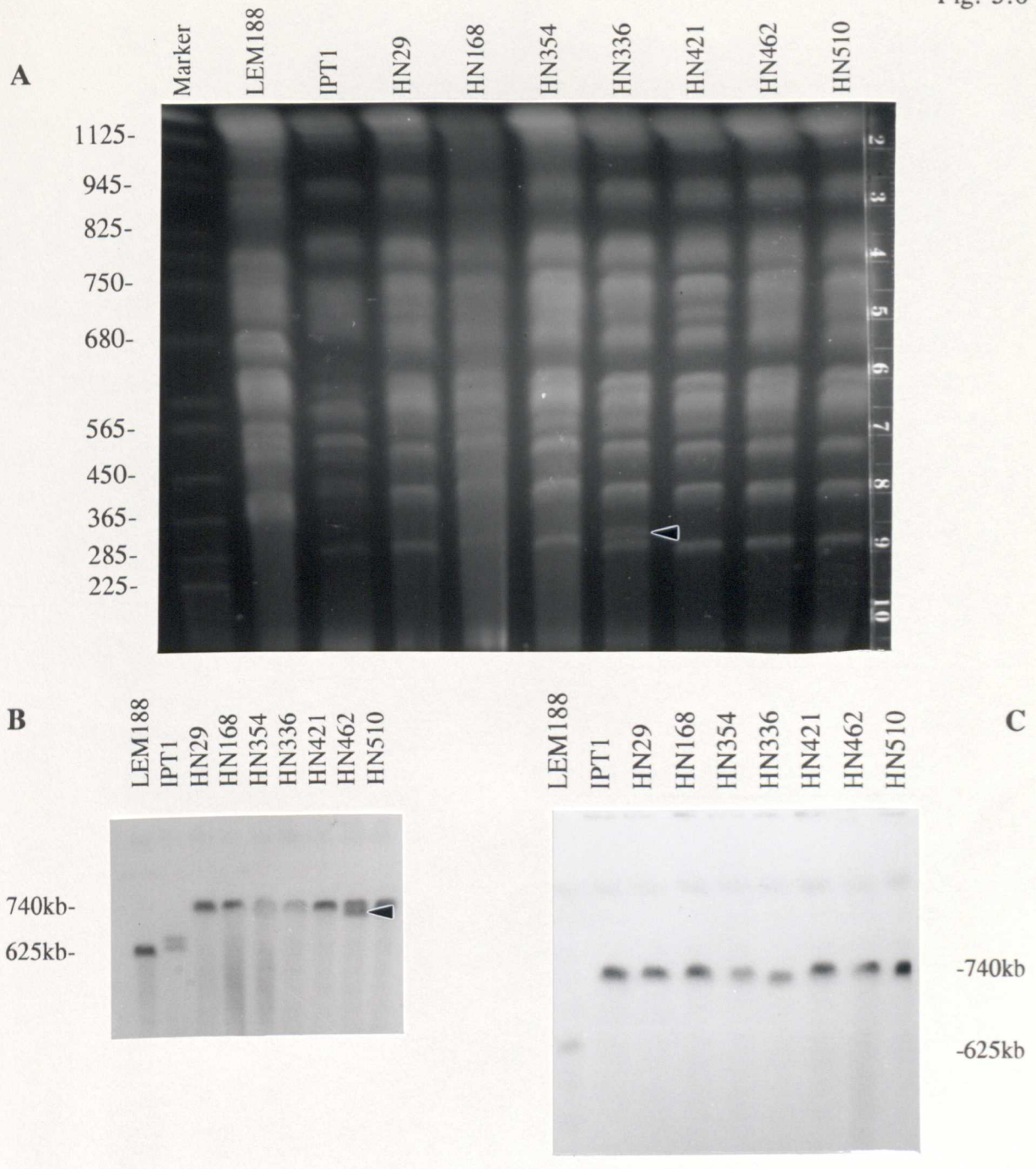


Figure 5.6A, 5.6B and 5.6C **A** Karyotype of Honduran *L. chagasi* test strains (HN) and *L. infantum* reference strains. The gel was run at 120V for 72 hours with a pulse time ramped from 120-160 seconds. The Honduran strains appear indistinguishable except for the duplicated small chromosome at about 300kb in HN336 (arrow). The *L. infantum* strains LEM188 and IPT1 are different from each other and from the *L. chagasi*. **B** Southern blot of the gel in A probed with a gp63 probe and washed in 1xSSC, 0.1%SDS, 42°C. The gp63 gene is located on two different sized chromosomes in HN462 and IPT1. **C** Southern blot of a pulsed field gel of *L. infantum* LEM188 and eight Honduran *L. chagasi* test strains probed with the gp63 probe and washed in 0.1xSSC 42°C and exposed for 18hours.

6. RFLP ANALYSIS OF THE PCR AMPLIFIED SSU rRNA GENE FOR THE IDENTIFICATION AND CLASSIFICATION OF MEMBERS OF THE *LEISHMANIA/ENDOTRYPANUM* CLADE

6.1. RESULTS

6.1.1. RFLPs of the SSU rRNA gene for trypanosomatid identification

Restriction endonucleases that would distinguish between the published sequences of *L. donovani* (GB:X07773), *L. tarentolae* (GB:X53917) and *E. monterogeii* (GB:X53911) SSU rDNA were identified by analyzing the aligned sequences with DM5 (DNA protein and sequence analysis programs version 5.06. Copyright 1987. Genetics Software Centre. Dept. Molecular and Cellular Biology, Biosciences West, University of Arizona, Tucson, Arizona, 85721, USA). DM5 can identify the restriction sites of 92 endonucleases. 81 enzymes were predicted to cleave at least one of the sequences at least once and thirty four enzymes were predicted to distinguish one or more strains (Table 6.1 and Appendix 6.1).

Table 6.1
Restriction enzymes predicted to distinguish between *L. donovani*, *L. tarentolae* and *E. monterogeii*

Taxa resolved:	Enzymes
L. tarentolae, *L. donovani* and *E. monterogeii*	***Sau96I, HaeIII, NspBII, RsaI***
L. donovani	*BinI, EcoPI, GsuI, HaeI, HinfIII, HphI, PflMI, PvuII, MnlI*
L. tarentolae	*BbvI, TaqII*
E. monterogeii	***AluI***, *CauII, Cfr10I, CfrI, Eco47III, FokI, GdiII, HaeII, HgaI, HgiAI,* ***HhaI, HpaII,*** *MnlI, NaeI, Nla1V,* ***SacI,*** *ScrFI,* ***TaqII,*** *XmaIII*

Table 6.1 Enzymes that were predicted to generate a unique fingerprint pattern in the strains indicated. Unique fingerprint patterns are produced either because a given strain contains a restriction site not present in the other strains or because it does not contain a site that is present in both the other strains. Enzymes in bold were used in the present study. Not all enzymes are commercially available e.g. *TaqII*.

6.1.2. RFLP analysis of a 561bp variable region of the SSU rRNA gene.

A minimal region of the SSU rRNA gene, between bases 901 and 1461 in the *L. donovani* (GB:X07773) sequence, was identified that would produce a dendrogram of the gene similar to published classifications (Para. 2.4.4)

Primers SSU561F and SSU561R (Table 2.5) efficiently amplified an approximately 561bp fragment from all trypanosomatid genera tested;- *Leishmania*, *Endotrypanum*, *Crithidia*, *Herpetomonas* and *T. cruzi*. The only species that could be distinguished on agarose gels was *T. cruzi* which has a 70bp insertion (not shown). Restriction digests of these products were analyzed on agarose gels.

Using *HaeIII* and *HpaII* all mammalian and lizard *Leishmania* were indistinguishable with the exception of the *L. hertigi* complex which shared the *E. monterogeii* banding pattern with the *HpaII* and had a unique fingerprint with *HaeIII* (Fig. 6.1). Using *AluI* a 300bp product was found in *L. (Leishmania)* and all lizard *Leishmania*, except LV108, that distinguished them from *L. (Viannia)* and *Endotrypanum*, however *L. (Viannia)* and *Endotrypanum spp.* appeared identical with *AluI*. *L. major* (LV305), *L. tropica* (ARD), and *L. gymnodactyli* appeared to have a doublet at about 300bp, although this was not well resolved on agarose gels, and these strains lacked or only had a very weak product at 150bp (Fig. 6.2b).

SacI has a six base recognition site (GAGCTC) which contains the *AluI* recognition site (AGCT) within it. *SacI* therefore recognises a subset of the *AluI* sites. *SacI* recognised a single site within the 561bp SSU rDNA region which was present in all lizard *Leishmania* except *L. tarentolae* (LV108) and M379 the only *L. (Leishmania)* tested (Fig. 6.2a).

6.1.3. RFLP analysis of the complete SSU rRNA gene

The PCR reaction was less efficient when using primers WSSUF and WSSUR which amplified almost the complete SSU ribosomal gene (2112/2205bp). Restriction digests of WSSUF and WSSUR products were analyzed on silver stained polyacrylamide gels.

HaeIII, HinfI, HpaII, RsaI and *Sau96I* restriction digests of the complete SSU rRNA gene amplified from representatives of *Leishmania* and *Endotrypanum* were compared in order to find fingerprints suitable for the identification of these genera (Appendix 6.1 and Fig 6.3). *HaeIII* discriminated the lizard and mammalian *Leishmania* and the *Endotrypanum*, but produced a complex fingerprint with only slight differences between strains (6.3c & 6.3d). *RsaI* was predicted to generate a product of 1101bp in *L. tarentolae* and two products of 1021bp and 78bp in *L. donovani* but neither of these latter were found (Fig. 6.3e). In a repeat *RsaI* produced the 1021bp product in *L. donovani* and *L. infantum* but not other

Leishmania species (Fig. 6.4). *HpaII* produced a fragment at 319bp in G755, LV344, *Endotrypanum spp.* and *L. deanei* and a fragment at 240bp in all other lizard and mammalian *Leishmania* (Fig. 6.3a). *HinfI* produced the same fingerprint pattern in all *Leishmania* and *Endotrypanum* tested and the outgroup G755 was the only strain distinguished by this enzyme (Fig. 6.3f). However *HinfI* is unlikely to be a suitable marker for the *Leishmania/Endotrypanum* clade as *C. fasciculata* and *Leptomonas* are predicted to have very similar fingerprints to *Leishmania* with this enzyme (Appendix 6.1). The putative *L. herreri* strain LV344 appeared identical to *E. monterogeii* and *E. schaudinni* with all these enzymes. The enzyme that most clearly resolved the mammalian and lizard *Leishmania* and *Endotrypanum* was *Sau96I*. With this enzyme a product at 1009bp was found in *Endotrypanum* and LV344, a product at 656bp was found in the lizard species and products at 452 and 205bp were found in the mammalian *Leishmania*. *L. deanei* had a unique restriction pattern with a fragment at 1200bp (Fig. 6.3b).

6.2. RFLPS OF THE SSU rRNA GENE FOR THE CLASSIFICATION OF TRYPANOSOMATIDS

A dendrogram was prepared from the data obtained from *HaeIII, HinfI, HpaII, RsaI* and *Sau96I* restriction digests of the complete SSU rRNA gene and the *AluI, HhaI* and *SacI* digests of the 561bp region by calculating Nei's distance from the presence/absence of bands, the distances were entered into a distance matrix (Fig. 6.5), from which dendrograms were prepared by UPGMA and FITCH programmes in PHYLIP (Fig. 6.6). The restriction fragments used in this calculation are shown in Appendix 6.2.

To prepare the data set for analysis it was necessary to compare the predicted and observed restriction patterns, as partial digestion could give rise to additional bands that would increase the observed level of similarity. The presence of the predicted cleavage products of a larger fragment was a reliable indicator of partial digestion. For example *Sau96I* is predicted to cleave the 2112bp PCR product of *Endotrypanum* at 713, 1724 and 1929bp producing products of 713, 1011, 205 and 183bp and to cleave *L. tarentolae* at 715, 1275 and 1931bp generating products of 715, 560, 656 and 181bp. In Figure 6.3b the predicted products are clearly visible, however some lizard *Leishmania* have additional products at about 900 and 1000bp. The product at about 900bp could be a result of partial restriction at the 1931bp site leading to a product of 937bp, the origin of the 1000bp product is not clear. As the expected products are present and their sizes add up to the 2112bp length of

the PCR product these anomalous bands were disregarded in the calculations of similarity. For species for which there is no published sequence the sum of the sizes of the products was checked, as was the intensity of the bands relative to their size. This procedure led to the selection of the following *L. deanei Sau96I* bands for inclusion in the similarity analysis:- 1200bp, 718bp and 180bp adding up to 2098bp, bands that were weak for their size at 1000bp and above 1200bp were disregarded.

Three dendrograms were compiled using the data from the RFLPs. Figure 6.6a shows a dendrogram prepared using UPGMA on the distance matrix in figure 6.5. Figure 6.6b shows a dendrogram using the FITCH parsimony method on the distance matrix in figure 6.5. A dendrogram prepared using the parsimony programme MIX on the raw presence absence data (Appendix 6.2) had essentially the same topology (not shown) as that found by UPGMA (Fig. 6.6a). A bootstrap analysis with 100 replicates was performed on the raw presence absence data which was then analyzed using Wagner parsimony in the MIX programme. This procedure yielded the bootstrap values shown at the nodes in figure 6.6a.

In the dendrograms (Fig. 6.6) *L. herreri* was indistinguishable from *Endotrypanum*, and the lizard *Leishmania* were at the tip of the *Leishmania* clade.

Attempts to amplify the complete SSU rRNA gene of *L. hertigi*, using primers WSSUF and WSSUR, failed but the 561bp variable region of the *L.hertigi* and *L. deanei* SSU rRNA genes were compared with 3 enzymes (*HaeIII* and *HpaII* in Fig. 6.1, and *AluI* Fig. 6.2b) and were identical with all of them. The complete SSU rDNA sequence of *L. deanei* did amplify and, depending on how the data was analyzed, *L. deanei* either clustered with the *Leishmania* or the *Endotrypanum* (Fig. 6.6). 90% of the bootstrap replicates supported the clustering of *L. deanei* with *Endotrypanum* but there was still one out of ten trees in which *L. deanei* would cluster with *Leishmania*. In the distance matrix (Fig. 6.5) *L. deanei* is about equidistant from *Endotrypanum* (0.44), and *Leishmania* (0.48) and is closest to the lizard *Leishmania* (0.39).

The sequence of the *L. hertigi* (LV42) 561bp region was obtained in order to clarify the classification of this species complex. This sequence is shown in Appendix 6.3 and the alignment of the sequence with other trypanosomatids is shown in Appendix 7.2. Classifications of this sequence were prepared by a distance matrix method using DNADIST

followed by FITCH in PHYLIP (Fig. 7.8 & 7.9) and by a parsimony method using DNAPARS in PHYLIP (Fig. 7.9). The classifications show that this sequence of *L. hertigi* is more closely related to the corresponding sequence in *Endotrypanum* than to that of *Leishmania*, which supports the classification based on the RFLPs of the SSU rRNA gene using the MIX and UPGMA programmes. The classification of *L. hertigi* with *Endotrypanum* was supported in 91% of bootstrap replicates using the parsimony method and 94% of the replicates using the distance method.

The relationships between members of the *Endotrypanum/L. herreri* group were investigated using RAPD with primer M13 (Fig. 6.7). The fingerprints indicated that *E. schaudinni* (LV58) was more similar to *E. monterogeii* than to *E. schaudinni* (LV59). *L. herreri* LV341 and LV342 were similar to each other and to *E. monterogeii* and *E. schaudinni* (LV59), whereas *L. herreri* LV344 appeared distinct from all other *Endotrypanum* strains by this method. The absence of variation between nine *L. infantum* and *L. chagasi* strains is shown for comparison.

The crosshybridisation of kinetoplast variable region DNA from lizard and mammalian *Leishmania* together with putative *L. herreri* strains and *Endotrypanum* strains was investigated using dot blots (Table 6.2 and Fig. 6.8). A LV341 derived probe hybridised strongly to 1ng of kDNA from LV341 and LV342 but only to 10ng of LV344 kDNA. A LV344 derived probe only hybridised strongly to 1ng of LV344 kDNA (not shown). An *E. monterogeii* LV88 probe also only hybridised strongly to 1ng homologous kDNA and not to other *Endotrypanum* strains. (Fig. 6.8b).

A *L. deanei* (LV402) probe hybridised strongly to the homologous kDNA, less strongly to the *L. hertigi* (LV42) kDNA despite higher loading, and very weakly to all other *Leishmania* and *Endotrypanum* strains (6.8c).

A *L. tarentolae* (LV414) kDNA probe hybridised strongly proportionately to loading to 1ng of all other lizard *Leishmania* tested, weakly to 10ng of all *L. (Leishmania)* tested and did not hybridise to *L. (Viannia)* (Fig. 6.8d).

	A	B
1)	*L. donovani* (LV9; **Ld**)	*L. adleri* (LV30; **Lad**)
2)	*L. infantum* (IPT1; **Li**)	*L. hoogstraali* (LV31; **Lho**)
3)	*L. mexicana* (M379; **Lm**)	*L. gymnodactyli* (LV247; **Lgy**)
4)	*L. amazonensis* (LV78; **Lam**)	*L. tarentolae* (LV108; **Lta**)
5)	*L. major* (LV305; **Lma**)	*L. tarentolae* (LV414; **Lta**)
6)	*L. major* (LV561;C22; **Lma**)	*L. herreri* (**LV344**)
7)	*L. tropica* (ARD; **Ltr**)	*L. herreri* (**LV342**)
8)	*L. tropica* (LV357; **Ltr**)	*E. schaudinni* (**LV58**)
9)	*L. panamensis* (LS94; **Lpa**)	*E. schaudinni* (**LV59**)
10)	*L. guyanensis* (M4147; **Lg**)	*E. monterogeii* (**LV88**)
11)	*L. braziliensis*/*L. guyanensis* hybrid (P2; **Lbg**)	*L. deanei* (LV402; **Lde**)
12)	*L. braziliensis* (LbV; **Lb**)	*L. hertigi* (LV42; **Lht**)

Table 6.2 Parasite strains on dot blots of kDNA in figure 6.8. Abbreviations or strain numbers in bold are used to label the dots in the figure.

6.3. DISCUSSION

6.3.1. RFLPs OF THE SSU rRNA GENE FOR THE IDENTIFICATION OF MAMMALIAN AND LIZARD *LEISHMANIA* AND *ENDOTRYPANUM*

6.3.2. PCR primers for the SSU rRNA gene

There are numerous published primers that will amplify all or part of the SSU rRNA gene (Clark et al., 1995; Du & Chang, 1994; Fernandes, A.P. et al., 1993; Marché et al., 1995; van-Eys et al., 1992), however none of these were used, because either primers appeared unnecessarily long or they could not be identified in the coding sequence of *Leishmania* SSU rRNA. The two pairs of primers used in the present study were designed for distinct purposes. The primers for the 561bp variable region were designed to produce a fragment short enough to be sequenced in a single pass on an automated DNA sequencing machine whilst still suitable for the classification of trypanosomatids. The primers for the complete SSU rRNA gene were designed to produce DNA suitable for RFLPs which could be used to identify genera. In practice both sequences were used for both classification and identification.

Primers SSU561F and SSU561R reliably produced concentrated PCR products ($>20ng\ \mu l^{-1}$) even from the dry waxy pellets of old (>1 year) crude lysates. These PCR products were very suitable for RFLP analysis. Although some workers have purified PCR products by ethanol precipitation prior to digestion (Avila et al., 1990) this was not found to be necessary and $10\mu l$ of PCR product mixed with $10\mu l$ of 2x enzyme mixture gave reliable results. Enzymes that would produce characteristic fingerprints suitable for the identification of a number of taxa were identified (Table 6.1).

The amount of information available in RFLPs of the 561bp sequence is clearly less than in the complete gene sequence even though the complete sequence contains extensive conserved regions. Therefore for classification purposes it was considered important to examine the complete SSU rRNA gene. PCR using the WSSUF and WSSUR primers designed to amplify the complete 2240bp SSU rRNA gene was less efficient and less reliable than PCR using the SSU561F and SSU561R primers. WSSUF and WSSUR products were not obtained from some samples and the concentration of product was variable ($0\text{-}15ng\ \mu l^{-1}$) and lower than that obtained from SSU561F and SSU561R primers (approximately $20ng\ \mu l^{-1}$). The differences in amplification efficiency may be due to the location of the two sets of primers in different types of secondary structure of the SSU rRNA gene. The SSU561F and SSU561R primers

anneal to loop regions of the gene whilst the WSSUF and WSSUR primers anneal to stem regions of the gene. The stem regions of the gene would be involved in intra strand annealing and it is possible that the WSSUF and WSSUR primers would have to compete for these stem binding sites. Since the self annealing process is likely to be more efficient than the binding of short oligonucleotides, PCR amplification might be severely restricted by such a process. As the loop regions have no self complementary sequence on the same strand no such competitive inhibition of amplification would affect the SSU561F and SSU561R primers. However when primer pairs WSSUF/SSU561R and SSU561F/WSSUR were used amplification was as efficient as with the SSU561 primers alone (Fig. 6.4), therefore PCR with WSSUF/WSSUR may be less efficient because *Taq* polymerase efficiency is inversely proportional to length of product. It is possible that other published primers or primers that anneal to sequences flanking the SSU rRNA gene would be more efficient and more satisfactory than the WSSUF and WSSUR primers but this point was not investigated.

Silver stained polyacrylamide gels were necessary to detect all the fragments produced by digestion of these PCR products. Silver stained polyacrylamide gels offer higher resolution and greater sensitivity than agarose gels, however they are more laborious to prepare, and as migration in polyacrylamide gels is sensitive to the quantity of salts present it is necessary to standardise the DNA concentrations of the digests so that equal volumes of each digest can be loaded.

6.3.3. RFLPs of PCR amplified SSU rDNA for identification of genera

Digests of the WSSUF and WSSUR products with a number of enzymes could reliably distinguish between *Endotrypanum*, lizard and mammalian *Leishmania*. The most suitable enzyme for this purpose was *Sau96I* (Fig. 6.3b) which produced a fingerprint characteristic of each genus, except that *L. deanei* had a unique fingerprint. A number of enzymes could also distinguish between *L. (Leishmania)* and *L. (Viannia)*. *HhaI* (*CfoI*) has previously been described for this purpose (Meredith et al., 1993), but the mobility difference between the diagnostic fragments was slight and *L. hertigi* and *Endotrypanum* gave the same restriction pattern as *L. (Viannia)* (Fig. 6.4a). *AluI* and *SacI* produced greater mobility differences between the *Leishmania* subgenera but one *L. tarentolae*, (LV108), and *Endotrypanum* also gave the same restriction pattern as *L. (Viannia)* (Fig. 6.2). Consequently this method should

be used with care for the identification of *Leishmania* subgenera until enzymes that can generate subgenus specific patterns are found.

6.3.4. The identity of the putative *L. herreri* strains

The RFLPs of the complete SSU rRNA gene clearly show that the putative *L. herreri* strain LV344 is an *Endotrypanum* (Fig. 6.3). A dendrogram of the putative *L. herreri* strains and *Endotrypanum* reference strains was compiled (Fig. 6.9). The dendrogram is based on the RFLPs of SSU rDNA, dot blots and RAPD, presented here, together with DNA buoyant densities (Croft et al., 1980), and the sequence of the 561bp region of LV344 (GB:U50043) (Perez Camps, 1995). The dendrogram follows that of Lopes *et al.* (1990) who examined RFLPs of whole nuclear DNA of 15 *Endotrypanum* strains including LV88, LV59 and LV58 and found that they fell into three groups (A, B, C). The RFLP based groups were consistent with two groups identified by kDNA and nDNA buoyant densities of some of the same strains (Croft et al., 1980). A classification of the RNA polymerase II gene of *Endotrypanum* and *Leishmania* species also classified *L. herreri* with *Endotrypanum* (Croan & Ellis, In Press)

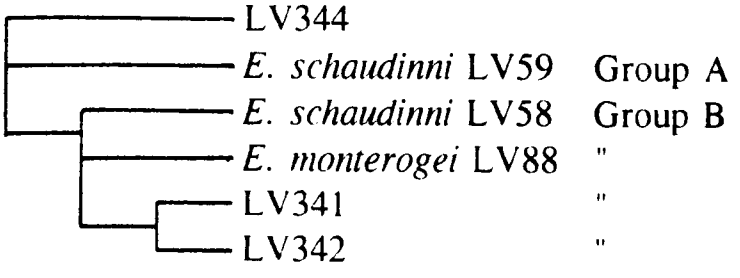


Figure 6.9 Dendrogram of *Endotrypanum* strains and the putative *L. herreri* strains included in this study and based on the RAPD results (Fig. 6.7), dot blots (Fig. 6.8) and previously described data (Croft et al., 1980; Lopes et al., 1990; Perez Camps, 1995). The groups are the same as those described by Lopes *et al.* (1990) who included LV58, LV59 and LV88 in their study.

LV341, LV342, *E. monterogeii* (LV88) and *E. schaudinni* (LV59) were all similar by RAPD (Fig 6.7). LV341 had the same kDNA and nDNA buoyant densities (1.698gml^{-1} and 1.712gml^{-1}) as *E. monterogeii* (LV88) and *E. schaudinni* (LV59) (Dr Chance, personal

communication). Therefore the combination of RAPD and buoyant density data indicate that LV341 and LV342 are members of Lopes' group B. The sequence of the 561bp variable region of LV344 is significantly different from the published sequence of *E. monterogeii* (Perez Camps, 1995) with a Kimura distance of 0.0082 compared with the distance between *L. guyanensis* and *L. donovani* of 0.0040 (Figs 7.8 and 7.9). LV344 is unlike members of group B by both RAPD and SSU rDNA sequence and unlike group A (LV58) by RAPD. A study of the miniexons of four *Endotrypanum* strains found two distinct subgroups within group A (Fernandes, O. et al., 1993) and the difference between LV344 and LV59 by RAPD may therefore reflect variation within group A. LV344 could alternatively be a member of group C, no reference strains for this group were included in the study. However group C has only been isolated in Brazil whereas group A and B strains are found in both Central and South America. LV344 may therefore be a member of group A or C or represent a previously undescribed group.

A more detailed study of a larger number of *Endotrypanum* isolates will be required to resolve the problems of the classification of the *Endotrypanum* and to classify LV344 with confidence. The biochemical, serological and DNA data do not support the classification of *E. monterogeii* as a separate species (Croft et al., 1980; Lopes & McMahon-Pratt, 1989; Lopes et al., 1990). This was unexpected since *E. monterogeii* is a trypomastigote in the sloth erythrocyte whilst *E. schaudinni* is an epimastigote (Shaw, 1969). It is possible that LV88, which is the only *E. monterogeii* strain that has been characterised, has been cross contaminated with *E. schaudinni*. LV88 was isolated in the 1960s and has passed through a number of different laboratories.

Although the group affiliation of LV344 could not be determined, it is clearly very different from the reference strains included in this study and may therefore represent a valid species. However the genetic distance between species varies from one genus to another. The distance between the SSU rDNA sequence of two *T. cruzi* strains (Fig. 7.8 & 7.9) is greater than between the two *Leishmania* subgenera or between LV344 and *E. monterogeii*. The genetic distance at which it is appropriate to create new species is therefore a matter of opinion. There are significant differences in the biology of *E. schaudinni* and *E. monterogeii* which justified the creation of separate species, however nothing is known of the biology of the putative *L. herreri* strains in the sloth and, as Lopes *et al.* (1990) point out, until

significant biological differences are found, the creation of additional *Endotrypanum* species must be questionable.

6.3.5. Potential applications of genus specific markers for the *Leishmania* and *Endotrypanum*

The search for genus specific markers was prompted by the problems of identification of trypanosmatids supposedly isolated from mammals but with RAPD fingerprints that did not conform to those of any *Leishmania* reference strains (Chap. 7). There are a number of cases in the literature of unidentified or monoxenous trypanosomatids being isolated from mammals including humans (Dedet et al., 1995; Mebrahtu et al., 1992; Schnur et al., 1992). In addition to the strains described in chapter 7, a trypanosomatid isolated from the spleen of a dog in India (MCAN/IN/XX/LV701) was also found not to be a *Leishmania* by RFLPs of the SSU rRNA gene (not shown). However genus specific markers are likely to be most useful for the identification of trypanosomatids from sandflies. Sandflies are known to transmit various *Trypanosoma* and unidentified trypanosomatids are occasionally reported (Ryan, et al., 1987; Williams, 1991), numerous other cases go unreported (Prof. Ward, University of Keele, personal communication). Primers SSU561F and SSU561R may be suitable for amplifying trypanosomatid DNA from crude preparations of sandfly DNA. Although these primers were not tested for cross-reactivity with sandfly DNA, they have only 50% and 25% homology respectively with the corresponding sequences in *Aedes albopictus* (GB:X57172), so it is unlikely that any cross reaction would occur. The identification of parasites isolated from sandflies to the genus level could provide a useful primary screen in studies of the epidemiology and ecology of *Leishmania*, particularly in the Neotropics where a considerable range of very diverse *Leishmania* parasites are found. Higher resolution fingerprinting techniques require large numbers of reference strains and the generic status of isolates which are very different from reference strains can be difficult to determine.

The *C. fasciculata* and *C. luciliae* strains tested in the present study had different SSU rDNA restriction profiles (Fig. 6.1) and consequently the monoxenous parasites of sandflies are likely to have greater intrageneric variability than the *Leishmania*. Further work would therefore be required to find genus specific markers for these parasites. However the characterisation of unknown trypanosomatids by SSU rRNA fingerprinting should make it

possible to identify distinctive clusters of the more common parasites of sandflies and to make an estimate of their diversity.

6.4. RFLPS OF PCR AMPLIFIED SSU rDNA FOR CLASSIFICATION

6.4.1. The classification of lizard *Leishmania*

The classification of mammalian and lizard *Leishmania*, and *Endotrypanum* (Fig. 6.6) was consistent with previous classifications produced from isoenzymes and the sequence of the SSU rRNA gene, in so far as they include the species used in this study (Cupolillo et al., 1994; Marché et al., 1995; Thomaz Soccol, 1993; Thomaz Soccol et al., 1993b) (Fig. 7.9). Lizard *Leishmania* species have not been included in any published classifications of *Leishmania* except those based on SSU rDNA sequences, and in those only one *L. tarentolae* sequence has been used (Marché et al., 1995). Classifications based on the sequence of the SSU rRNA gene have been unable to resolve *L. tarentolae* from the mammalian *Leishmania* (Fig. 7.9) and (Marché et al., 1995), however in the dendrogram based on RFLPs of the SSU rRNA gene (Fig. 6.6) the lizard *Leishmania* form a distinct group within the *Leishmania*. The higher resolution of the RFLPs over the sequence data may be a consequence of the improved signal to noise ratio obtained by selecting only restriction enzymes that would be informative at the level of the *Leishmania/Endotrypanum* group. However as the RFLP classification is based on a much smaller amount of information the higher resolution may be an artefact of the smaller dataset or the particular set of restriction enzymes used.

The buoyant densities of both nuclear and kinetoplast DNA of the lizard *Leishmania* span the range of those for *L. (Leishmania)* but generally exceed those of *L. (Viannia)*. The buoyant density values are very variable with the nuclear DNA ranging from 1.716 to 1.720g ml^{-1} and the kDNA from 1.700 to 1.706g ml^{-1}. The corresponding values for *L. (Leishmania)* are nDNA 1.718-1.719g ml^{-1} and kDNA 1.697-1.704g ml^{-1} and for *L. (Viannia)* nDNA 1.716-1.717g ml^{-1} and kDNA 1.691-1.694g ml^{-1} (Chance et al., 1974). These values indicate that the lizard *Leishmania* have a closer relationship to *L. (Leishmania)* than to the *L. (Viannia)* which is consistent with the SSU rRNA based dendrogram (Fig. 6.6). The pattern of cross hybridisation in the dot blots is also consistent with a close relationship between lizard *Leishmania* and *L. (Leishmania)* since the *L. tarentolae* probe cross hybridised weakly with *L. (Leishmania)* but not at all with *L. (Viannia)* (Fig. 6.8d).

The *L. chagasi* probe also hybridised more strongly with the lizard *Leishmania* than with *L. (Viannia)* (Fig. 4.7).

L. tarentolae (LV108) had the same restriction pattern as *L. (Viannia)* with *SacI* and *AluI* (Fig. 6.2). *L. (Viannia)* is predicted to be discriminated from *L. (Leishmania)* by these enzymes but none of the published sequences predict that *L. tarentolae* (LV108) might have these *SacI* or *AluI* sites. The *L. tarentolae* LV414 kDNA variable region probe hybridised equally to the LV414 and LV108 dots. The presence of the *AluI* restriction site in LV108 may therefore be of only minor significance. The difference between *L. tarentolae* (LV108) from Senegal, and *L. tarentolae* (LV414) which was isolated in Algeria in 1939 and maintained by weekly subpassage, may reflect the different geographical origins of these strains or the different ways in which they have been maintained. However the only detailed comparative study of a number of lizard and mammalian *Leishmania* species found that *L. tarentolae* from Algeria and France were quite different (Gomez-Eichelmann et al., 1988), suggesting that there may be significant diversity within the *Leishmania* parasites isolated from *Tarentola spp.*

There are two other partial *L. tarentolae* and *L. adleri* sequences in Genbank (GB:M81414 and GB:M80291) (van-Eys et al., 1992), these are identical to each other and more similar to mammalian *Leishmania* sequences (Kimura distances 0.0034-0.0068) than they are to the homologous region of the complete published *L. tarentolae* sequence (Kimura distance 0.0091). Briones *et al.* (1992) concluded that lower resolution methods would be needed to accurately classify the lizard *Leishmania* and establish the position or positions of the reptilian parasites relative to the mammalian *Leishmania*. In a classification of the RNA polymerase II gene of *Leishmania* and *Endotrypanum* species the lizard *Leishmania* clustered with the mammalian *Leishmania* but external to the pathogenic mammalian species (Croan & Ellis, In Press). This is consistent with the hypothesis that the mammalian *Leishmania* evolved from parasites of lizards as early as the Jurassic (Lainson & Shaw, 1987), but is not consistent with the SSU rDNA sequence data or the kDNA dot blots.

It is possible that *Sauroleishmania* is not a valid genus. Firstly because the SSU rDNA sequence data and the DNA buoyant density data suggest that they form at least two distinct groups with different relationships to the mammalian species. Secondly because, even if they form a single distinct group, the SSU rDNA RFLPs and the kDNA cross hybridisation

experiments indicate that they render the genus *Leishmania* paraphyletic. The genus *Sauroleishmania* was created primarily because the lizard *Leishmania* parasitise a different class of vertebrates and a different genus of sandflies from the *Leishmania* (Killick-Kendrick et al., 1986). The difference in hosts is not necessarily inconsistent with the lizard parasites being in the same genus as mammalian *Leishmania*. The classification based on the polymerase II gene however is consistent both with the lizard *Leishmania* being members of the same genus as the mammalian parasites and with the lizard *Leishmania* being in a separate genus provided that the *L. hertigi* complex is included in the *Endotrypanum* or in a new genus of its own.

It has been assumed that the lizard *Leishmania* have been associated with the same class of hosts since the Jurassic (Lainson & Shaw, 1987; Saf'janova, 1986). The classification shown in figure 6.6 suggests that the lizard parasites are derived from the mammalian parasites and not the reverse. This would imply a much later date for the separation of the mammalian and lizard groups. The cross infectivity of mammalian and lizard parasites to hosts of the other group tends to support a relatively close relationship. *L. adleri* is capable of producing transient infections that last up to five weeks in hamsters (Adler, 1964) and one week in humans (Manson-Bahr & Heisch, 1961), although other workers have failed to infect mammals with other lizard parasites (Belova, 1971). Mammalian parasites from visceral cases will also occasionally infect lizards (Belova, 1971).

Lizard parasites will also generate a cell mediated immune response in humans that will cross react with mammalian antigens in the Montenegro skin test up to at least two years later (Wilson & Southgate, 1979). Therefore prevalence of infections with mammalian parasites in surveys with the Montenegro test may be overestimates, but it is also possible that the lizard parasites may have some real epidemiological significance, if the cross reactivity is indicative of some cross protection. A fuller appreciation of the relationship between the lizard and mammalian *Leishmania* may lead to inclusion of these parasites in studies of the epidemiology, immunology and taxonomy of *Leishmania* with possibly interesting results.

6.4.2. The classification of the *L. hertigi* complex

The RFLPs of *L. deanei* and the sequence of the 561bp variable region of the *L. hertigi* SSU rRNA gene both showed that the *L. hertigi* complex is more closely related to *Endotrypanum* than to *Leishmania* in over 90% of bootstrap replicas (Fig. 6.6 & 7.9). In the only isoenzyme based classification that has included the *L. hertigi* complex it was placed in the *L. (Leishmania)* but at the base of the tree close to the node separating *L. (Leishmania)* from *L. (Viannia)* and an alternative data analysis placed the *L. hertigi* complex in the *L. (Viannia)* (Thomaz Soccol, 1993; Thomaz-Soccol et al., 1993b). As no outgroup was used in that study it was not possible to determine the position of the root, but if the root is placed on the branch leading to *L. hertigi* then that dendrogram would have the same topology as both of those in figure 6.6. In the classification based on the Polymerase II gene *L. hertigi* was classified with *Endotrypanum* in 76% of bootstrap replicates (Croan & Ellis, In Press).

The biology of the *L. hertigi* complex is poorly known. These parasites are found in the upper dermis, spleen and liver of tree porcupines (*Coendu spp.*), but are not easy to detect except by culture (Herrer, 1971). The particular cell type parasitised has not been reported but there are no reports that they infect macrophages, nor do the parasites cause any cell or tissue reaction.

The lizard *Leishmania* develop in the hindgut of *Sergentomyia* sandflies, a tropism that has been characterised as primitive. If the lizard *Leishmania* have evolved recently from mammalian species then the posterior development of the lizard *Leishmania* must be a feature of the particular parasite invertebrate relationship and not an indicator of a primitive origin for that relationship. *L. hertigi* and *Endotrypanum* which form a single clade in the phenograms in figures 6.6a and 7.1 but not 6.6b have suprapylarian and mostly rectal infections respectively (Shaw, 1985). Therefore both major branches of the phenograms contain parasites with a full range of tropisms in the sandfly, suggesting that this tropism is not a useful character for long range classifications.

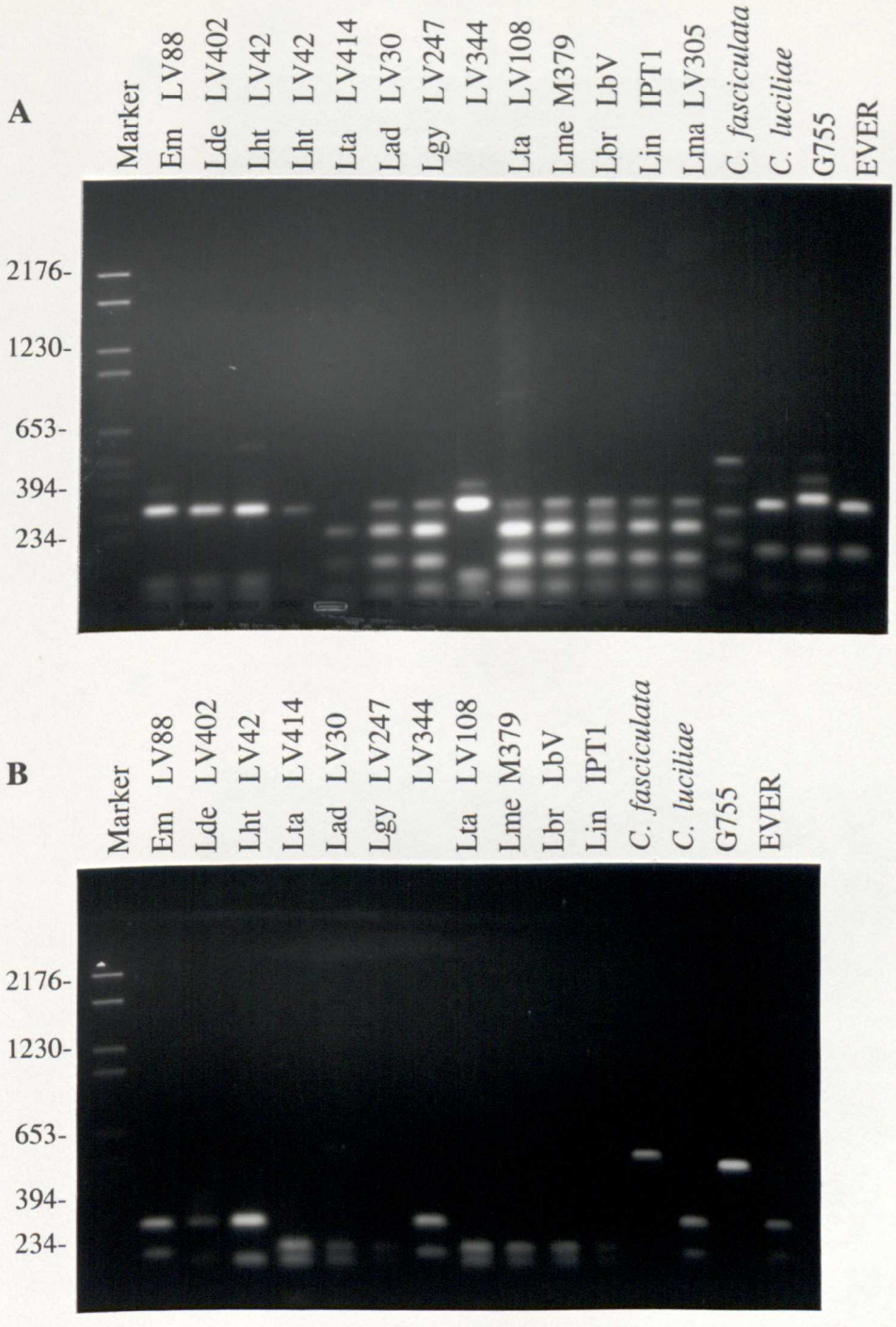


Figure 6.1A and 6.1B. **(A)** *HpaII* and **(B)** *HaeIII* restriction digests of the 561bp variable region of the SSU rRNA gene amplified with primers SSU561F and SSU561R. *E. monterogei* (LV88) (Em); *L. deanei* (LV402) (Lde); *L. hertigi* (LV42) (Lht); *L. tarentolae* (LV414 and LV108) (Lta); *L. adleri* (LV30) (Lad); *L. gymnodactyli* (LV247) (Lgy); *L. mexicana* (M379) (Lme); *L. braziliensis* (Lbv) (Lbr); *L. infantum* (IPT1) (Lin); *L. major* (LV305) (Lma); *C. fasciculata*; *C. luciliae*; G755; EVER.

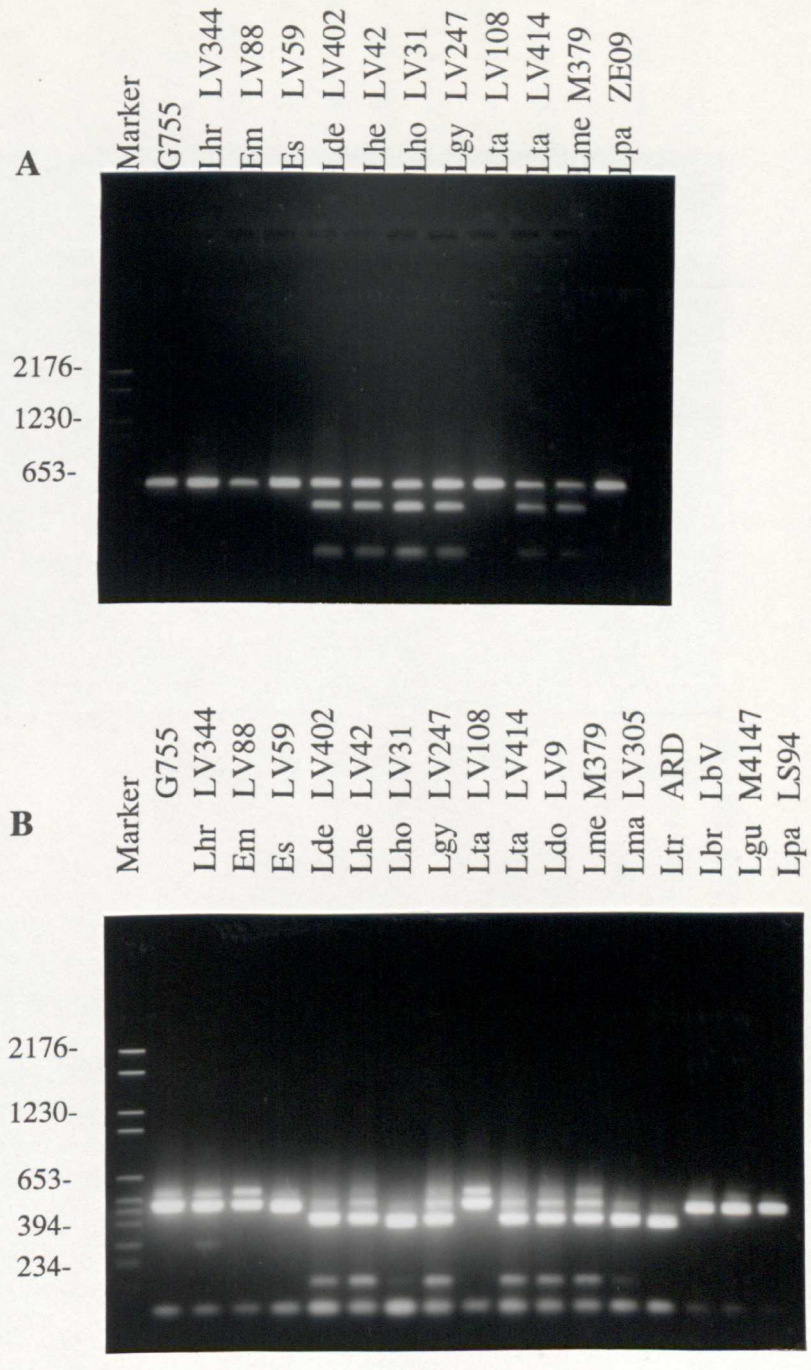


Figure 6.2A and 6.2B Restriction digests of the 561bp variable region of the SSU rRNA gene amplified with primers SSU561F and SSU561R. *L. herreri* (LV344) ((Lhr); *E. monterogei* (LV88) (Em); *E. schaudinni* (LV59) (Es); *L. deanei* (LV402) (Lde); *L. hertigi* (LV42) (Lht); *L. tarentolae* (LV414 and LV108) (Lta); *L. hoogstraali* (LV31) (Lho); *L. gymnodactyli* (LV247) (Lgy); *L. donovani* (LV9) (Ld0); *L. mexicana* (M379) (Lme); *L. major* (LV305) (Lma); *L. tropica* (ARD) (Ltr); *L. braziliensis* (LbV) (Lbr); *L. guyanensis* (M4147) (Lgu); *L. panamensis* (ZE09) (Lpa). Digested with **(A)** *SacI* and **(B)** *AluI*.

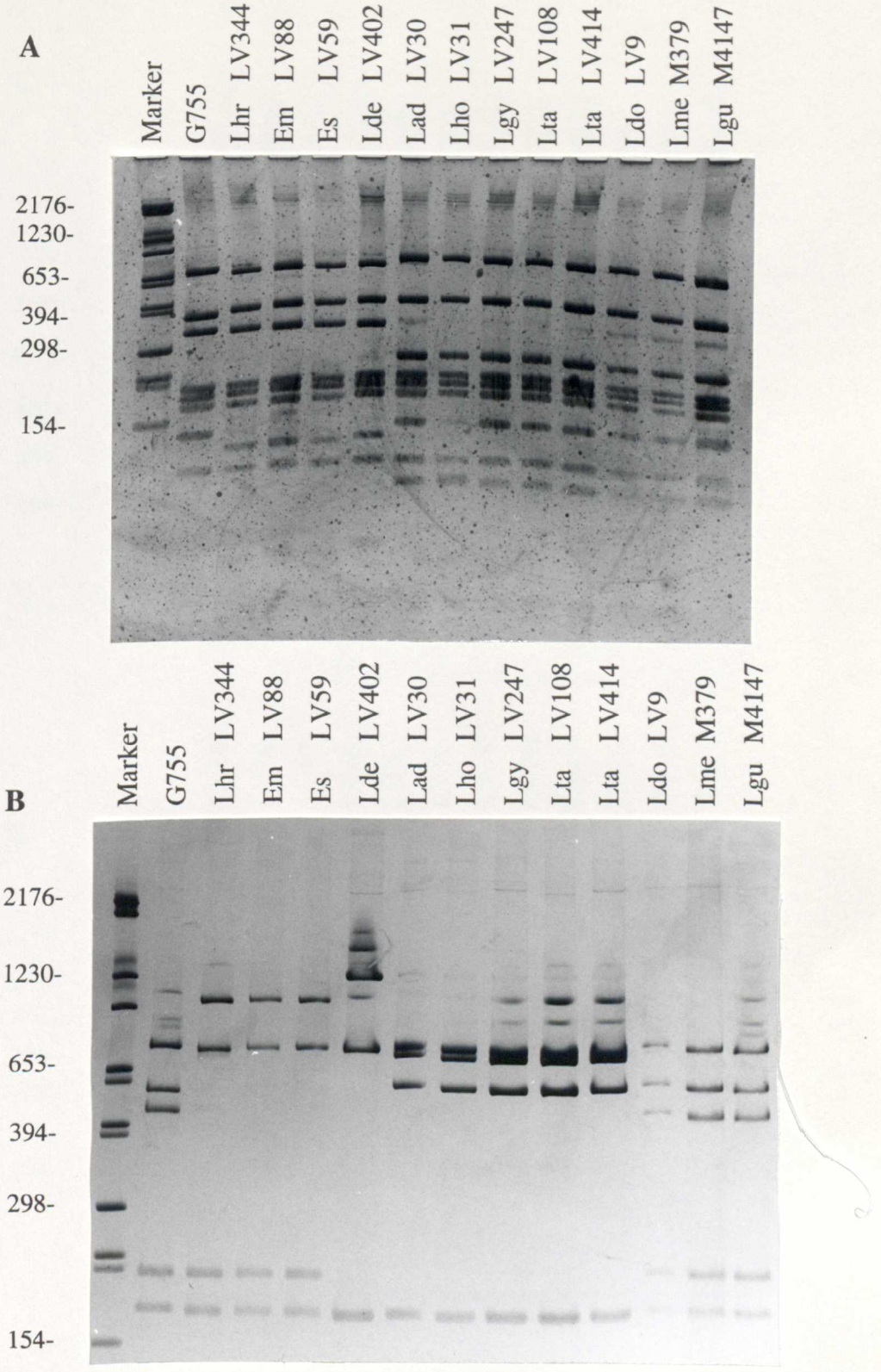


Figure 6.3A and 6.3B **(A)** *HpaII* and **(B)** *Sau96I* restriction digests of the complete SSU rRNA gene amplified with primers WSSUF and WSSUR. Guatemalan leptomonad (G755); *E. monterogei* (LV88) (Em); *E. schaudinni* (LV59) (Es); *L. herreri* (LV344) ((Lhr); *L. deanei* (LV402) (Lde); *L. adleri* (LV30) (Lad); *L. hoogstraali* (LV31) (Lho); *L. gymnodactyli* (LV247) (Lgy); *L. tarentolae* (LV414 and LV108) (Lta); *L. donovani* (LV9) (Ldo); *L. mexicana* (M379) (Lme); *L. guyanensis* (M4147) (Lgu).

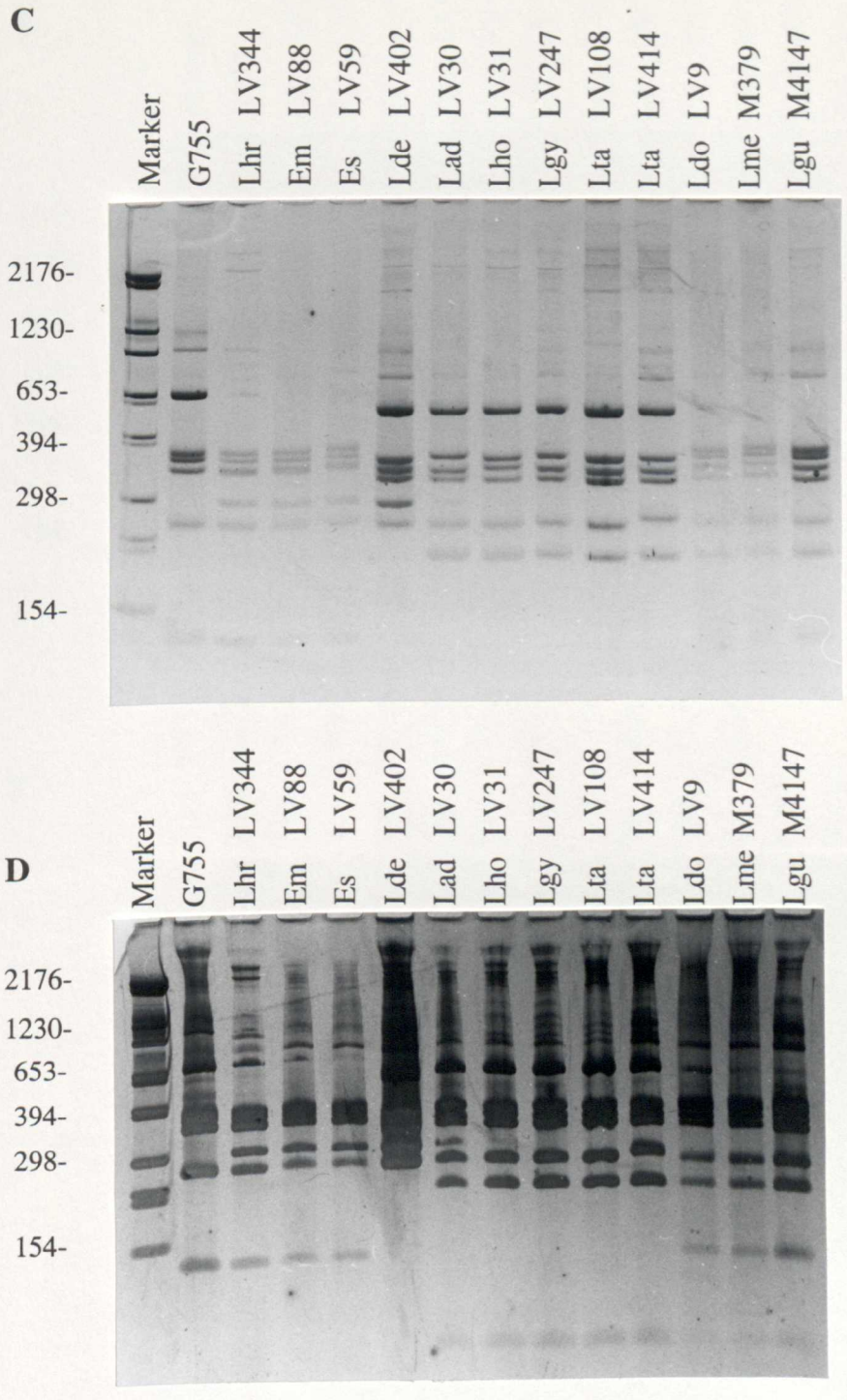


Figure 6.3C and 6.3D *HaeIII* restriction digests of the complete SSU rRNA gene amplified with primers WSSUF and WSSUR. Guatemalan leptomonad (G755); *E. monterogei* (LV88) (Em); *E. schaudinni* (LV59) (Es); *L. herreri* (LV344) ((Lhr); *L. deanei* (LV402) (Lde); *L. adleri* (LV30) (Lad); *L. hoogstraali* (LV31) (Lho); *L. gymnodactyli* (LV247) (Lgy); *L. tarentolae* (LV414 and LV108) (Lta); *L. donovani* (LV9) (Ldo); *L. mexicana* (M379) (Lme); *L. guyanensis* (M4147) (Lgu). **(C)** Developed to show strong bands, **(D)** developed to show weak bands.

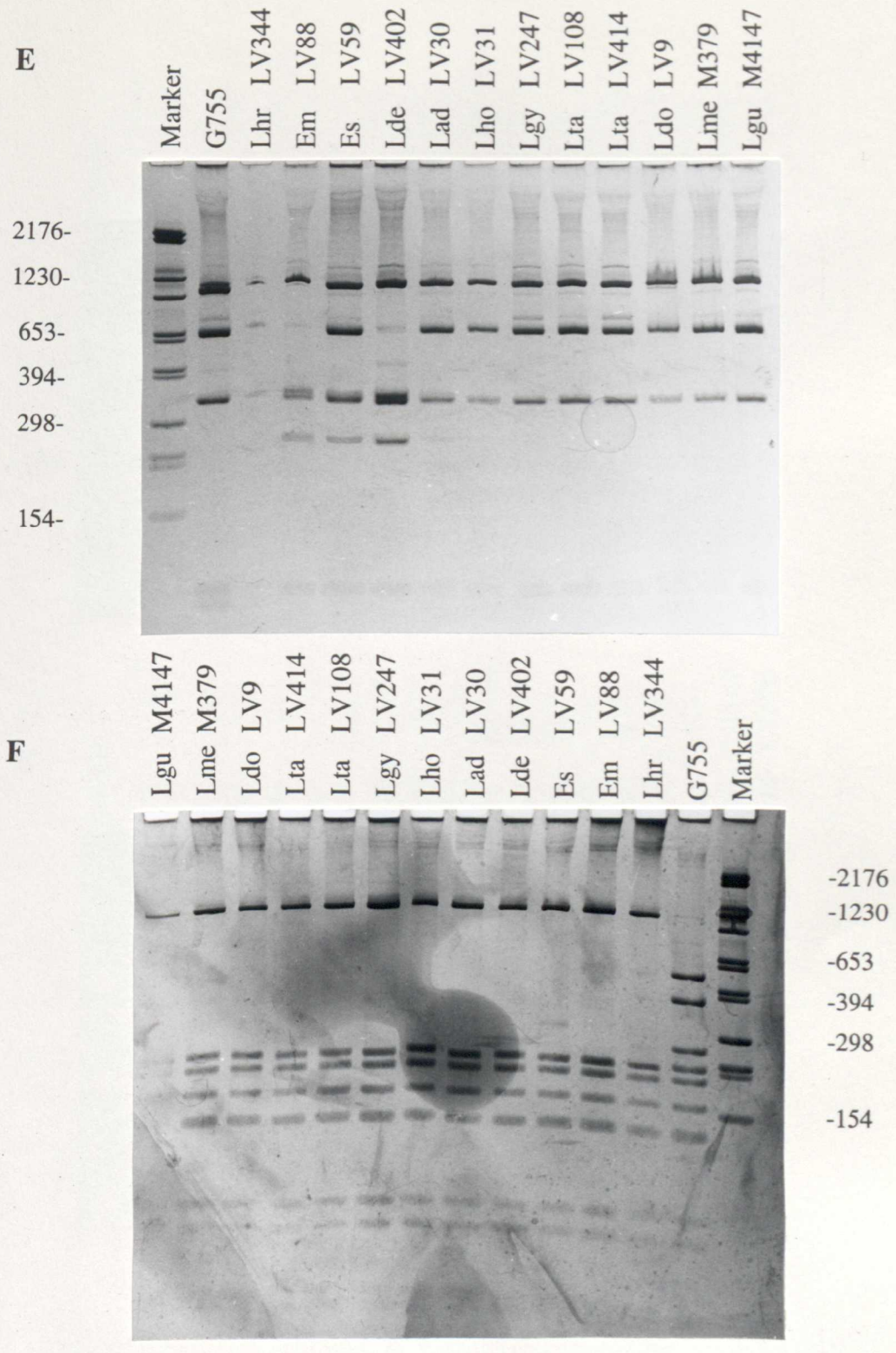


Figure 6.3E and 6.3F **(E)** *RsaI* and **(F)** *HinfI* restriction digests of the complete SSU rRNA gene amplified with primers WSSUF and WSSUR. Guatemalan leptomonad (G755); *E. monterogei* (LV88) (Em); *E. schaudinni* (LV59) (Es); *L. herreri* (LV344) ((Lhr); *L. deanei* (LV402) (Lde); *L. adleri* (LV30) (Lad); *L. hoogstraali* (LV31) (Lho); *L. gymnodactyli* (LV247) (Lgy); *L. tarentolae* (LV414 and LV108) (Lta); *L. donovani* (LV9) (Ldo); *L. mexicana* (M379) (Lme); *L. guyanensis* (M4147) (Lgu).

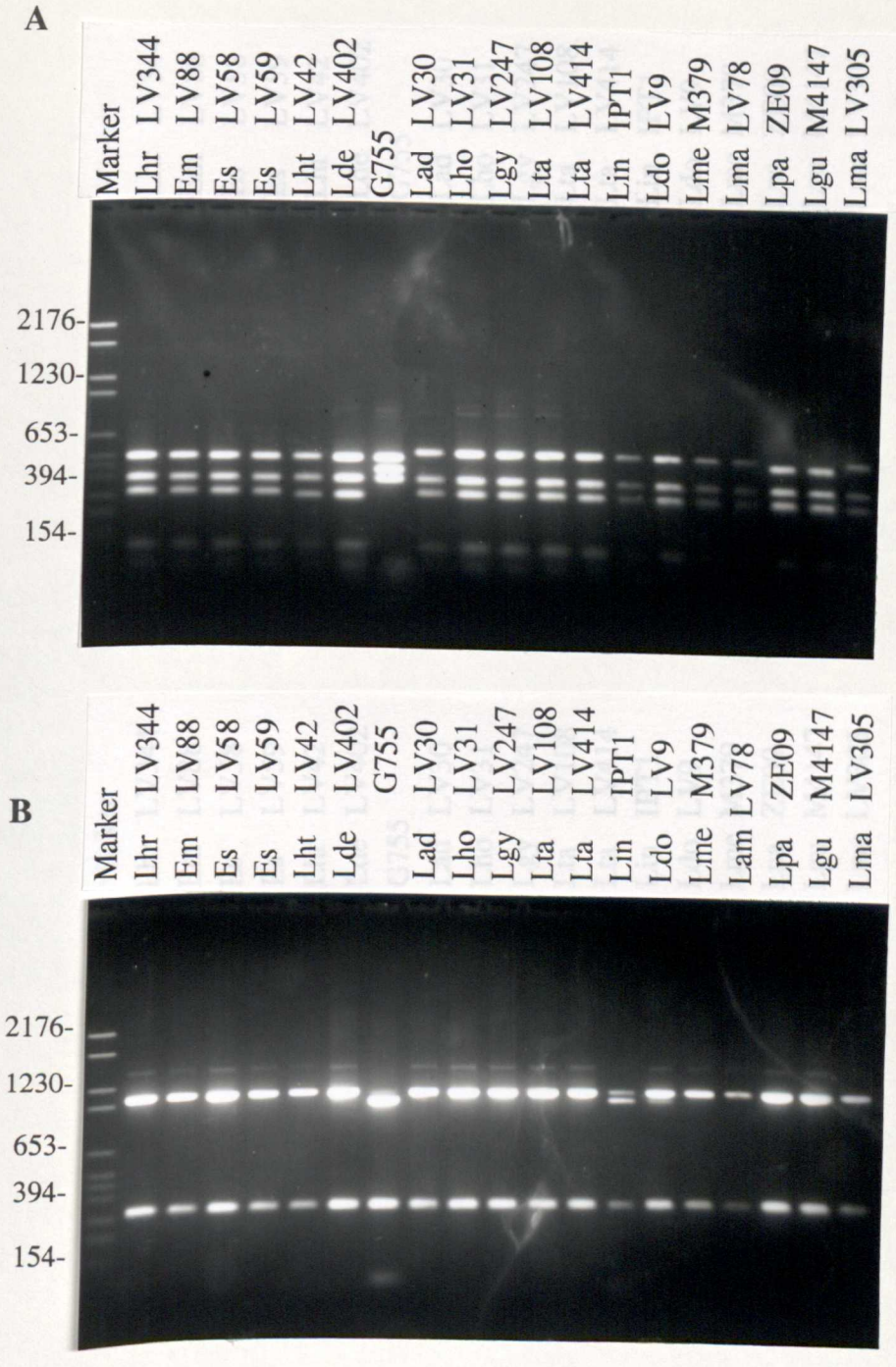


Figure 6.4A and 6.4B **(A)** *HhaI* and **(B)** *RsaI* restriction digests of part of the SSU rRNA gene amplified with primers WSSUF and SSU561R. Guatemalan leptomonad (G755); *E. monterogei* (LV88) (Em); *E. schaudinni* (LV59 and LV59) (Es); *L. herreri* (LV344) ((Lhr); *L. hertigi* (LV42) (Lht); *L. deanei* (LV402) (Lde); *L. adleri* (LV30) (Lad); *L. hoogstraali* (LV31) (Lho); *L. gymnodactyli* (LV247) (Lgy); *L. tarentolae* (LV414 and LV108) (Lta); *L. infantum* (IPT1) (Lin); *L. donovani* (LV9) (Ldo); *L. mexicana* (M379) (Lme); *L. amazonensis* (LV78) (Lam); *L. panamensis* (ZE09) (Lpa); *L. guyanensis* (M4147) (Lgu); *L. major* (LV305) (Lma).

	G755	LV344	EM	ES	LDE	LA	LH	LG	LV108	LV414	LD	LM	LB
G755	0.0												
LV344	0.45	0.0											
E. monterogei	0.45	0.0	0.0										
E. schaudinni	0.45	0.0	0.0	0.0									
L. deanei	0.80	0.44	0.44	0.44	0.0								
L. adleri	0.80	0.72	0.72	0.72	0.39	0.0							
L. hoogstraali	0.80	0.72	0.72	0.72	0.39	0.0	0.0						
L. gymnodactyli	0.80	0.72	0.72	0.72	0.39	0.0	0.0	0.0					
L. tarentolae 108	0.80	0.72	0.72	0.72	0.39	0.0	0.0	0.0	0.0				
L. tarentolae 414	0.68	0.60	0.60	0.60	0.58	0.15	0.15	0.15	0.15	0.0			
L. donovani	0.65	0.52	0.52	0.52	0.48	0.25	0.25	0.25	0.25	0.45	0.0		
L. mexicana	0.65	0.52	0.52	0.52	0.48	0.25	0.25	0.25	0.25	0.45	0.0	0.0	
L. braziliensis	0.47	0.35	0.35	0.35	0.61	0.50	0.50	0.50	0.50	0.40	0.2	0.2	0.0

Figure 6.5 Nei's distances calculated from presence/ absence data of restriction fragments (Appendix 6.2). Guatemalan leptomonad (G755); *E. monterogei* (LV88) (Em); *E. schaudinni* (LV59) (Es); putative *L. herreri* (LV344) (Lh); *L. deanei* (LV402) (Lde); *L. adleri* (LV30) (La); *L. hoogstraali* (LV31) (Lh); *L. gymnodactyli* (LV247) (Lg); *L. tarentolae* (LV414 and LV108) (Lt); *L. donovani* (LV9) (Ld); *L. mexicana* (M379) (Lm); *L. guyanensis* (M4147) (Lg).

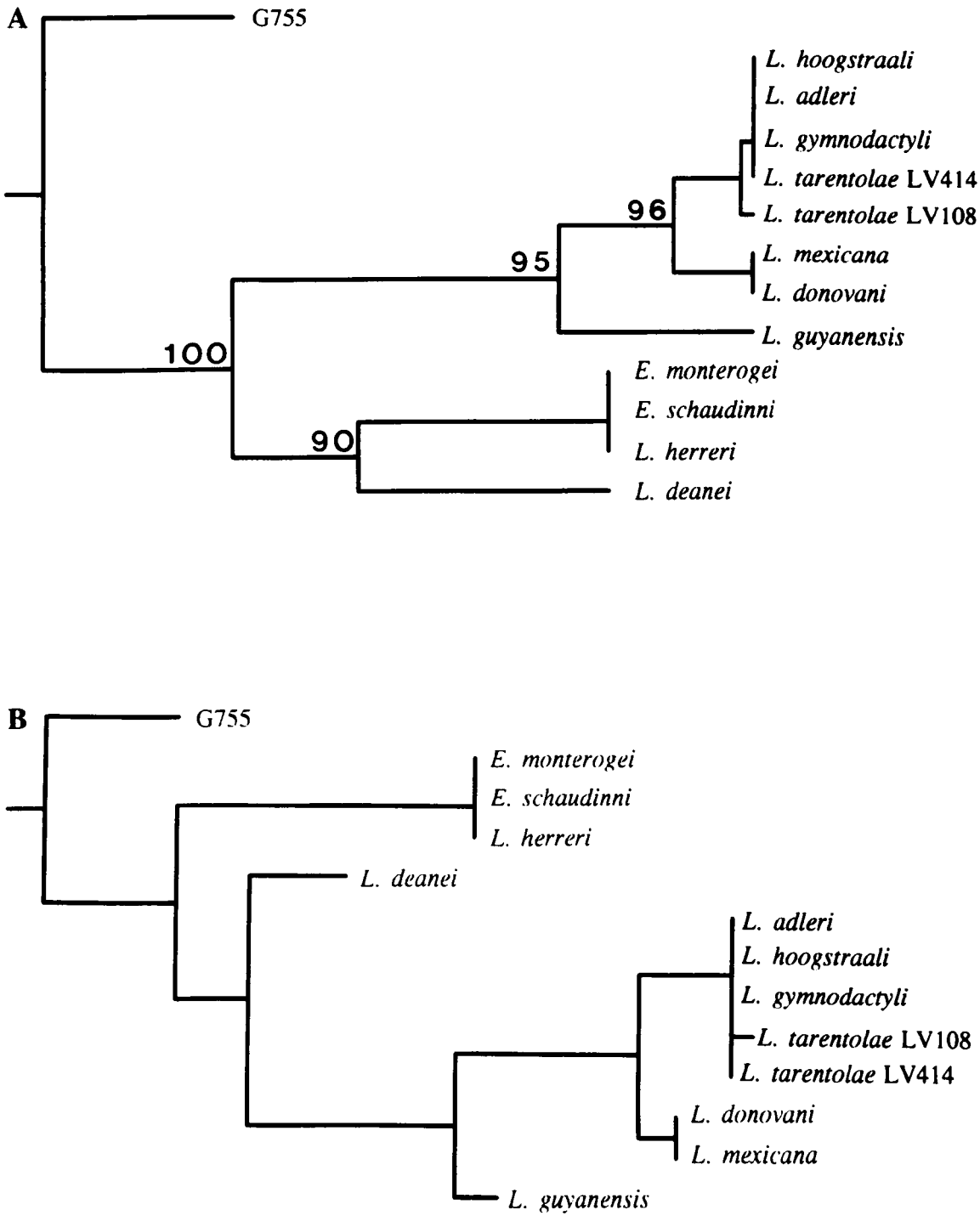


Figure 6.6A and 6.6B **(A)** Dendrogram prepared using the UPGMA option in NEIGHBOR in PHYLIP on the distance matrix in Figure 6.5 with the import order randomised (random number generator 21) and jumbled five times. **(B)** Dendrogram prepared using FITCH in PHYLIP on the distance matrix in Figure 6.5 with the import order randomised (random number generator 21) and jumbled five times. The data was obtained from *HaeIII, HinfI, HpaII, RsaI* and *Sau96I* restriction digests of the whole SSU rRNA gene (Fig 6.3), the *AluI,* and *SacI* digests of the 561bp region (Fig. 6.2) and the *HhaI* digest of 1400bp region amplified by WSSUF and SSU561R (Fig 6.4a). Bootstrap values in (A) calculated from 100 replicates using the MIX programme.

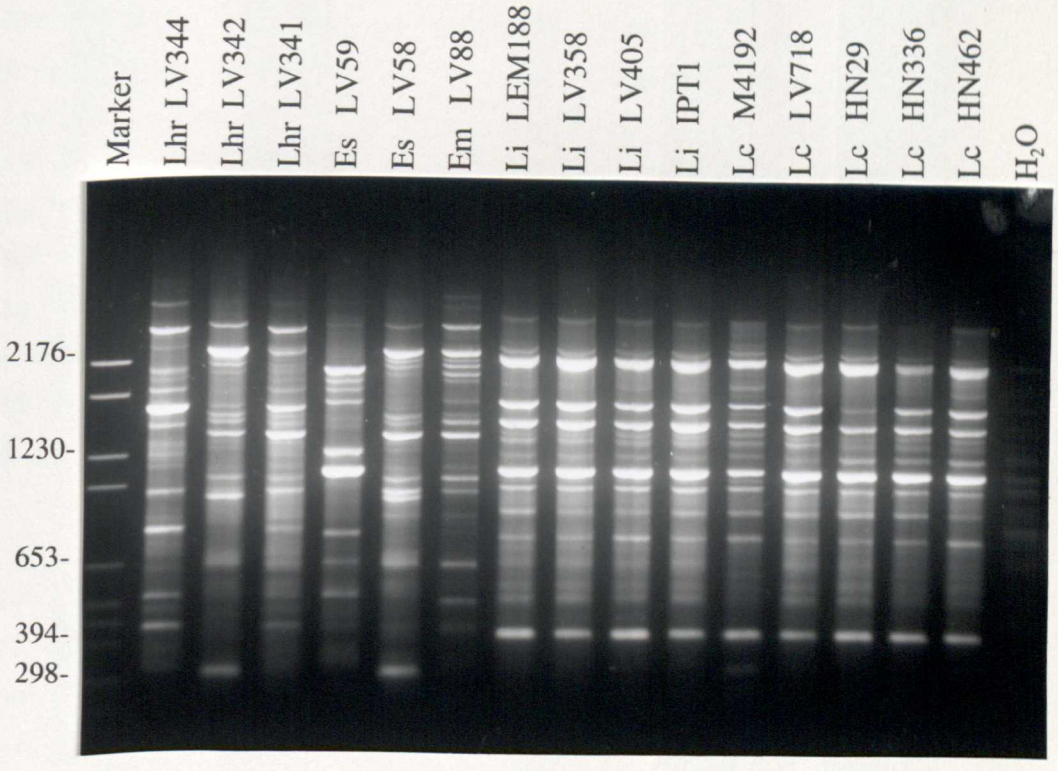


Figure 6.7 RAPD fingerprints with primer M13 of *L. herreri* (LV344, LV342 and LV341) (Lhr); *E. monterogei* (LV88) (Em); *E. schaudinni* (LV59 and LV58) (Es); *L. chagasi* (M4192, LV718, HN29, HN336 and HN462) (Lch); *L. infantum* (LEM188, LV358, LV405 and IPT1) (Lin).

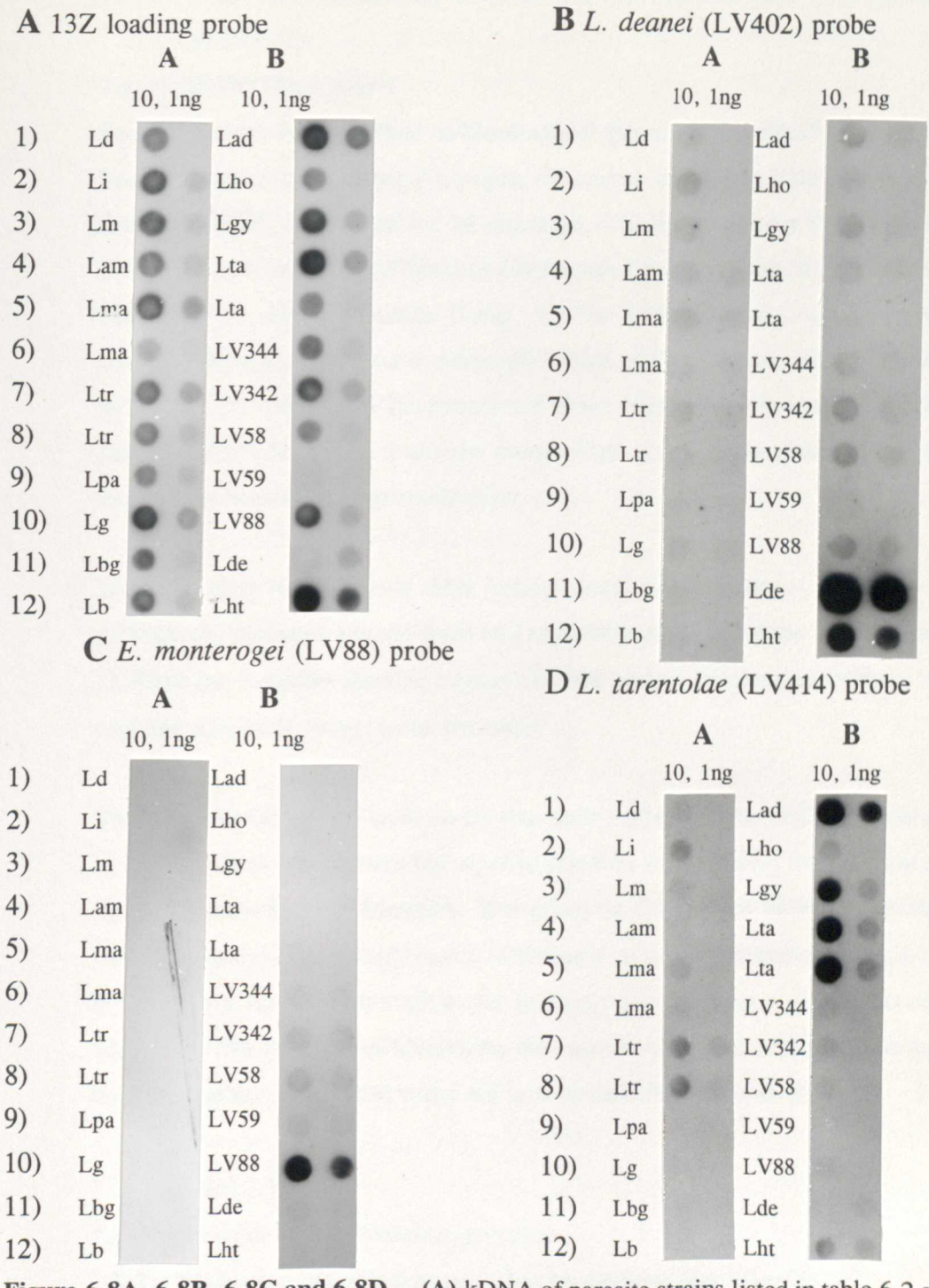


Figure 6.8A, 6.8B, 6,8C and 6.8D **(A)** kDNA of parasite strains listed in table 6.2 amplified with primers 13Z and LiR dotted on to an Amersham N^+ membrane and probed with PCR primer 13Z which hybridises to the universal minicircle origin of replication to show kDNA loading. The blot was washed in 1xSSC, 0.1%SDS at 37°C and exposed for 2 hours. **(B)** The same dot blot as in (A) probed with kDNA PCR amplified from *L. deanei* (LV402) using primers 13Z and LiR. The blot was washed in 0.1M NaOH at 25°C and exposed for 20 hours. **(C)** The same dot blot as in (A) probed with kDNA PCR amplified from *E. monterogei* (LV88) using primers 13Z and LiR. The blot was washed in 0.1M NaOH at 25°C and exposed for 20 hours. **(D)** The same dot blot as in (A) probed with kDNA PCR amplified from *L. tarentolae* (LV414) using primers 13Z and LiR. The blot was washed directly in 0.1% SDS at 42°C and exposed for 2 hours. See table 6.2 for abbreviations.

7. IDENTIFICATION OF UNKNOWN TRYPANOSOMATIDS FROM CENTRAL AMERICA

7.1. INTRODUCTION

Parasite isolates were received on blood agar slopes with Schneider's insect medium overlay from colleagues in Panama, Nicaragua, Honduras and Costa Rica (Table 2.1). Parasites from Guatemala were received as stabilates. The isolates from Panama, Nicaragua and Honduras were readily identifiable as *Leishmania* by comparison of their RAPD fingerprints with those of reference strains (Chap. 3). The isolates from Costa Rica and Guatemala however did not correspond to reference strains of Central American *Leishmania* parasites by RAPD. Parasites from Guatemala and Costa Rica grew vigorously as promastigotes in culture and the latter had a stumpy morphology at stationary phase, suggesting that they might be monoxenous trypanosomatids.

The difficulties in identifying these isolates raised two problems:-

1) Were the parasites a novel form of *Leishmania* or monoxenous trypanosomatids?

2) Were the parasites genuine clinical isolates or had *Leishmania* cultures become cross contaminated with monoxenous parasites?

The group in Guatemala were aware that their parasites were unlike any reference strains by isoenzyme electrophoresis but reported that they were isolated from human cases and that they were infectious to hamsters. The group in Costa Rica however reported that their parasites were similar to *Leishmania* reference strains by isoenzyme electrophoresis and that if they could not be identified it was probably a consequence of cross contamination in Liverpool. The problem of identifying the two unknown trypanosomatid isolates therefore was approached in different ways and will be described separately below.

7.2. RESULTS

7.2.1. Analysis of Guatemalan parasites.

7.2.2. Molecular biological approaches to parasite identification

Thirty two frozen stabilates were supplied by Dr B. Arana and F. Arana (University del Valle, Guatemala). Three strains (G338, G536 and G707) were chosen at random and recovered into liquid culture. Their RAPD fingerprints were compared with *L. mexicana* (BEL21) and *L. braziliensis* (M2903), the two agents of ACL in Guatemala (Fig. 7.1a). G338, G536 and G707 were also tested by PCR with *L. (Viannia)* specific primers MP1H

and MP3L and primers for all *Leishmania* 13A and 13B (Fig. 7.1b and 7.1c). The Guatemalan isolates were unlike *L. mexicana* and *L. braziliensis* by RAPD and they did not produce any product with the *L. (Viannia)* specific primers. With the *Leishmania* primers 13A and 13B they produced the expected product at 120bp for the conserved region of the minicircle but in addition they generated a number of higher molecular weight products that were not seen in *Leishmania* reference strains but were produced by reference strains of *Crithidia* and *Herpetomonas* (Fig. 7.1c).

Five additional stabilates (U120, G408, G505, G523, G735) were recovered and found to have a similar RAPD fingerprint to the first group (Fig. 7.1d). In order to preclude the possibility of cross contamination, a third group of stabilates (U139, U154, G519, G755) were recovered and maintained in a separate laboratory but were also found to be identical by RAPD to the previous batches (Fig. 7.1d).

DNA of U265, U276 and G755 was submitted to Dr Barkers group at the Molteno Laboratories, Cambridge who reported that they were negative by all their PCR tests for *Leishmania*.

Two isolates (G536 and G707) were compared by PFGE with three strains of monoxenous trypanosomatid that were being maintained at the Liverpool School at the same time, but no similarity between the Guatemalan isolates and the other trypanosomatids was found (Fig. 7.1e).

7.2.3. Infectivity to rodents.

Pairs of Balb/C mice were inoculated with four strains (U139, U154, G519, G755) of parasites. The mice were examined regularly for the development of skin lesions. No lesions were found by visual inspection and after four months the mice were sacrificed. The spleens were placed in Evans' semi solid medium but no parasites had developed after four weeks.

7.2.4. Morphology and culture characteristics

The Guatemalan parasites grew vigorously in culture to high densities ($> 10^8$ parasites ml^{-1}). The parasites' growth was slowed by maintaining them at 16°C. Cultures maintained at 5°C could not be recovered after one month but cultures maintained at 16°C in monophasic Schneider's insect medium have been recovered after 5 months without subculture. G755

showed weak growth in the ATCC *Crithidia* selective medium. The parasites were viable after three weekly subpassages but not after four. *L. mexicana* survived two weekly subpassages in the same medium.

The normal form in culture was a promastigote (Fig. 7.2b), but in older cultures a wider range of forms was observed:- round forms with flagella, apparently syncytial agglomerations and some choanomastigote like forms with large flagellar pockets. Giemsa stained preparations were examined for the presence of opisthomastigotes but none were found.

Promastigotes of G755 were examined by electron microscopy, electron dense inclusions were visible but the parasites were similar to *Leishmania* preparations (Fig. 7.3a and 7.3b). The ratio of the length to the breadth of the kinetoplast of G755 (3.0-5.5) was less than that for *L. braziliensis* (Table 7.1, Chapter 7 p134). Electron micrographs of G755 were submitted to Professor Vickerman at the Department of Zoology, Glasgow who reported that there was nothing in the micrographs to indicate that the parasites were not *Leishmania*.

7.2.5. Analysis of second batch of Guatemalan parasites.

It was reported from Guatemala that new clinical isolates had an anomalous isoenzyme profile unlike *L. braziliensis* or *L. mexicana* and were infective to rodents. Six of these isolates were reisolated from hamsters and sent to Liverpool without sub passage. This second batch of parasites consisted of hamster biopsies in cacodylate buffer for electron microscopy (U265, U267, U276, U277 and U280) and hamster biopsies on blood agar slopes with Schneider's overlay for culture (U265, U266, U276, U277, U280).

Electron microscopy of the biopsy of the U265 lesion showed numerous amastigotes (Fig. 7.4a). The number of microtubules of one of these amastigotes was 116 (Fig. 7.4b), which is similar to the mean of *L. mexicana* complex species (*L. aristidesi* 118±3.5, *L. amazonensis* 114±2.6).

7.2.6. Infectivity to rodents and sandflies.

Parasites from the blood agar slopes of U265, U266 and U276 were inoculated into the footpads of Syrian hamsters and the base of the tail of Balb/C mice. Lesions were visible on the feet of the hamsters after one month. The lesions in the hamsters grew rapidly to 10-

12mm diameter at six weeks at which time the hamsters were sacrificed. The lesions consisted of dense masses of amastigotes (Fig. 7.5) which readily transformed in culture to promastigotes. Lesions were visible in the mice after two months.

Twelve *Lu. longipalpis* sandflies were allowed to feed on footpads of a hamster infected with U265 and seven on a hamster infected with U266. Five flies survived for five days at which time three flies were dissected. All three flies were infected, in one the infection was restricted to the midgut, in one there was a heavy midgut infection with some unattached parasites visible in the hindgut and in the third fly the preparation did not permit the determination of the site of infection. The remaining two flies were fixed and sectioned and the sections stained with Giemsa. In both these flies the infection appeared to be restricted to the mid gut (Fig. 7.6).

7.2.7. Fingerprinting parasites from rodents

The parasite behaviour in rodents and sandflies was similar to that of parasites of the *L. mexicana* complex, however colleagues in Guatemala reported that, in their laboratory, the isoenzyme profile of this second batch of parasites was similar to the profile prior to hamster passage and did not correspond to *L. mexicana* reference strains. A duplicate set of isolates (U265, U266, U276, U277, U280), that had been characterised by enzyme electrophoresis after hamster passage, was sent to Liverpool for comparison with the same strains maintained here.

RAPD on strains U276, U265 and U266 passaged through mice in Liverpool produced a *L. mexicana* like fingerprint (Fig. 7.7a). RAPD on the strains that had been passaged in Liverpool and the same strains that had been maintained and characterised in Guatemala showed that the profiles of the two batches of the same parasites were quite different. The parasites maintained in Liverpool had a *L. mexicana* like profile whilst those maintained in Guatemala had a profile similar to the original batch of parasites that had proved difficult to identify (Fig. 7.7b).

In order to test the extent of the cross contamination with unidentifiable parasites, duplicates of two of the original group of strains (G755 and G339) were obtained from the cryobank of Guatemalan stocks held at the Centre for Disease Control in Atlanta. When tested by RAPD these isolates were characterised as *L. mexicana* (Fig. 7.7c).

7.2.8. SSU rDNA sequence analysis of unknown Guatemalan parasite G755.

As G755 did not match any reference strain tested either by RAPD or by RFLPs of the SSU rRNA gene (Chapter 6), the sequence of the 561bp region was obtained in order to classify this parasite (Appendix 7.1). The length of sequence obtained was 563bp between positions 936 and 1450 in the *L. donovani* (GB:X07773) sequence. The excess length was due principally to the presence of a 47bp insertion in G755 between positions 994 and 995 in the *L. donovani* sequence (positions 58-127 Appendix 7.2) and not present in any other trypanosomatid for which sequence data is available except *T. cruzi*. A dendrogram of the region between positions 936 and 1450 in the *L. donovani* SSU RRNA gene sequence was prepared using the DNADIST programme in PHYLIP to classify G755 with 10 trypanosomatid species (Figs 7.8 and 7.9). In this dendrogram G755 is classified between the *Crithidia/Leptomonas* clade and *Endotrypanum*. In a dendrogram compiled using the parsimony programme DNAPARS G755 lay outside the *Crithidia/Leptomonas* clade (not shown). The different results are a consequence of the distance programme treating the 47bp insertion as a single difference and the parsimony programme treating each base in the insertion as a difference. The distance matrix (Fig. 7.8) shows that the leptomonad G755 is approximately equidistant from *Leishmania*, *Endotrypanum*, *Crithidia* and *Leptomonas*.

Although the distance between the leptomonad G755 and the established genera within in the same clade appears substantial, the significance of this cannot be evaluated until the homologous sequence of more species of *Endotrypanum*, *Crithidia* and *Leptomonas* are published. When this information becomes available it will be possible to determine if G755 comes within the normal range of variation of one of these genera.

7.3. ANALYSIS OF COSTA RICAN PARASITES

Twelve parasite strains were received, reportedly isolated from cases of cutaneous leishmaniasis in the Department of Liberia, Costa Rica and believed to be due to *L. chagasi* (Zeledon et al., 1989). RAPD-PCR comparison of the Costa Rican strains with a group of reference strains showed more similarity with *Crithidia luciliae* than with any of the *Leishmania* reference strains tested (Fig. 7.10a).

The parasites grew vigorously in culture to high density ($>5x10^8ml^{-1}$) and could survive in Schneider's insect medium for 8 months at 16°C without subpassage, but did not survive in Home and Newton's 1958 (Taylor and Baker, 1968) *Crithidia* medium and only survived four weekly subpassages in the ATCC selective medium for *Crithidia*. At high densities

numerous choanomastigotes with large flagellar pockets were seen (Fig. 7.11). Electron microscopy showed that the parasites had kinetoplasts quite distinct from those seen in *Leishmania*. The kinetoplast of the Costa Rican parasites was very broad in relation to its length (Table 7.1, Fig 7.12) (cf Fig 7.3b).

A duplicate set of the same twelve isolates was obtained from Costa Rica, these parasites had identical morphology and growth characteristics to the first batch and identical RAPD profile (Fig. 7.10b).

A comparison of the *HpaII* and *HaeIII* restriction patterns of the 561bp variable region of the SSU rRNA gene of the Costa Rican parasite EVER with some monoxenous trypanosomatids showed that EVER had a similar fingerprint to *Crithidia luciliae* (Figs 6.1a and 6.1b).

Table 7.1 Dimensions of parasites

Strain No.	Gua4	Gua4	G755	G755	G755	EVER	EVER
Micrograph	D190	D192	D195	D197	D202	D200	D200
kDNA length	0.67	1.08	0.85	0.55	0.83	0.704	0.648
kDNA width	0.077	0.092	0.155	0.185	0.173	0.285	0.248
Ratio l/w	8.7	11.7	5.5	3.0	4.8	2.4	2.6

Table 7.1 Dimensions of parasites (μm) taken from electron micrographs. Gua4 *L. braziliensis*; G755 Unknown Guatemalan leptomonad; EVER Unknown Costa Rican trypanosomatid.

7.4. DISCUSSION

It is clear that the parasites from Costa Rica and Guatemala are cross contaminants of culture collections as in both cases parasites originally identified as *Leishmania* by isoenzymes later changed phenotype (Para. 7.2.7 and 7.3). Cross contamination is probably more common than is acknowledged in print (Keil & Griffin, 1994; Shaw & Camargo, 1995) and raises a number of difficulties each time. It may, as here, require a considerable effort to demonstrate that cross contamination has occurred, and it also frequently raises intriguing hypotheses about the origin of the contaminants.

7.4.1. Costa Rican trypanosomatids

The Costa Rican parasites produced large numbers of choanomastigotes in culture and were identified as *C. luciliae* by both RAPD and RFLPs of SSU rDNA (Figs 7.10 and 6.1). Although the Costa Rican parasite EVER had a *C. luciliae* like fingerprint in the RFLP of the 561bp variable region of the SSU rRNA gene the extent of variability of this sequence within the *Crithidia* is not known. The *Leishmania* are monomorphic with these enzymes for this sequence so it is possible that EVER is not *C. luciliae* but another closely related parasite. RAPD however generates species specific patterns within the *Leishmania* and may do the same within the *Crithidia* but it is not possible to be confident of this without testing a wider range of well characterised reference strains. The electron micrographs showed that these parasites had the short and broad kinetoplast characteristic of a number of *Crithidia* (Brooker, 1971; Ismaeel, 1994) and the width of kinetoplast (285 and 248nm; Table 7.1, Fig. 7.12) was similar to that reported for *C. luciliae* (280nm) (Freymuller & Camargo, 1981). The failure to flourish in *Crithidia* selective media may indicate that different *Crithidia* have different growth requirements.

As EVER has the growth characteristics and morphology of *Crithidia* and has fingerprints similar to *C. luciliae* it can be concluded that EVER is a *Crithidia* closely related to *C. luciliae*.

C. luciliae has been isolated from *Musca domestica*, and a number of calliphorids - *Calliphora sp*, *Lucilia sp* and *Phaenicia sericata* in England and the USA. In the laboratory both *Phlebotomus papatasi* and *Oncopeltus fasciatus* have been infected with *C. luciliae* (Wallace, 1966). As this parasite is so widely distributed in common diptera it is possible that the original contamination was caused by a fly infecting culture media, however it is

also possible that the parasite was recovered from a lesion and then spread through the culture collection. There have been a number of records of *Crithidia* being isolated from both visceral and cutaneous lesions of mammals including humans (Dedet et al., 1995; Mebrahtu et al., 1992; Schnur et al., 1992) and (Para 6.3.5). Monoxenous trypanosomatids are transmitted between hosts by contamination (Wallace, 1966). Since calliphorids feed principally on carrion and decaying matter of all kinds and are attracted to festering wounds, *Crithidia luciliae* presumably must be capable of surviving in this material. In festering wounds it may be possible for *Crithidia* to avoid contact with the immune system but still be isolated into culture by biopsy. It is more difficult to account for the reports of *Crithidia* isolated from the viscera.

7.4.2. Guatemalan trypanosomatids

The Guatemalan leptomonads proved more difficult to identify as their morphology was consistent with *Leishmania*, *Leptomonas*, *Endotrypanum*, *Herpetomonas* and *Crithidia*. They were unlike any of the limited number of monoxenous trypanosomatids available at Liverpool by both RAPD and the much lower resolution SSU rDNA RFLPs. The only genus specific markers for *Leishmania* known to the writer are the kinetoplast PCR primers of Dr Barker's group at the Molteno Laboratories Cambridge (Dr Barker personal communication), these primers were negative with strains of the Guatemalan leptomonad sent to Dr Barker's laboratory for testing, but the specificity of these primers has not been published. Uliana *et al.* (1991) described a genus specific marker for *Leishmania* based on a *PvuII* restriction site in the SSU rRNA gene, however this marker was only tested against *Trypanosoma* species. A search for this site in the trypanosomatid sequences used in this study showed that *T. cruzi* (GB:M12676) is the only one which is not cut by *PvuII*. As it was reported that the Guatemalan leptomonads were repeatedly isolated from humans and laboratory animals (Dr Arana, University del Valle, Guatemala) the possibility remained that they represented a novel taxon of digenetic parasites.

The dendrogram (Fig. 7.9) prepared from the partial SSU rDNA sequence (Appendix 7.1) shows that the G755 isolate lies between the *Leishmania/Endotrypanum* and *C. fasciculata/Leptomonas* groups. The distance matrix calculated from the same sequence shows that G755 is approximately equidistant between *Leishmania*, *Leptomonas*, *Endotrypanum*, and *Crithidia* with Kimura distances in the range of 0.0662-0.0860. In comparison the differences in this region of the SSU rDNA gene in the *Leishmania* are

0.0000 to 0.0108; between *Endotrypanum* and *Leishmania* 0.0445 to 0.0490; and between *C. fasciculata* and *Leptomonas* 0.0377 (Fig7.8).

The distances between G755 and other taxa are greater than between any other genera in this clade. Although there is very little difference between the *Leishmania* sequences it does not necessarily follow that other genera are as homogeneous and when further sequences become available for *Crithidia* and *Leptomonas* it may be found that G755 lies within the range of intrageneric variation of these groups. The differences in RFLPs of *C. fasciculata* and *C. luciliae* (Fig. 6.1) indicates that differences do in fact exist in the SSU rRNA gene of different *Crithidia* species at least. It seems unlikely that G755 will be within *Endotrypanum* as this parasite is exclusive to sloths which are not found in Guatemala. The behaviour of G755 in culture, particulary its ability to survive for several months without subpassage, suggests that it is a monoxenous insect parasite as these presumably must be capable of surviving in nutrient poor conditions between hosts. The morphology of G755 is more consistent with *Leptomonas* than *Crithidia* but it is not clear how reliable morphology is for identification in this rather variable group.

The Guatemalan group that isolated these parasites have done extensive vector studies and isolated a number of unknown trypanosomatids from sandflies (Arana et al., 1991). It is possible that these sandfly isolates may be the source of the cross contamination, but samples have not been available in time to include in the present study. Alternatively these parasites may have been isolated from lesions and cross contaminated other stocks at the Guatemalan laboratory.

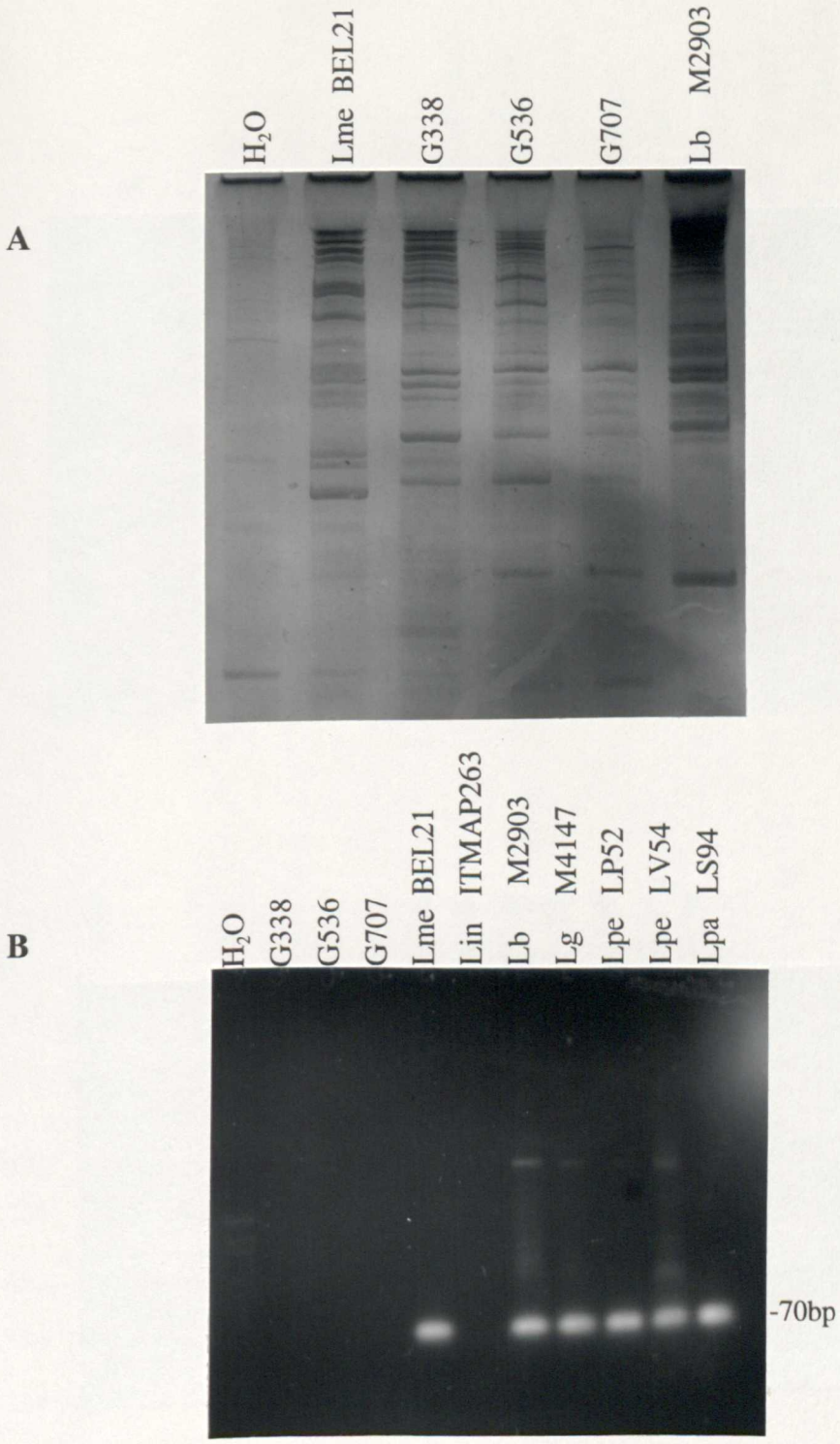


Figure 7.1A and 7.1B **(A)** RAPD fingerprints generated using primer OPA4 on *L. mexicana* (M379) (Lm); Guatemalan leptomonads (G338, G536 and G707); *L. braziliensis* (M2903) (Lb). **(B)** PCR using primers MP1L and MP3H for conserved region of kDNA of the *L. braziliensis* complex. Guatemalan leptomonads (G338, G536 and G707); *L. mexicana* (BEL21) (Lme); *L. infantum* (ITMAP263) (Lin); *L. braziliensis* (M2903) (Lbr); *L. guyanensis* (M4147) (Lgu); *L. peruviana* (LP52 and LV54) (Lpe); *L. panamensis* (LS94) (Lpa). The *L. mexicana* strain BEL21 was later confirmed to be contaminated with *L. braziliensis* DNA

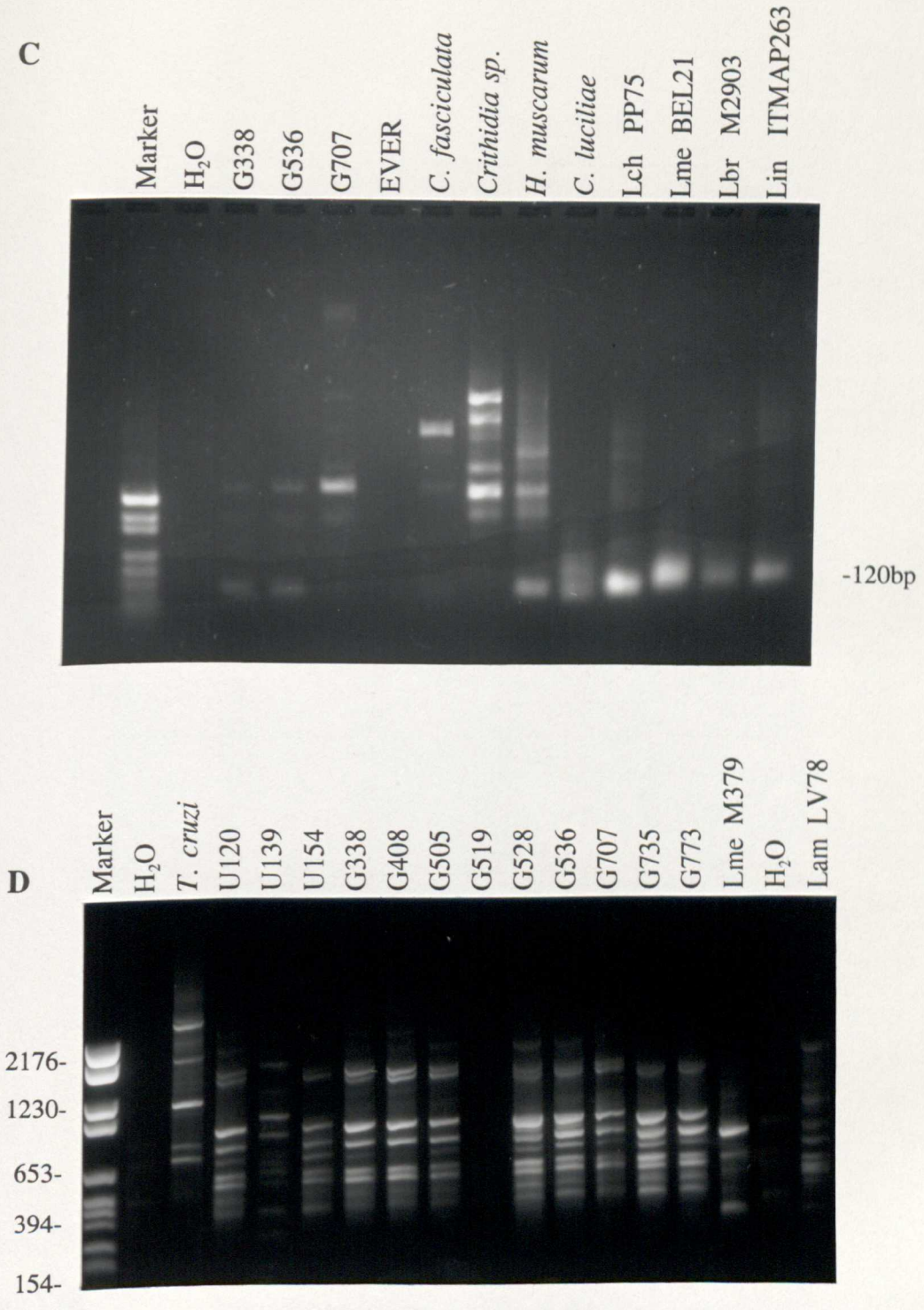


Figure 7.1C and 7.1D **(C)** PCR using primers 13A and 13B for the conserved region of kinetoplast minicircles. Guatemalan leptomonads (G338, G536 and G707); Costa Rican trypanosomatid (EVER); *C. fasciculata*; *Crithidia sp*; *H. muscarum*; *C. luciliae*; *L. chagasi* (PP75) (Lch); *L. mexicana* (BEL21) (Lme); *L. braziliensis* (M2903) (Lbr); *L. infantum* (ITMAP263) (Lin). **(D)** RAPD fingerprints generated by primer M13 with *T. cruzi*; Guatemalan leptomonads (U120, U139, U154, G338, G408, G505, G519, G528, G536, G707, G735, G773); *L. mexicana* (M379) (Lme); *L. amazonensis* (LV78) (Lam).

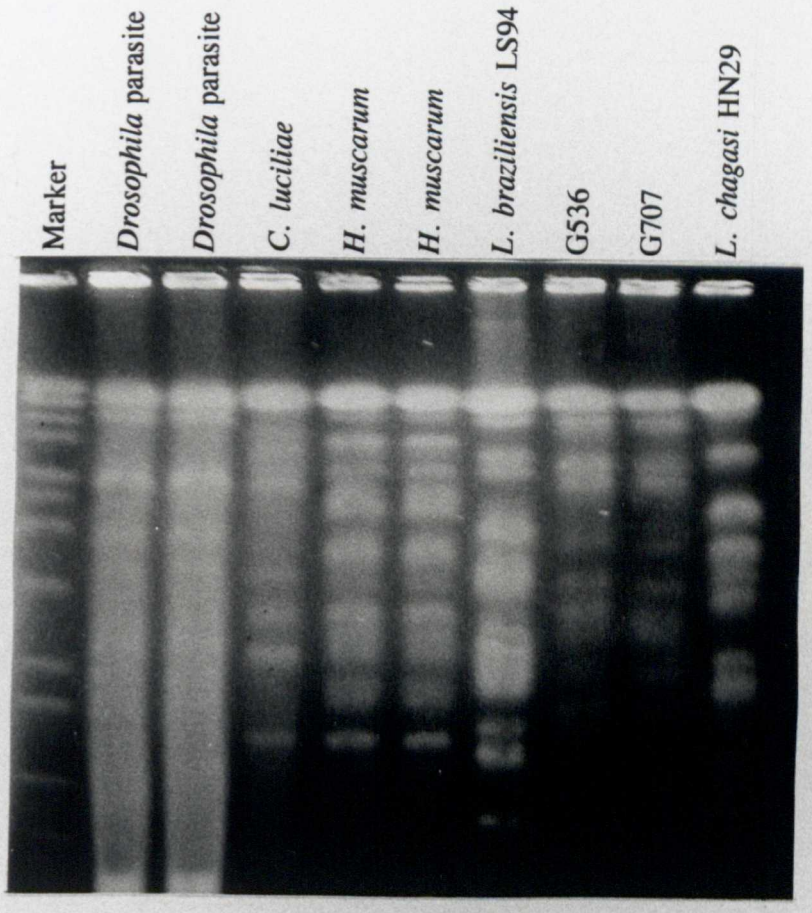
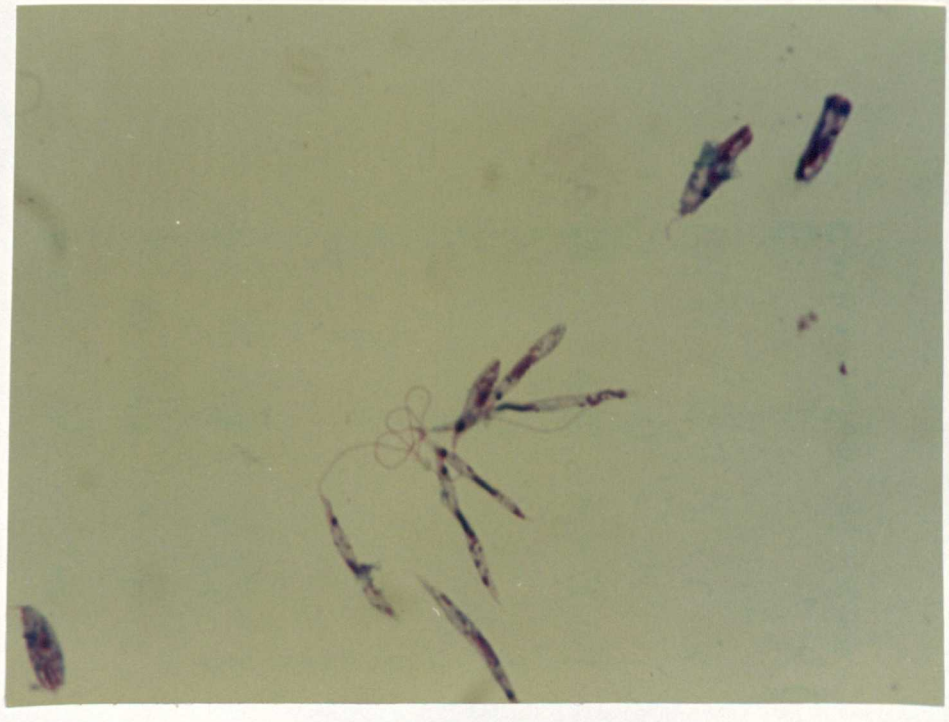


Figure 7.1E Molecular karyotypes of monoxenous trypanosomatids being maintained at the Liverpool School of Tropical Medicine contemporaneously with Guatemalan leptomonads. *Crithidia sp* isolated from *Drosophila*; *C. luciliae*; *H. muscarum*; *L. panamensis* (LS94) (Lpa); Guatemalan leptomonads (G536 and G707); *L. chagasi* (HN29) (Lc). The gel was run for 72 hours at 160V with a pulse time ramped from 72 seconds to 120 seconds. The pulsed field gel was run by A. Ismael (LSTM) using Guatemalan parasites lysed *in situ* in agarose blocks prepared by the present author.

A

B



10μm

Figure 7.2A and 7.2B **(A)** Giemsa stained *L. braziliensis* promastigotes (x1,600). **(B)** Giemsa stained Guatemalan leptomonads (G755) (x1,600).

Fig. 7.3

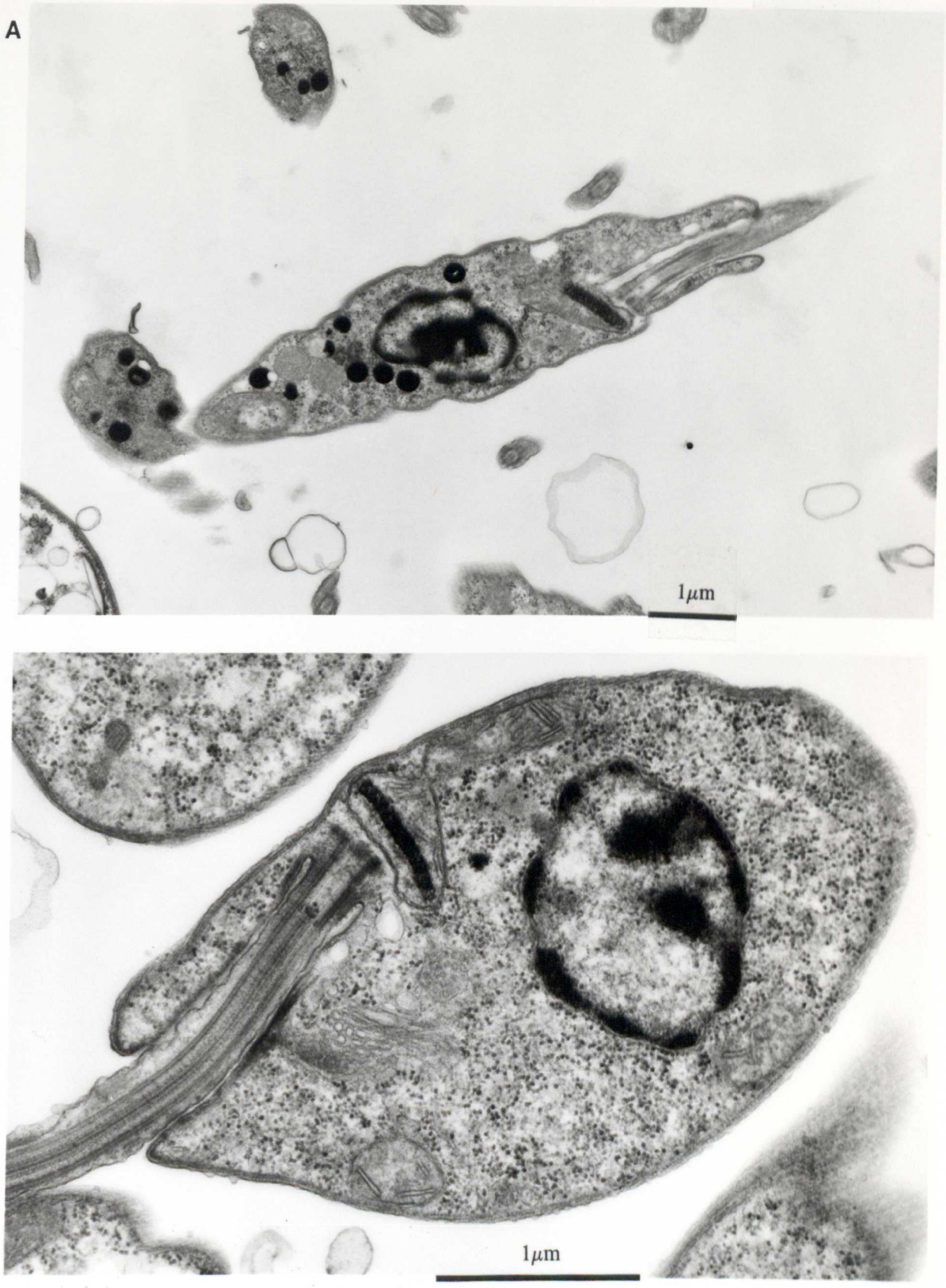


Figure 7.3A and 7.3B **(A)** Electron micrograph of Guatemalan leptomonad (G755) (x6,600) (D.1.9.5). **(B)** Electron micrograph of *L. braziliensis* (Gua4) (x15,500).

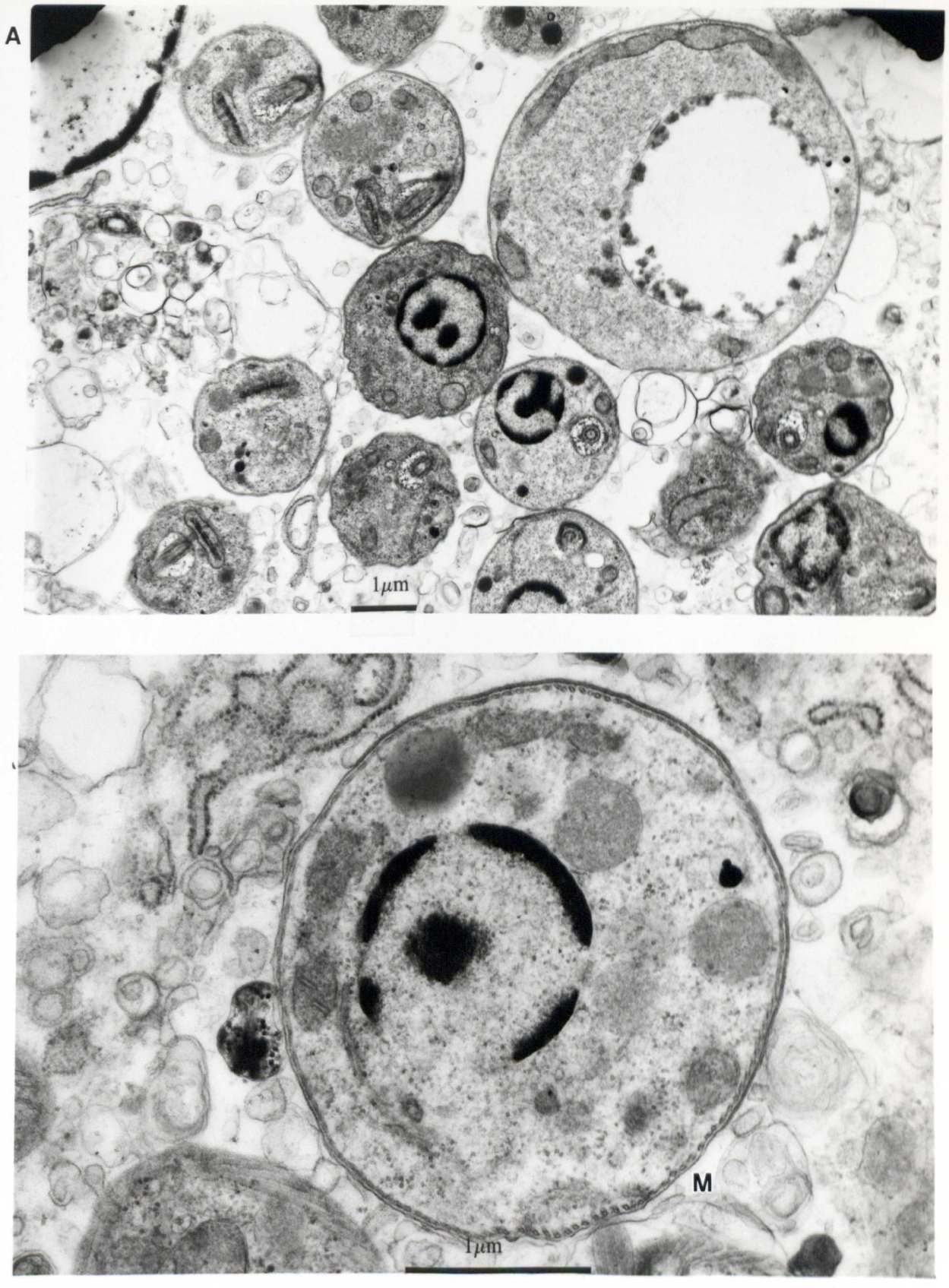
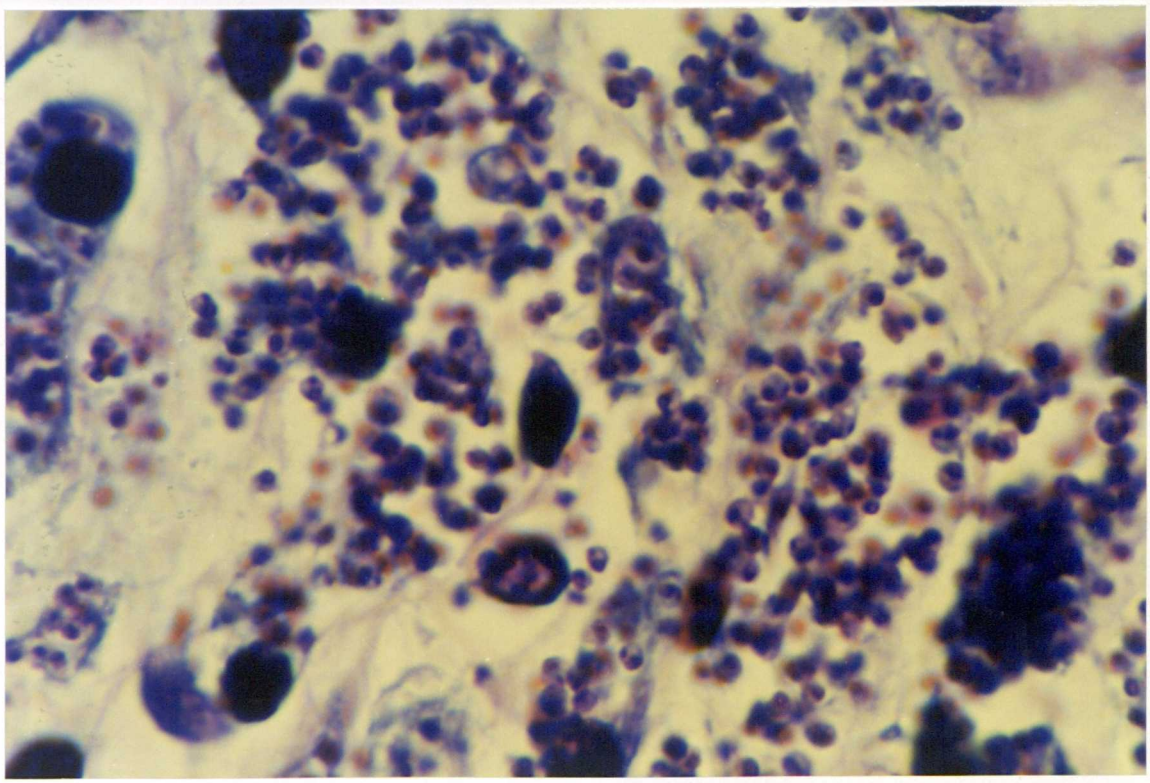


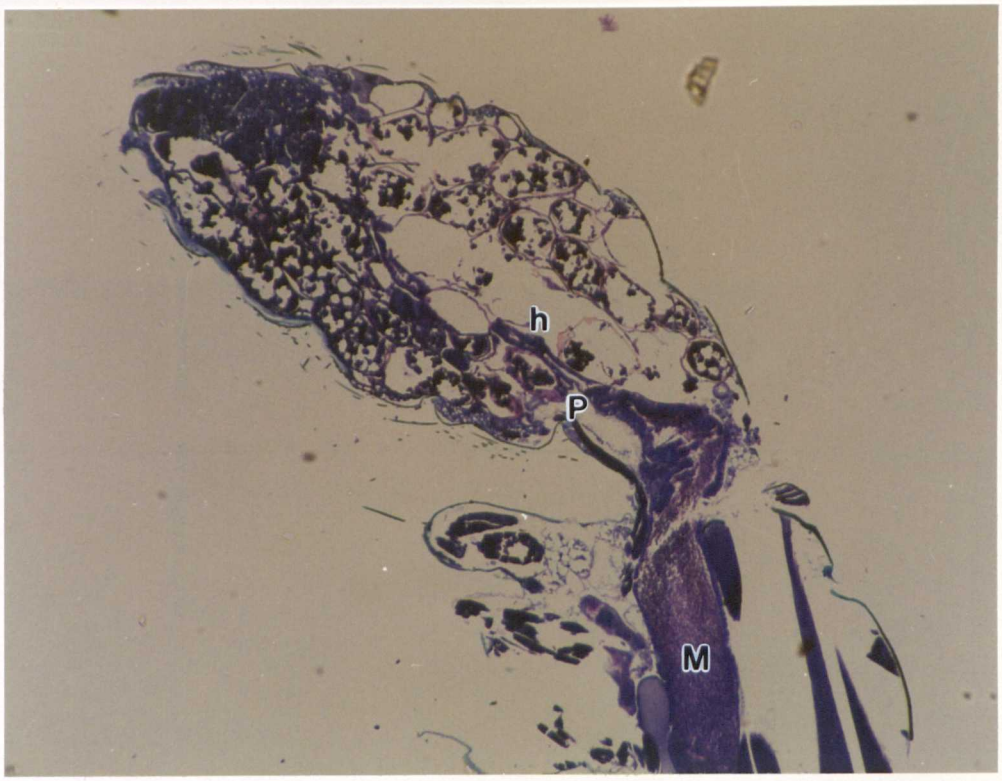
Figure 7.4A and 7.4B **(A)** Electron micrograph of amastigotes from a lesion caused by U265 in a hamster foot. The hamster was infected in Guatemala and a biopsy was sent to Liverpool in cacodylate buffer (x5,200). (EM D.4.5.4.)
(B) Amastigote from the same lesion showing 116 microtubles (m) (x15,500). (EM D.4.5.3.)



10μm

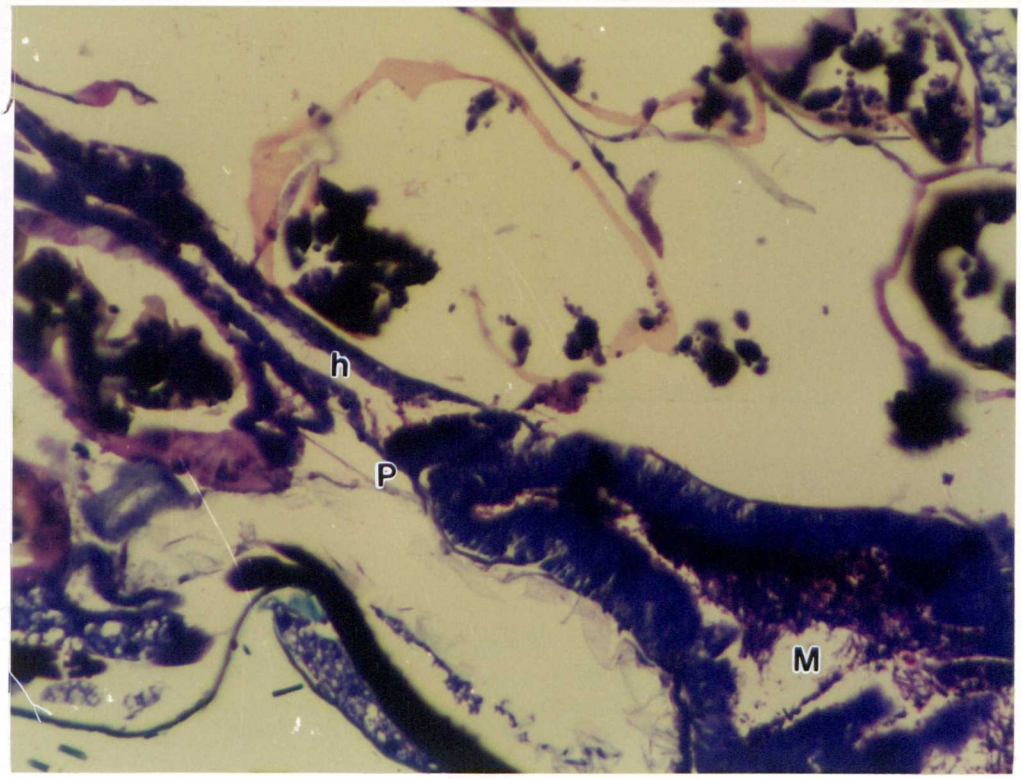
Figure 7.5 Giemsa stained amastigotes of Guatemalan U266 parasites in hamster foot lesion (x1,600).

A



300μm

B



50μm

Figure 7.6A and 7.6B **(A)** Giemsa stained section of thorax and abdomen of *Lu. longipalpis* infected with U266 from Guatemala showing heavy midgut infection (x64). **(B)** Giemsa stained section shown in Figure 7.6A at higher magnification (x320) showing pylorus. Note the heavy infection in the midgut and absence of parasites from the hindgut. **M** midgut; **P** Pylorus; **H** hindgut.

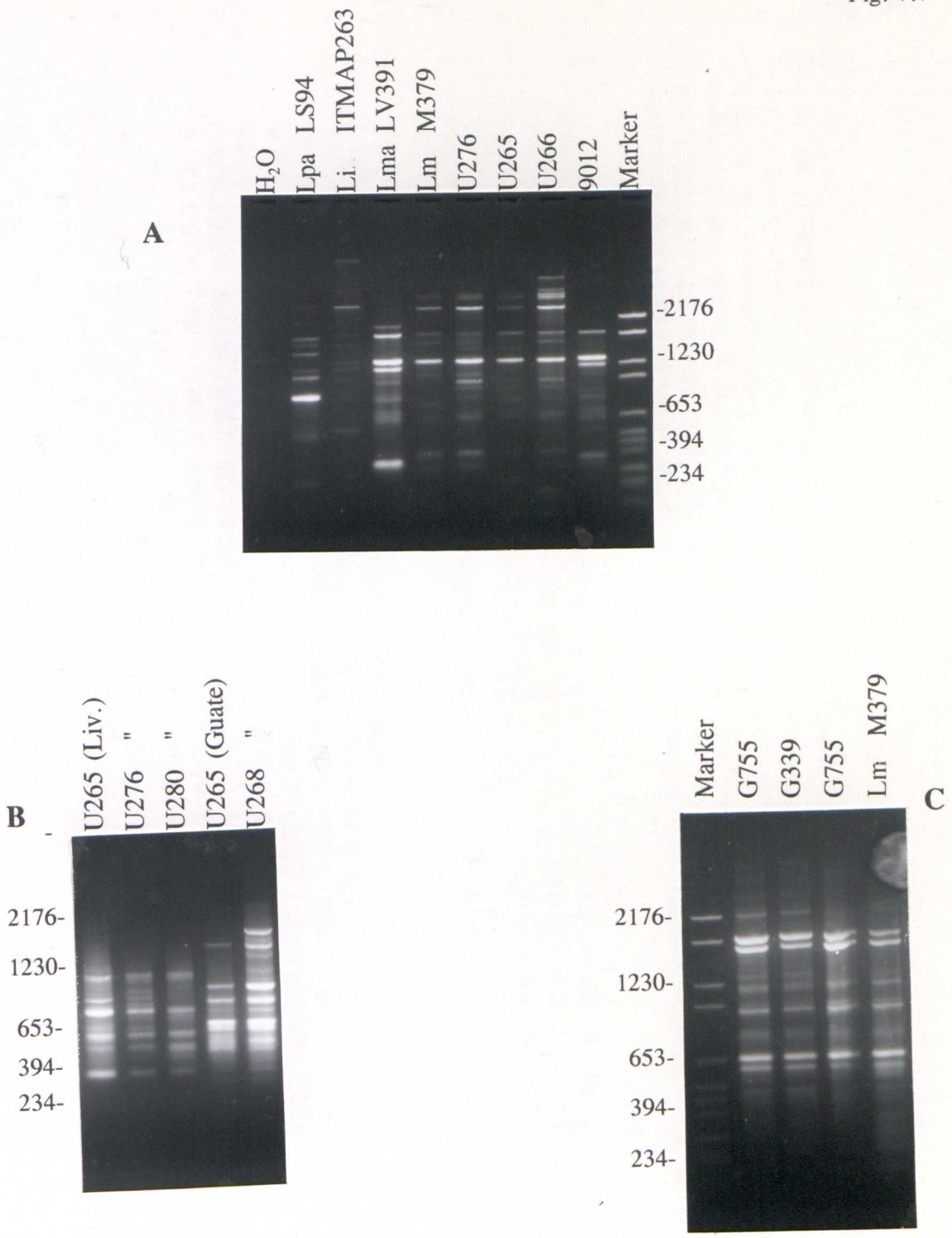
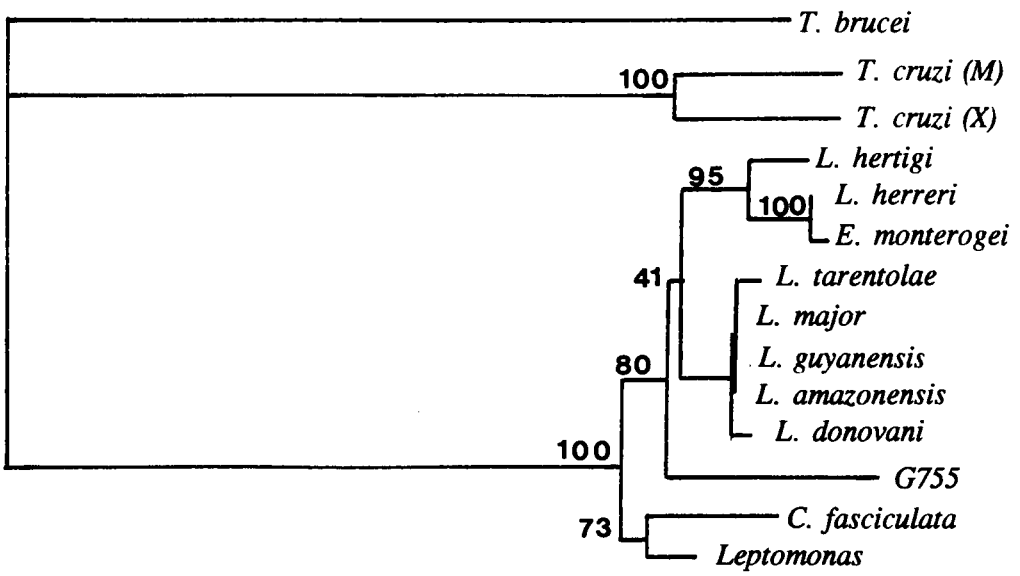


Figure 7.7A, 7.7B and 7.7C **(A)** RAPD fingerprints generated by primer 3301 on *L. panamensis* (LS94) (Lpa); *L. infantum* (ITMAP263) (Li); *L. major* (LV391) (Lma); *L. mexicana* (M379) (Lm); Guatemalan parasites recovered from mouse and hamster lesions (U276, U265 and U266. Putative *L. mexicana* (9012). **(B)** RAPD fingerprints generated by primer M13 from strains isolated in Liverpool from hamster biopsies sent from Guatemala (U265, U276 and U280) and parasites from the same batch isolated from hamster biopsies in Guatemala and cultured there before being sent to Liverpool (U265 and U268). **(C)** Duplicates of unidentified leptomonads recovered from the CDC cryobank and sent to Liverpool for processing (G755, G339, G755); *L. mexicana* (M379) (Lm).

	L.ma	L.am	L.ta	L.do	E.mo	L.herr	L.hert	L.gu	C.fa	Lep	G755	T.crX	T.crM	T.br
L. major	0.0000	0.0000	0.0065	0.0043	0.0445	0.0432	0.0362	0.0000	0.0564	0.0426	0.0663	0.3566	0.3549	0.3402
L. amazonensis	0.0005	0.0000	0.0065	0.0043	0.0445	0.0431	0.0361	0.0000	0.0563	0.0425	0.0662	0.3599	0.3581	0.3437
L. tarentolae	0.0038	0.0043	0.0000	0.0108	0.0469	0.0455	0.0428	0.0065	0.0635	0.0495	0.0736	0.3559	0.3542	0.3410
L. donovani	0.0019	0.0024	0.0057	0.0000	0.0490	0.0475	0.0405	0.0043	0.0586	0.0425	0.0616	0.3591	0.3574	0.3391
E. monterogei	0.0167	0.0172	0.0177	0.0186	0.0000	0.0063	0.0276	0.0445	0.0830	0.0638	0.0761	0.3697	0.3662	0.3425
L. herreri	-	-	-	-	-	0.0000	0.0279	0.0431	0.0755	0.0571	0.0735	0.3498	0.3432	0.3264
L. hertigi	-		-	-		-	0.0000	0.0361	0.0746	0.0634	0.0730	0.3476	0.3434	0.3376
L. guyanensis	0.0019	0.0024	0.0047	0.0028	0.0167	-	-	0.0000	0.0563	0.0425	0.0662	0.3599	0.3581	0.3437
C. fasciculata	0.0215	0.0220	0.0255	0.0220	0.0314	-	-	0.0215	0.0000	0.0377	0.0860	0.3520	0.3574	0.3422
Leptomonas spp	0.0201	0.0206	0.0230	0.0211	0.0260	-	-	0.0201	0.0201	0.0000	0.0743	0.3389	0.3478	0.3483
G755	-	-	-	-	-	-	-	-	-	-	0.0000	0.4509	0.4336	0.3533
T. cruzi X	0.1244	0.1255	0.1256	0.1249	0.1255	-	-	0.1244	0.1244	0.1216	-	0.0000	0.0760	0.3613
T. cruzi M	0.1266	0.1277	0.1277	0.1271	0.1275	-	-	0.1266	0.1277	0.1261	-	0.0202	0.0000	0.3604
T. brucei	0.1584	0.1596	0.1585	0.1583	0.1561	-	-	0.1572	0.1539	0.1571	-	0.1479	0.1495	0.0000

Figure 7.8 Upper right quadrant:- distance matrix compiled from the sequences of the region of the SSU rRNA gene spanned by the available sequences of the Gutemalan leptomonad G755, *L. hertigi* (LV42) (L.hert) and *L. herreri* (LV344) (L.herr) using DNADIST in PHYLIP. Lower left quadrant:- distance matrix compiled from the whole sequence of the SSU rRNA gene of the indicated taxa in the same way. *Leishmania major* (GB:X53915) (L.ma); *L. amazonensis*(GB:X53912) (L.am); *Leishmania tarentolae* (GB:X53916) (L.ta); *L. donovani* (GB:XO7773) (L.do); *Endotrypanum monterogei* (GB:X53911) (E.mo); *L. guyanensis* (GB:X53911) (L.gu); *Crithidia fasciculata* (GB:X03450) (C.fa); *Leptomonas spp* (GB:X53914)(Lep); *T.cruzi* (GB:X53917) (T.crX); *T.cruzi* (GB:M31432) (T.crM); *T.brucei* (GB:M12676) (T.br); *Bodo caudatus* (GB:X53910) (B.ca).

A



B

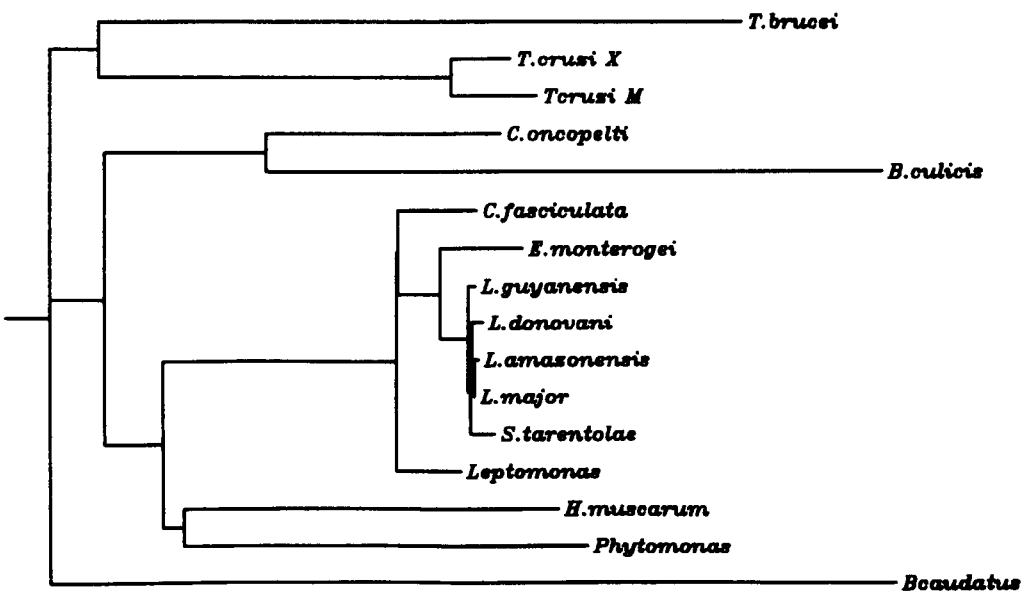


Figure 7.9A and 7.9B (A) Dendrogram of the available sequence of the SSU rRNA gene of the Guatemalan leptomonad (G755); *L. hertigi* (LV42); *L. herreri* (LV344) (GB:U50043) with the same sequence from representative trypanosomatids (Appendix 7.2), prepared using FITCH on the output from DNADIST (Fig 7.8). *Leishmania major* (GB:X53915); *L. amazonensis* (GB:X53912); *Leishmania tarentolae* (GB:X53916); *L. donovani* (GB:XO7773); *Endotrypanum monterogei* (GB:X53911); *L. guyanensis* (GB:X53911); *Crithidia fasciculata* (GB:X03450); *Leptomonas spp* (GB:X53914); *T.cruzi* (GB:X53917); *T.cruzi* (GB:M31432); *T.brucei* (GB:M12676). Numbers at nodes were bootstrap values calculated from 205 replicates.

(B) Dendrogram of the published sequences of the whole SSU rRNA gene prepared using FITCH on the output for DNADIST (Fig. 7.8). The same strains as in (A) were used with the addition of *H.muscarum* (GB:L18872); *Phytomonas sp.* (GB:L35076); *Crithidia oncopelti* (GB:L29264); *Blastocrithidia culicis* (GB:X53910); *Bodo caudatus* (GB:X53910). Species for which only partial sequences were available were not used.

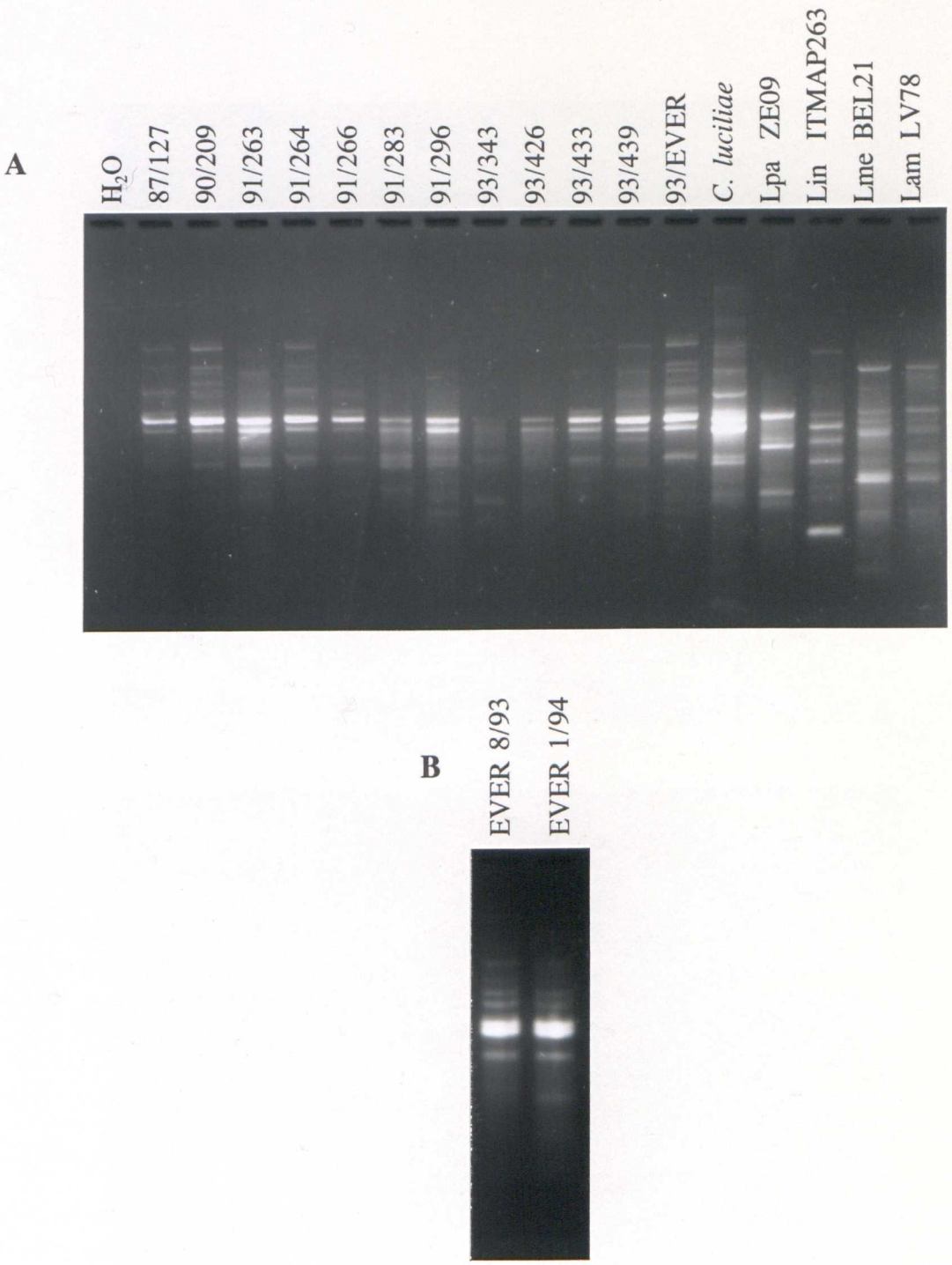
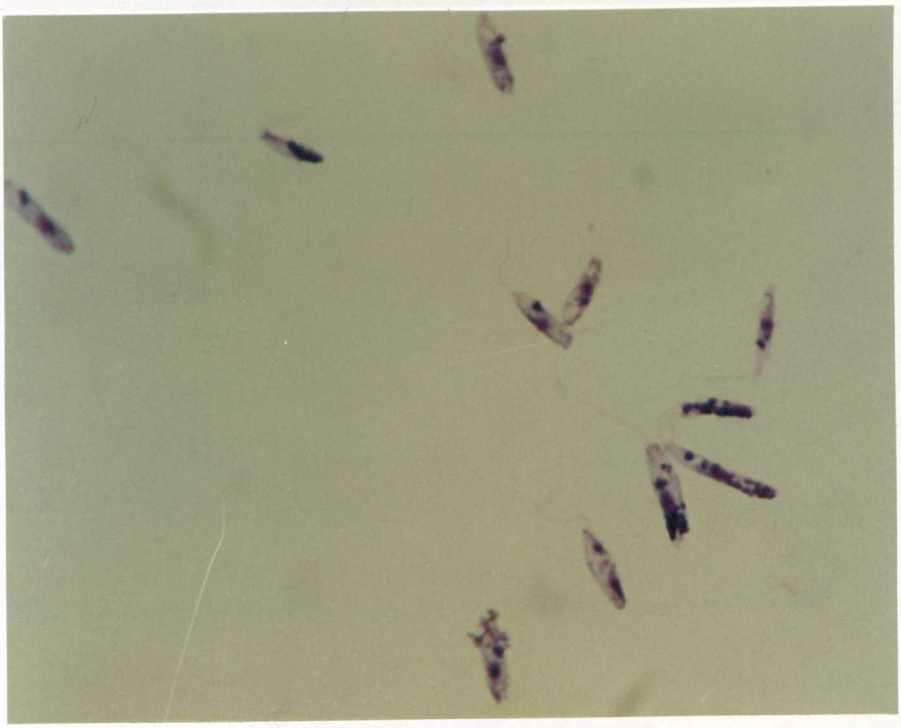


Figure 7.10A and 7.10B **(A)** RAPD fingerprints generated by primer M13 with Costa Rican trypanosomatids 87/127; 90/209; 91/263; 91/64; 91/266; 91/283; 91/296; 92;343; 92/426; 93/433; 93;439; 93;EVER; *C. luciliae*; *L. panamensis* (ZE09) (Lpa); *L. infantum* (ITMAP263) (Lin); *L. mexicana* (BEL21) (Lme); *L. amazonensis* (LV78) (Lam). **(B)** RAPD fingerprints generated by primer M13 on Costa Rican trypanosomatids 93/EVER recieved in August 1993 and of a duplicate of the same strain recieved in January 1994.

A



B

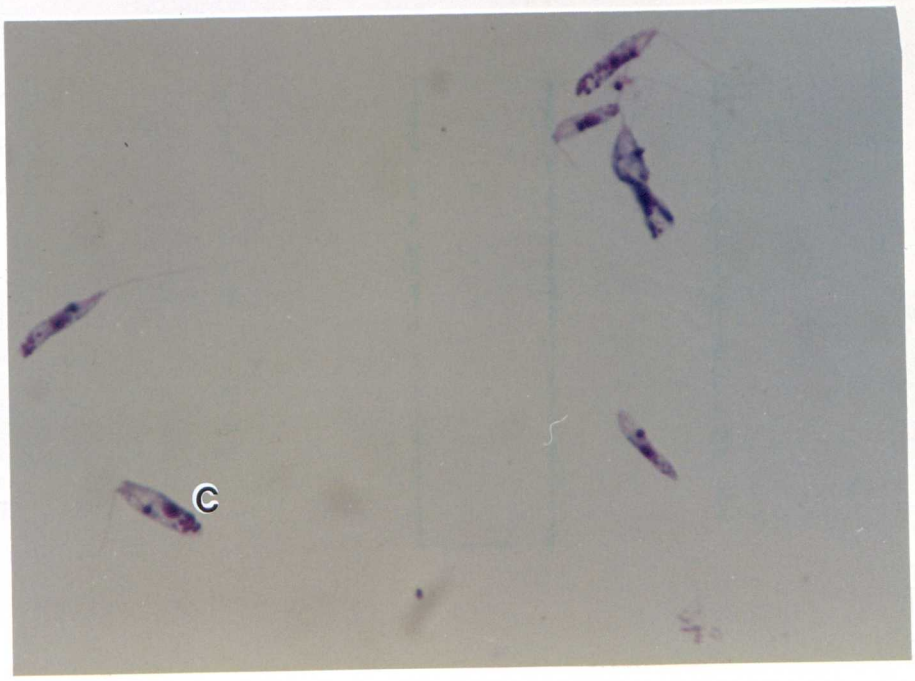


Figure 7.11A and 7.11B **(A)** and **(B)** Giemsa stained promastigotes of Costa Rican trypanosomatids (EVER) showing some choanomastigote forms (x1,600). C choanomastigote.

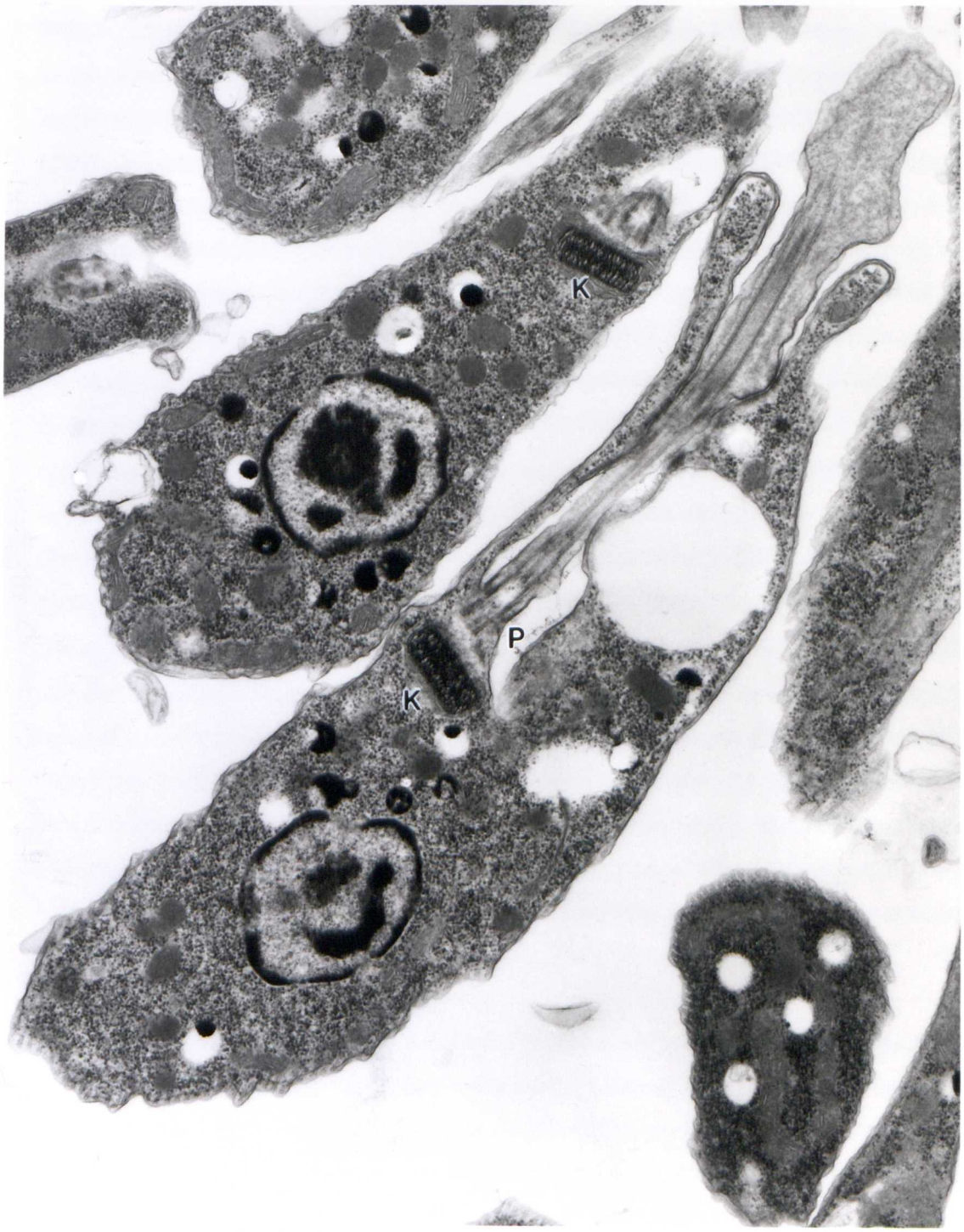


Figure 7.12 Electron micrograph of Costa Rican trypanosomatid EVER (x8,900), showing broad kinetoplast (k), and deep flagellar pocket (p).

8. DISCUSSION AND CONCLUSIONS

8.1. DNA BASED APPROACHES TO THE IDENTIFICATION AND CLASSIFICATION OF *LEISHMANIA*

Five PCR based techniques for the identification and classification of *Leishmania* were used in the present study. In approximate order of increasing resolution the five methods were as follows:- partial sequences of the SSU rRNA gene; RFLPs of PCR amplified SSU rDNA; kDNA variable region probes; RAPDs and lastly RFLPs of kinetoplast DNA (schizodemes). The five methods have only partially overlapping applications. The sequence of the SSU rRNA gene cannot resolve the mammalian or lizard *Leishmania* presumably because the few informative differences in this gene are disguised by homoplasy. However the sequence of this gene is useful for the identification and classification of other trypanosomatids such as the Guatemalan leptomonad G755 (Para. 7.2.8) which would be difficult to identify by fingerprint techniques in the absence of reference strains. RFLPs of the SSU rRNA gene appear to give higher resolution classifications than the complete gene sequence. This may be because enzymes were selected that were known to detect polymorphisms in this gene (Para 6.2). *HaeIII* or *HpaII* digests of the 561bp fragment of the SSU rRNA gene produced genus specific markers for all members of the genus *Leishmania* tested except *L. hertigi* (Para. 6.1.2). RFLPs of the SSU rRNA gene also provided markers for the identification of lizard and mammalian parasites using *Sau96I* (Para. 6.1.3) and the *L. (Viannia)* and *L. (Leishmania)* subgenera using *AluI* or *SacI* (Para. 6.1.2). RAPD was found to be useful for identifying *Leishmania* species (Para. 3.1.3) and for classifying *L. (Viannia)* species (Para. 3.4). kDNA variable region probes were useful for identifying most *L. (Leishmania)* species and examining the relationships within the major groups (Para. 4.4). PCR based schizodemes of kinetoplast DNA were useful for identifying *L. chagasi* populations (Para. 5.1.3).

The methods described above offer techniques for approaching the majority of problems in the identification and classification of *Leishmania*, however one important area is not adequately resolved by any of the methods described here or elsewhere. The relationship between the various *Leishmania* complexes cannot be resolved by SSU rDNA based approaches which are too low resolution or by RAPD, schizodemes or isoenzymes which are too high resolution. Tibayrenc *et al.* (1993) used RAPD and isoenzymes to investigate the relationship between *L. braziliensis*, *L. guyanensis*, *L. amazonensis*, *L. mexicana* and *L. infantum* and also between various *T. cruzi* strains. Using both techniques they found

Jaccard distances between the major groups in excess of 0.8 on a scale from 0 (no difference) to 1 (no similarity). However the distance matrix and dendrogram derived from SSU rDNA sequence data (Fig. 7.8 & 7.9) shows that the two major groups of *T. cruzi* parasites are at least as distant from each other as *Leishmania* is from *Endotrypanum* whilst the *Leishmania* complexes are almost indistinguishable by this method. This comparison suggests that, at a Jaccard distance of 0.8, isoenzymes and RAPD are both saturated with change and cannot give a useful indication of the distance between species or strains (Para. 1.13.1.1). The restriction analysis of the SSU rRNA gene (Chap. 6) highlighted the difficulty of classifying the lizard *Leishmania* and the *L. hertigi* complex, both these groups appear to lie outside the zone of optimum resolution of the existing methods.

The RFLPs of the SSU rRNA gene did not permit the determination of the generic affiliation of the *L. hertigi* complex with confidence although the partial sequence of the same gene supported a closer relationship with *Endotrypanum* than *Leishmania* (Fig. 6.6 & 7.9). Consequently it seems likely that this group of parasites diverged from either *Endotrypanum* or *Leishmania* soon after the two genera separated. Such short lineages deep within a dendrogram are difficult to resolve with confidence by any method (Felsenstein, 1988). The existing high resolution methods produce a Jaccard similarity coefficient between the *Leishmania* subgenera of less than 0.2 (Cupolillo et al., 1994), at this low level of the similarity homoplasy is likely to be significant. SSU rDNA sequences show a similarity of 0.989 between *Endotrypanum* and *Leishmania* (Fernandes, et al., 1993), at this high level of similarity there is insufficient information to be confident of relationships. The dendrogram in figure 7.9 suggests that the *L. tarentolae* may be evolving faster than the mammalian parasites, a possibility that is further supported by the nDNA buoyant density of this species which exceeds that of all mammalian *Leishmania*. The relatively rapid evolution of the DNA of the lizard parasites would be consistent with their adaption to a different class of host and a different genus of vector. If the lizard *Leishmania* are evolving at a different rate this will further compound the difficulties of classification, particularly when examining relationships that are beyond the zone of optimal resolution of the method being used (Para. 1.13). DNA sequences or methods that show approximately 60-80% similarity between the *L. (Leishmania)*, the *L. (Viannia)* and the lizard *Leishmania*, or between *Leishmania* and *Endotrypanum* should help to resolve the classification of the lizard parasites and the *L. hertigi* complex.

Mitochondrial genes of metazoa evolve at approximately 2% per million years, approximately 200 times faster than the SSU rRNA genes, and these sequences have been used to examine the relationships within a number of metazoan families (Nigro, 1988). Sequences or RFLPs of these genes might be useful for examining relationships between *Leishmania* complexes. A phylogeny of the Trypanosomatidae has been compiled from the sequences of the mitochondrial 9S and 12S rRNA but the mitochondrial rRNA genes appear to be as conserved as the nuclear genes (Lake et al., 1988). The editing of trypanosomatid mitochondrial genes may however be an obstacle to the use of these genes for classification as editing can introduce greater heterogeneity into the protein product of these genes than is present in the mitochondrial DNA (Landweber & Gilbert, 1993). As *Leishmania* mitochondrial genes are only 5' edited (Maslov et al., 1994), the analysis of the unedited 3' regions may provide reliable classifications of the *Leishmania*.

A 260bp repetitive sequence has been used to construct a phylogeny of eight mammalian *Leishmania* species from the Old World which shows relatively little difference between strains within a species and sufficient difference between species to resolve the *L. tropica*, *L. major* and *L. donovani* complexes with confidence (Piarroux et al., 1995). The sequence of that repetitive unit for various lizard species might provide a useful insight into their relationship to the mammalian species. The sequence of a polymerase gene from a number of *Leishmania* and *Endotrypanum* species including *L. hertigi* and some lizard species produced a dendrogram similar to those presented here with the exception of the lizard *Leishmania* which lay outside the *L. (Viannia)* and *L. (Leishmania)* subgenera but inside the *L. hertigi* complex. The similarity between *L. donovani* and *L. braziliensis* was 92% and between *L. donovani* and *L. gymnodactyli* was 90% (Croan & Ellis, In Press). This is a much more useful level of difference between the subgenera than provided by any other available data. Other sequences that have been proposed for the classification of *Leishmania* are ribosomal spacers and the dihydrofolate reductase - thymidylate synthase gene (DHFR-TS) (Briones et al., 1992). Restriction digests of the internal transcribed spacers (ITS) between the SSU and LSU rRNA genes suggest that this region will give a similar resolution to that obtained with isoenzymes (Cupolillo et al., 1995), so it will probably not be useful for examining the relationship between complexes. The DHFR-TS gene only showed 6.6% homology between *L. amazonensis* and *L. major* (Nelson et al., 1990), which is about what would be expected by chance, so whilst this gene might be useful for examining relationships within complexes it is unlikely to resolve different species complexes.

Although the DNA and isoenzyme based classifications do not permit the classification of the *L. hertigi* complex and the lizard *Leishmania* with confidence, biogeographical evidence can be interpreted to support the dendrogram in figure 6.6 which places the *L. hertigi* complex external to both the *L. (Leishmania)* and *L. (Viannia)* complexes and places the lizard *Leishmania* at the tip of the *Leishmania* tree. These observations are consistent with a Neotropical origin of the *Leishmania/Endotrypanum* clade, a possibility that is discussed in the next section.

8.2. EVIDENCE FOR A SOUTH AMERICAN ORIGIN OF *LEISHMANIA*

8.2.1. Introduction

Few authors have discussed the particular host parasite systems in which digenetic *Leishmania* might have evolved, nor when and where conditions might have been suitable for such a transition. The only existing hypothesis is that *Leishmania* appeared as a digenetic parasite of reptiles and early mammals at about the time that the first true sandflies arose in the Jurassic (136-190 MYA) and that the Old and New World mammalian species developed in the early Tertiary (65-5MYA) at about the time the genera *Phlebotomus* and *Lutzomyia* formed (Saf'janova, 1986). Lainson and Shaw (1986) also proposed a Jurassic origin for the lizard *Leishmania*, which they regarded as a separate genus - the *Sauroleishmania*. The same authors also suggested that the mammalian *Leishmania* might have evolved in edentates in the Neotropics with migration to the Old World in more recent times. Gondwana was still separating from Laurasia in the Jurassic and it would be possible for the host parasite systems to become distributed world wide. The absence of reptilian *Leishmania* from the New World is ascribed to the co-evolution of these parasites with *Sergentomyia* sandflies which are restricted to the Old World.

An analysis of ribosomal gene sequences has recently suggested that the trypanosomatids developed a digenetic lifecycle on at least four occasions (Fernandes, et al., 1993). One of the groups that made an independent switch from monogenetic to digenetic lifecycles was the *Leishmania/Endotrypanum* clade which appeared at about the time of the mammalian radiation towards the end of the Cretaceous (135-65MYA). Other analyses of the SSU rRNA gene have shown that the lizard parasite *L. tarentolae* cannot be resolved from the mammalian *Leishmania* on the complete sequence of this gene although there are significant differences between *Leishmania* species and *Endotrypanum* Chapter 6 and (Briones et al., 1992). The nuclear DNA GC compositions of the lizard *Leishmania* (58-61%) are within the

range of the values for the mammalian parasites (57-60%) whilst the GC composition of *Endotrypanum* (53%) is quite distinct (Chance et al., 1974; Croft et al., 1980). Since the *Endotrypanum* are restricted to the New World, this molecular evidence would be consistent with a Neotropical origin for the *Leishmania/Endotrypanum* clade, with the evolution of the lizard parasites occurring after the *Leishmania* lineage had migrated to the Old World.

8.2.2. Evidence from phylogenies

In the isoenzyme based dendrogram of Thomaz Soccol *et al.* (1993, 1993a) the mammalian *Leishmania* are divided into two groups. One group consists of the *L. (Viannia)* subgenus which is restricted to the Neotropics, the other group consists of the *L. (Leishmania)* which is found worldwide. The first two branches off the *L. (Leishmania)* lineage are the *L. hertigi* complex and the *L. mexicana* complex both of which are indigenous to the Neotropics, whilst at the tip of this branch are all the Old World mammalian species. The location of exclusively neotropical species adjacent to the root of the *Leishmania* tree and Old World species at the tip of one of the major branches has not been considered significant for the origin of the clade, because it has been believed that the lizard *Leishmania* would be an outgroup to all the mammalian *Leishmania* (Killick-Kendrick et al., 1986). However the dendrograms in figure 6.6 and the published sequence of the *L. tarentolae* SSU rRNA gene provide strong evidence that the lizard species separated from the *Leishmania* lineage after the *Endotrypanum* and the *L. hertigi* complex branched off and possibly after the *L. (Viannia)* branched off. As the lizard *Leishmania* are restricted to the Old World the most parsimonious explanation of this data is that the *Endotrypanum/Leishmania* clade evolved in the Neotropics and that the lizard parasites diverged from the *L. (Leishmania)* after the migration of members of that lineage to the Old World.

There are a number of problems with molecular clocks for determining the timing of evolutionary events (Para. 1.12) and the available evidence is sufficiently imprecise to support a Tertiary (65-5MYA) or Cretaceous (135-66MYA) origin of the *Leishmania/Endotrypanum* clade. The divergence between the ribosomal genes of the *Leishmania* subgenera has been estimated at about 0.3%. The divergence between mammals and amphibians is about 3.2% and occurred approximately 340 MYA (Fernandes, et al., 1993). This would be consistent with the separation of the *Leishmania* subgenera about 30 MYA. However small differences between large numbers are often unsatisfactory and Fernandes *et al.* (1993) used the same data to support a separation of the subgenera before

the separation of the Neotropics and Africa about 90 MYA. In the same study the distance between *Endotrypanum* and *Leishmania* was 1.1% indicative of a separation about 110MYA. However the vertebrates may not be a very satisfactory phylum to use to calibrate the trypanosomatid molecular clock. In the present study the Kimura distance between the complete SSU rRNA gene of *E. monterogeii* and *Leishmania* species was between 0.0167 and 0.0186 and between the homologous gene from two strains of *T. cruzi* was 0.0202 (Fig. 7.13). If the earliest date for the separation of two major *T. cruzi* lineages is set at about the time the marsupials and edentates appeared in the Neotropics at the end of the Cretaceous about 65 MYA, then this would imply that the *Leishmania* and *Endotrypanum* separated about 60 MYA and the *Leishmania* subgenera separated about 15 MYA (Kimura distance 0.0019-0.0047).

The GC content of the South American species appears to resemble the primitive state more closely than that of the Old World species. GC contents of individual genes and whole genomes are remarkably stable within both the mammals and the birds over large genetic distances but is much more variable in the protozoa and bacteria. GC content is believed to be determined by two pressures, firstly the relative abundance of different tRNAs which will be most significant for highly expressed genes and secondly a mutational bias which will be most significant for genes expressed at low levels and for non-coding regions. High GC content DNA also tends to be more stable at higher temperatures and code for amino acids such as alanine and arginine that are more thermostable. The *Leishmania* genes for heat shock proteins for example have GC compositions in excess of 90% at the third codon position (Alvarez et al., 1994; Bernardi et al., 1988).

All trypanosomatids tend towards GC richness for highly expressed genes. The GC contents in the flanking regions has been estimated (Table 8.1) and shows a rising trend through *T. brucei*, *T. cruzi*, *C. fasciculata*, *L. donovani*, *L. major*. The trend in GC content of the flanking regions follows the branching order of the trypanosomatids in the phylogeny of Fernandes *et al.* (1993) (Fig. 2), consequently low GC content may be the primitive state.

L. major	61%
L. donovani	59%
C. fasciculata	52%
T. cruzi	42%
T. brucei	41%

Table 8.1 GC composition of gene flanking regions for five trypanosomatids (Alvarez et al., 1994).

The overall GC content for a large number of mammalian and lizard *Leishmania* and *Endotrypanum*, species is available. The GC content of the parasites rises through *Endotrypanum*, *L. hertigi*, *L. braziliensis*, *L. mexicana*, *L. major*, *L. donovani*, *L. tropica* *L.aethiopica*. Lizard *Leishmania* species fall into two groups, *L. adleri* and *L. hoogstraali* have values similar to members of the *L. braziliensis* complex at the low end of the range whilst *L. agamae* and *L. tarentolae* have values higher than any *Leishmania* (Chance et al., 1974; Croft et al., 1980). The GC composition of kinetoplast DNA is lower than that of nuclear DNA and is more variable with different strains of the same species having different values, but a similar rising trend is apparent from *L. braziliensis* through *L. mexicana* to the Old World species. Once again the lizard *Leishmania* have no clear pattern but *L. hertigi* has kDNA GC composition which is in the same range as the *L. mexicana* complex values. For both kDNA and nuclear DNA the Neotropical *Leishmania* appear to have a more primitive GC content than the Old World parasites.

8.2.3. Evidence from palaeontology

As the phylogenies, parasite distribution and GC content all point to an origin in South America it is appropriate to consider possible host parasite systems in which digenetic parasitism might have arisen.

During the Cretaceous (65-135 MYA) Gondwana split into two major fragments one of which became Africa and India and the other South America, Antarctica and Australia (Cox, 1974). At the same time the mammalian radiation was occurring (Novacek, 1992). It is believed that the marsupials evolved in South America and migrated to Australia and the Nearctic (Briggs, 1987). The placental mammals (Eutheria) probably evolved in Asia but

only the most primitive orders reached South America, principally the notoungulates, which are now extinct, and the edentates and no Eutheria reached Australia (Cox, et al., 1976).

Therefore by the Palaeocene (65.0-53.5 MYA) South America had become isolated from the other continents and had a primitive fauna whose sole surviving descendants are the Edentata (Xenarthra) represented by the armadillos, anteaters and sloths and the marsupials. South America then remained at least partially isolated from the other continents until the Isthmus of Panama formed in the Pliocene. Sometime around the Oligocene/Eocene boundary (37.5 MYA) Hystricomorpha and primates arrived, possibly by island hopping from the Nearctic (Briggs, 1987). The hystricomorphs diversified into at least twelve families including the guinea pigs and porcupines. The South American Chiroptera are presumed to be indigenous but the fossil record gives no indication of when they arrived. No other mammalian orders arrived until the Isthmus of Panama was formed in the Pliocene (5.0-2.5 MYA) (Patterson & Pascual, 1972; Savage, 1986). Representatives of all the surviving orders that were present in South America during the Oligocene and Miocene (37.5-5.0 MYA), except the Chiroptera, are at least occasionally infected with *Leishmania* and some are associated with highly specialised species of *Endotrypanum* or *Leishmania* that are restricted to South America and to specific hosts. Table 8.2 lists the families present in South America at the end of the tertiary that are still extant with an indication of whether or not they are hosts of *Leishmania*.

It is worth emphasising that marsupials in Australia and hystricomorphs and primates in Africa are not associated with *Leishmania* so it is unlikely that *Leishmania* migrated to South America in these groups.

Sandflies are believed to be endemic to the New World since almost modern sandflies have been found in Baltic amber from the Cretaceous so sandflies could have become distributed world wide before the break up of Gondwana (Lewis, 1982). Sandflies are well established as vectors of some bat and reptilian *Trypanosoma* and there are a number of reports of sandflies being infected with *Crithidia* or other unidentified monoxenous kinetoplastids (Robert et al., 1994; Shaw et al., 1987). Species of *Herpetomonas*, *Crithidia* and *Leptomonas* thrive in the scent glands of opossums (Jansen et al., 1988) and there are sporadic reports of mammals infected with what appear to be monoxenous insect parasites (Dedet et al., 1995; Mebrahtu et al., 1992; Schnur et al., 1992). *Leptomonas* and *Crithidia*

are both closely related to the *Leishmania/Endotrypanum* clade (Fig. 7.9), it is therefore possible that the latter clade evolved from a monogenetic trypanosomatid of sandflies. It is also possible that digenetic trypanosomatids evolved from monogenetic parasites of mammals (Maslov & Simpson, 1995; Vickerman, 1994).

Order	Family		*Leishmania* species
Marsupialia	Didelphidae	Opossums	*L. mexicana*
	Caenolestidae	Shrew-opossums	
Primates	Cebidae	Monkeys	*L. braziliensis*
	Callitrichidae	Marmosets	*L. braziliensis*
Xenarthra	**Dasypodidae**	Armadillos	*L. naiffi*
	Bradypodidae	Sloths	*L. braziliensis*
	Myrmecophagidae	Anteaters	*L. braziliensis*
Rodentia	Octodontidae	Degus	
	Abrocomidae	Chinchilla-rats	
	Echymidae	Spiny rats	*L. aristidesi*
	Chinchillidae	Chinchillas	
	Dasyproctidae	Agoutis, Pacas	*L. lainsoni*
	Dinomyidae	Pacarana	
	Caviidae	Guinea pigs	*L. enriettii*[1]
	Hydrochoeridae	Capybaras	
	Erethizontidae	Porcupines	*L. hertigi*

Table 8.2 Modern families of mammals that were also present in South America at the end of the tertiary, together with an indication of whether they are known to be hosts of *Leishmania*. List of mammal families from Patterson & Pascual, (1972), host status data from Herrer et al., (1973), Lainson et al., (1994), Shaw & Lainson, (1987). Chiroptera are excluded from Paterson & Pascual's list because their fossil record is so poor. [1]*L. enriettii* has only been isolated twice and only from domestic guinea pigs, its enzootic host is unknown. Mammalian families in bold are significant reservoir hosts.

As sloths are reservoir hosts of both *Endotrypanum* and *L. braziliensis* which are represented on both major branches of the *Endotrypanum/Leishmania* group it is possible that ancestors of modern sloths were early hosts of a *Leishmania* like organism. After the *Endotrypanum* and the *Leishmania* separated the *L. hertigi* complex were probably the next group to branch off (Chapter 6). The hystricomorph ancestors of tree porcupines, which are the exclusive hosts of *L. hertigi*, did not reach the Neotropics until the lower Eocene at the earliest. Therefore, assuming a molecular clock, the *Endotrypanum* and *Leishmania* lineages probably did not separate much before the Eocene.

8.2.4. Possible means of migration of *Leishmania* to the Old World

If it is accepted that the *Leishmania* evolved in South America it is necessary to consider when and how they reached the Old World. It has been suggested that the original *Leishmania* radiation occurred from Central Asia on the grounds of the present epidemiology and the historical record (Elgood, 1934; Lysenko, 1971). The radiation of the Old World *Leishmania* from Central Asia is consistent with an earlier arrival from the New World via Beringia. The most obvious dispersal route is via the Isthmus of Panama. *L. mexicana* like parasites could have spread up the Pacific coast of North America across Beringia and into North East Asia. However the Isthmus of Panama did not become a permanent land bridge between the Neotropics and Nearctic until the mid to late Pliocene (3-5 MYA) by which time Beringia may have been too cold for sandflies (Wolfe, 1994a, b). Two genera of fossil ground sloths have been identified in Miocene deposits about eight million years old from El Salvador, indicating that the ocean barrier between the Neotropics and the Nearctic was only partially effective by this time. The presence of a distinctive strain of *L. mexicana* in the Dominican Republic which may be indigenous (Barker et al., 1982; Kreutzer, 1990; Johnson et al., 1992; Schnur et al., 1983; Zeledon, 1992) suggests that *Leishmania* can cross open sea, either in the vector or a host, and become established in new habitats. *Leishmania* are not found in the Lesser Antilles with the possible exception of Martinique (Zeledon, 1992). However the endemic mammals of those islands have been largely extinguished by introduced mammals and habitat change since the conquest. Consequently *Leishmania* may have spread to the Nearctic via the Antilles during the Eocene or Miocene. Alternatively it may have been possible for *Leishmania* to have spread up the chain of islands that were to become Central America during the Miocene. The climate of the West Coast of the Nearctic and the Bering straits region reached a warm optimum in the Miocene about 13MYA, since which time temperatures at higher latitudes have declined although with several reversals and

temperatures in the tropics have slowly increased. The Arctic Ocean broke through the Bering Straits about 3MYA leading to a cooling of the Northern Pacific and the creation of a presumably insuperable water and climatic gap between the Nearctic and the Palaearctic for sandflies and hence for *Leishmania*. *Leishmania* may therefore have spread out of the Neotropics through the Nearctic into Asia sometime during the Miocene (Briggs, 1987; Brouwers, 1994; Spicer & Chapman, 1990; Wolfe, 1994a, b).

Both rodent hosts and sandfly vectors were available to transmit *Leishmania* to the Old World by the Bering route in the Pliocene. The major cricetid radiation occurred in the late Miocene/ Pliocene and *Sigmodon* and *Oryzomys*, which are modern hosts of *L. mexicana* in Central America, are both known from late tertiary North American fossils. *Sigmodon* and *Oryzomys* are also the only New World cricetids that have a range extending from Central America into the temperate zone to above 40°N (Savage, 1986). In the Texan focus of *L. mexicana* the parasite is enzootic in the woodrat, *Neotoma micropus*, in an arid environment similar to that inhabited by *L. major* and the gerbil *Rhombomys opimus* in Asia (Ashford, & Bettini, 1987; Kerr et al., 1995).

It has been suggested that the *Lutzomyia (Helcocertomyia)* which are now found in the Western Nearctic and Western Neotropics originated in Laurasia and spread into South America from the Nearctic after the formation of the Isthmus of Panama. *Lu. (H.) vexator* is associated with rodent burrows but has also been shown to feed on reptiles that shelter in those burrows and is a vector of *Trypanosoma (Megatrypanum)* of rodents and is a possible vector of *L. (Viannia)* species in Peru. So it seems likely that new hosts and new vectors which both had ranges that extend well into the temperate Nearctic arrived in South America when the Panama land bridge formed in the early Pliocene (Kerr et al., 1995; Killick-Kendrick, 1990; Lane, 1993; Williams, 1991; Young & Perkins, 1984). These same hosts may also have been infected by waif mammals or wind blown sandflies from South America in the Miocene.

Assuming that *Leishmania* arrived in Central Asia in the Miocene, possibly in cricetid rodents, then the lizards that share burrows with cricetids may have become infected. This could have lead to the development of the lizard *Leishmania* in Central Asia. The lizard parasites may have developed at a time when the climate was already too cold for these species to cross back across the Bering straits although they could spread south into Africa.

Mammalian parasites have been found to be infective to lizards and transient infections of mammals and man have been reported for certain lizard *Leishmania* (Adler, 1964; Belova, 1971; Manson-Bahr & Heisch, 1961). The presence of *Sergentomyia* sandflies may have permitted occasional infections of lizards with mammalian parasites to become an established cycle.

8.3. The classification and definition of *Leishmania* of lizards and mammals

There are three significant implications of a Neotropical origin of *Leishmania*. Firstly Vickerman's (1976) definition of *Leishmania* may need to be broadened, secondly *Sauroleishmania* renders *Leishmania* paraphyletic and thirdly the genus *Endotrypanum* may need to be amended. These three points are all interrelated.

If the classification of *L. hertigi* with *Endotrypanum* is confirmed then it will be necessary to reclassify one of them to regain monophyly. The cell type that *L. hertigi* amastigotes parasitise is not known and nor is the vector of this parasite. *Endotrypanum* is transmitted by sandflies but has been excluded from the *Leishmania* because it develops as intraerythrocytic epimastigotes or trypomastigotes in sloths, although it may multiply as amastigotes in macrophages in cell culture (Perez Camps 1995). The transfer of *L. hertigi* to *Endotrypanum* would create a monophyletic group without disturbing the *Leishmania*. The inclusion of *Endotrypanum* in *Leishmania* would create an evolutionary coherent group of digenetic intracellular parasites of mammals and reptiles transmitted by sandflies in which they develop as promastigotes.

The evidence from the SSU rRNA gene (Chapter 6) and DNA buoyant densities demonstrate a close relationship between the lizard and mammalian parasites. The SSU rRNA gene data may be consistent with the lizard parasites being members of a subgenus *L. (Sauroleishmania)* as proposed by Saf'janova in 1982 (Killick-Kendrick et al., 1984). However if the lizard parasites did develop in Central Asia from ancestors of members of the *L. (Leishmania)* subgenus then the creation of *L. (Sauroleishmania)* would render the *L. (Leishmania)* polyphyletic as the lizard parasites would be more closely related to the Old World *L. (Leishmania)* than to the *L. (Leishmania)* of the New World. If the lizard parasites are evolving more quickly, as suggested above (Para. 8.1), then the DNA evidence may support the subgeneric status of the lizard parasites even though the biogeographic evidence contradicts such a ranking. The phylogenetic position of the lizard parasites may therefore

be difficult to resolve until more suitable genes for phylogenetic analysis at this level are discovered.

Vickerman (1976) defines *Leishmania* as follows:- "Digenetic parasites multiplying in the mononuclear phagocytic cells of mammals and in the gut lumen of sandflies; characterised by intracellular amastigotes in the mammal and free or attached promastigotes in the vector." The host range specified in this definition would need to broadened to include the lizards if the lizard parasites are once again accepted as true *Leishmania*. The cell type parasitised may have to be broadened if *L. hertigi* is to be retained within the *Leishmania* unless intramacrophage parasites of this species are found *in vivo*. If *L. hertigi* is transferred to *Endotrypanum* then cell type parasitised can be retained as amastigotes have been seen in blood mononuclear cells of lizards (David, 1929; Pozio et al., 1986).

There is substantial biological, ecological and molecular evidence for a Neotropical origin of *Leishmania*. Further work is required on the lizard *Leishmania* to determine if they are a monophyletic group and their relationship to *Leishmania*. The PCR based methods we have developed for low resolution identification and classification of *Endotrypanum*, *Leishmania* and monoxenous trypanosomatids will facilitate further studies of parasites of sandflies and vertebrates that will give us a clearer understanding of the range of genera parasitising these groups and their ecology.

Jaccard Distance	Lg/Lpe	Lg/Lpa	Lg/Lb	Lpe/Lpa	Lpe/Lb	Lpa/Lb
AB1 (60%)	0.74	0.62	0.78	0.67	0.37	0.71
AB3 (70%)	0.83	0.67	0.92	0.87	0.29	0.94
AB4 (60%)	0.77	0.40	0.64	0.75	0.56	0.73
AB6 (70%)	0.73	0.6	0.78	0.76	0.667	0.667
AB12 (60%)	0.8	0.5	0.8	0.67	0.0	0.67
AB13 (70%)	0.89	0.25	0.92	0.85	0.54	0.87
AB15 (60%)	0.84	0.29	0.83	0.86	0.61	0.79
AB18 (60%)	0.84	0.33	0.86	0.85	0.31	0.86
AB19 (70%)	0.8	0.67	0.85	0.93	0.56	0.91
A1 (70%)	0.79	0.53	0.86	0.72	0.58	0.79
A4 (60%)	0.75	0.38	0.8	0.7	0.59	0.77
A7 (60%)	0.8	0.0	0.73	0.8	0.73	0.73
A8 (60%)	0.94	0.77	1.0	0.9	0.71	0.81
total	10.52	6.01	10.77	10.33	6.52	10.25
mean	0.81	0.46	0.83	0.79	0.50	0.79
S.D.(s)	0.059	0.21	0.091	0.089	0.206	0.088
ts/$\sqrt{n}$	0.036	0.129	0.055	0.053	0.124	0.052
Jaccard (pooled)	0.77	0.53	0.79	0.77	0.50	0.74
Jaccard 60% GC primers	0.81	0.41	0.80	0.77	0.48	0.76
Jaccard 70% GC primers	0.81	0.54	0.87	0.83	0.54	0.83

Appendix 3.1 Jaccard distance coefficients between *L. (Viannia)* strains for individual primers. The percentages in brackets after each primer are the % GC of that primer. The Jaccard (pooled) is the value of the Jaccard coefficient calculated from the pooled presence absence data.

Jaccard Similarity	Lg/Lpe	Lg/Lpa	Lg/Lb	Lpe/Lpa	Lpe/Lb	Lpa/Lb
AB1	0.42	0.54	0.36	0.5	0.78	0.45
AB3	0.29	0.5	0.14	0.23	0.83	0.12
AB4	0.375	0.75	0.5	0.4	0.61	0.43
AB6	0.43	0.53	0.36	0.39	1.0	0.5
AB12	0.33	0.75	0.3	0.5	0.43	0.5
AB13	0.2	0.86	0.15	0.27	0.61	0.23
AB15	0.29	0.83	0.3	0.33	0.56	0.35
AB18	0.29	0.8	0.25	0.29	0.82	0.25
AB19	0.33	0.5	0.25	0.14	0.73	0.25
A1	0.6	0.9	0.4	0.61	0.69	0.46
A4	0.47	0.71	0.4	0.47	0.58	0.4
A7	0.33	1.0	0.43	0.33	0.43	0.43
A8	0.125	0.625	0.0	0.33	0.44	0.3
TOTAL	4.47	9.3	3.88	4.80	8.59	4.67
MEAN	0.34	0.71	0.29	0.37	0.66	0.36
S.D.(s)	0.12	0.16	0.14	0.13	0.17	0.12
Jaccard (Pooled)	0.31	0.66	0.28	0.34	0.635	0.33

Appendix 3.1 Jaccard similarity coefficients between *L. (Viannia)* strains for individual primers. The Jaccard (Pooled) line is the value of the Jaccard similarity coefficient calculated from the pooled data.

Appendix 4.1 Sequence of minicircle conserved region of *L. infantum* (GB:Z35501), *L. tarentolae* (GB:K01978) and *L. braziliensis* (GB:M87315) together with the sequences of primers 13Y, 13Z, 13A, 13B (Rodgers *et al.* 1990), LU and LB (De Bruijn *et al.* 1992) and LiR (Para. 2.4.3). The sequences to which primers MP1L and MP3H (Lopez *et al.* 1993) anneal was not identified. Direction of amplification is indicated by arrowheads (< >). Primers marked (R) have been reversed for ease of comparison with the aligned sequence. Underlined sequences between nucleotides 1 & 21; 50 & 58; 104 & 125 correspond to conserved sequence blocks 1, 2 and 3.

```
        <LiR(R)              AGGGGCGTTC TGCGA
        <13Y(R)   CCCGTGGGGG AGGGGGCG
        >13A         GTGGGGG AGGGGCGTTC T
L. infantum     1 TCCTTCGGGT AGGGGCGTTC TGCGAAAACC GAAAAATGG. .GTGCAGAAA
L. tarentolae     TCCCTCAGGT AGGGGCGTTC TCCGAAAACC GAAAAATGCA T..GCAGAAA
L. braziliensis   GCCTCTGGGT AGGGGCGTTC TGCGAAAACC GAAAAATGG. CATACAGAAA


L. infantum    51 TCCCGTTCAA AAAATGCCCA AAAA..CGCC AATTTTGGCC TCCGGGGCGG
L. tarentolae     TCCCGTTCAA AAATCGGCCA AAA..TCGCC ATTTTTTC.A ATTTTCGTGT
L. braziliensis   TCCCGTTCAA AAATCCCCCA AAAT..TCGC GTTTTTTGGC CTCCCCGTGC
                                                  <LB(R)      C CTCCCCGTGC
        <LB(R)    ACAATTAG
        >LU              GGG GTTGGTGTAA TATAG
        >13Z          ACTGGGG GTTGGTGTAA AATAG
        <13B(R)     AACTGGGG GTTGGTGTAA AAT
L. infantum    101 AAAACTGGGG GTTGGTGTAA AATAGGGCCG GGTGGTCCGG GGAT.TTCC.
L. tarentolae     GAAACTAGGG GTTGGTGTAA AATAGGGGTG GGGCTCCCCG GGGT.AATT.
L. braziliensis   ACAATTAGGG GTTGGTGTAA TATAGTGGGC CGCGCACTCT CGCT.GAGG.
```

Appendix 6.1 Restriction sites of endonucleases that restrict *L. donovani* (GB:X07773) (Ld), *L. tarentolae* (GB:X53917)(Lt) and *E. monterogeii* (GB:X53911)(Em) together with limited data for *C. oncopelti* (Gb:L29266) (Co), *C. fasciculata* (Gb:X03450)(Cf), and *Leptomonas sp* (Gb:X53914) (Lp). Data was compiled using DM5 on unaligned sequences. Where no data is shown for any of *L. donovani*, *L. tarentolae*, or *E. monterogeii* the enzyme is not predicted to cut that species.

Species	Enzyme	Cleavage sites										
Ld	(*AluI*) AGCT	140,	330,	417,	605,	947,	1026,	1411,	1661,	1985,		
Em		140,	329,	416,	603,	945,	1407,		1657,	1981		
Lt		140,	330,	417,	605,	947,	1026,	1409,	1659,	1983		
Ld	(*BbvI*) GCTGC	1662,										
Em		1658,										
Lt		1660,	1728									
Ld	(*BinI*) GGATC	1267,	2168									
Em		1264,										
Lt		1266,										
Ld	(*CauII*) CCSGG	158,	1909									
Em		158,	1036,	1905								
Lt		158,	1907									
Ld	(*Cfr10I*) PCCGGY	288,	1278,	1359								
Em		287,	1355									
Lt		288,	1277,	1357								
Ld	(*CfrI*) YGGCCP	339,	1040,	1361,	1601							
Em		338,	1357,	1597								
Lt		339,	1040,	1279,	1599							
Em	(*Eco47III*) AGCGCT	957,										
Ld	(*EcoPI*) AGACC	1131,	1221									
Em		1129,	1219,	1918								
Lt		1131,	1221,	1920								
Ld	(*FokI*) CATCC	546										
Em		544,	1050,	2082								
Lt		546,										
Ld	(*GdiII*) YGGCCG	339,	1040,	1601								
Em		338,	1597									
Lt		339,	1040,	1599								
Ld	(*GsuI*) CTCCAG	172,										
Em	(*HaeI*) WGGCCW	1347,										
Lt		1349,										
Ld	(*HaeII*) PGCGCY	993,										
Em		957,	991									
Lt		993,										
Ld	(*HaeIII*) GGCC	340,	715,	1041,	1065,	1277,	1281,	1352,	1362,	1602,	1729,	2142
Em		339,	713,	1063,				1348,	1358,	1598,	1725,	2142
Lt		340,	715,	1041,	1065,	1276,	1280,	1350,		1600,		2142
Lp		349,	718,	724,	1072,			1357,	1367,	1607,	1734,	2142
Co		358,	682,	724,	1012,					1514,		2142
Cf		369,	738,	744,	1094,			1380,	1390,	1630,	1757,	2142
Ld	(*HgaI*) GCGTC	302,	802,	1014,	1111							
Em		301,	800,		1109							
Lt		302,	802,	1014,	1111							
Ld	(*HgiAI*) GWGCWC	1025,										
Lt		1025,										
Ld	(*HhaI*) GCGC	58,	444,	446,		994,	1315,	1836,	1958,	1981		
Em		58,	443,	445,	958,	992,	1311,	1832,	1954,	1977,	2050	
Lt		58,	444,	446,		994,	1313,	1834,	1956,	1979		
Ld	(*HinfI*) GANTC	34,	52,	265,	391,	1513,	1580,	1637,	1759,	1913,	1923	
Em		34,	52,	264,	390,	1509,	1576,	1633,	1755,	1909		

Lt		34,	52,	265,	391,	1511,	1578,	1635,	1757,	1911		
Cf		10,	63,	81,	294,	420,	1541,	1608,	1665,	1702,	1787,	1941
Lp		43,	61,	274,	400,	1518,	1585,	1642,	1764,	1918		
Ld	(*HinfIII*) ATTCG	1348,	1735									
Em			1731,									
Lt			1733,									
Ld	(*HpaII*) CCGG	158,	289,	299,	395,	1018,	1030,	1039,	1279,	1360,	1540,	1910, 2142
Em		158,	174,	288,	298,	394,	977,	1028,	1037,	1356,	1536,	1906, 2142
Lt		158,	289,	299,	395,	1018,	1030,	1039,	1278,	1358,	1538,	1908, 2142
Cf		187,	318,	328,	424,	1068,	1378,	1388,	1568			
Lp		167,	298,	308,	404,	1037,	1365,	1545,	1915			
Co		7,	204,	319,	413,	700,	968,	1231,	1452			
Ld	(*HphI*) GGTGA	334,	706,	1119,	1780,	2064,	2153					
Em			704,	1117,	1776,	2060						
Lt			706,	1119,	1778,	2062						
Ld	(*MboII*) TCTTC	1164,	1747,	2112								
Em		1162,	1743									
Lt		1164,	1745,	2110								
Ld	(*MnlI*)CCTC	29,	891,	977,	1067,	1180,	1380,	1815				
Em		29,	889,		1065,	1178,	1376,	1811				
Lt		29,	891,		1067,	1180,	1378,	1813				
Ld	(*MnlI*) GAGG	400,	433,	478,	569,	1002,	1082,	1117,	1417,	1551,	1938,	1965, 2121
Em		399,	432,	476,	567,		1080,	1115,	1413,	1547,	1934,	1961
Lt		400,	433,	478,	569,	1002,	1082,	1117,	1415,	1549,	1936,	1963, 2119
Ld	(*NaeI*) GCCGGC	288,	1278									
Em		287,										
Lt		288,	1277									
Ld	(*NlaIV*) GGNNCC	403,	581,	762,	993,			1479,	1932			
Em		402,	579,	760,	991,	1024,	1030,	1475,	1928			
Lt		403,	581,	762,	993,			1477,	1930			
Ld	(*NspBII*) CMGCKG	329,	592,		2142							
Em			590,		2142							
Lt			592,	1283,	2142							
Ld	(*PflMI*) CCANNNNNTGG		174,	1193,	1256							
Em				1191,	1253							
Lt				1193,	1255							
Ld	(*PvuII*) CAGCTG	329,	2163									
Ld	(*RsaI*) GTAC	1021,	1103,	1439,		2022						
Em			1101,	1435,	1681,	2018						
Lt			1103,	1437,		2020						
Lp		982,	1110,	1444,		2027						
Co		775,	1047,	1351,	1933	2142						
Cf		1002,	1132,	1467,		2050						
Ld	(*SacI*) GAGCTC	1025										
Lt		1025										
Ld	(*Sau96I*) GGNCC	715,	1276,	1728,	1933							
Em		713,		1724,	1929							
Lt		715,	1275,		1931							
Lp		718,	724,	1039,	1733,	1938						
Co		681,	702,	738,	939,	1075,	1639,	1790,	1844,	1991		
Cf		738,	744,	1061,	1756,	1961						
Ld	(*ScrFI*) CCNGG	158,	679,	712,	795,		1909					
Em		158,	677,	710,	793,	1036,	1905					
Lt		158,	679,	712,	795,		1907					
Ld	(*TaqII*) CACCCA	251,	1253,	1365								
Em		250,	1250,	1361								
Lt		251,	1252									
Ld	(*XmaIII*) CGGCCG	1040										
Lt		1040										

Appendix 6.2 Restriction fragments used in the preparation of Nei's index (Fig. 5.10)

Enzyme	G755	LV344	LV88	LV58	LV402	LV30	LV31	LV247	LV108	LV414	LV9	M379	M4147
HaeIII													
628	1	0	0	0	0	0	0	0	0	0	0	0	0
542	0	0	0	0	1	1	1	1	1	1	0	0	0
417	1	1	1	1	0	0	0	0	0	0	1	1	1
374	1	1	1	1	1	1	1	1	1	1	1	1	1
350	1	1	1	1	0	0	0	0	0	0	0	0	0
339	0	0	0	0	1	1	1	1	1	1	1	1	1
326	0	0	0	0	1	1	1	1	1	1	1	1	1
275	0	1	1	1	1	0	0	0	0	0	0	0	0
250	0	0	0	0	0	0	0	0	0	1	0	0	0
240	1	1	1	1	1	1	1	1	1	0	1	1	1
212	0	0	0	0	0	1	1	1	1	1	1	1	1
127	1	1	1	1	0	0	0	0	0	0	1	1	1
70	0	0	0	0	0	1	1	1	1	1	1	1	1
HinfI													
1120	0	1	1	1	1	1	1	1	1	1	1	1	1
480	1	0	0	0	0	0	0	0	0	0	0	0	0
380	1	0	0	0	0	0	0	0	0	0	0	0	0
260	1	0	0	0	0	0	0	0	0	0	0	0	0
	6	6	6	6	6	6	6	6	6	6	6	6	6
HpaII													
620	1	0	0	0	0	1	1	1	1	1	1	1	1
580	0	1	1	1	1	0	0	0	0	0	0	0	0
370	1	1	1	1	1	1	1	1	1	1	1	1	1
319	1	1	1	1	1	0	0	0	0	0	0	0	0
240	0	0	0	0	0	1	1	1	1	1	1	1	1
232, 180, 158		3	3	3	3	3	3	3	3	3	3	3	33
131	1	0	0	0	0	1	1	1	1	1	1	1	1
114	0	1	1	1	1	0	0	0	0	0	0	0	0
96	1	1	1	1	1	1	1	1	1	1	1	1	1
81	0	0	0	0	0	1	1	1	1	1	1	1	1
RsaI													
1021	1	1	1	1	1	1	1	1	1	1	1	1	1
563	1	0	0	0	0	1	1	1	1	1	1	1	1
337	0	1	1	1	1	0	0	0	0	0	0	0	0
334	1	1	1	1	1	1	1	1	1	1	1	1	1
240	0	1	1	1	1	0	0	0	0	0	0	0	0
78	1	1	1	1	1	1	1	1	1	1	1	1	1
Sau96I													
1200	0	0	0	0	1	0	0	0	0	0	0	0	0
1009	0	1	1	1	0	0	0	0	0	0	0	0	0
713	1	1	1	1	1	1	1	1	1	1	1	1	1
656	0	0	0	0	0	1	1	1	1	1	0	0	0
560	0	0	0	0	0	1	1	1	1	1	1	1	1
500	1	0	0	0	0	0	0	0	0	0	0	0	0
452	1	0	0	0	0	0	0	0	0	0	1	1	1
205	1	1	1	1	0	0	0	0	0	0	1	1	1
180	1	1	1	1	1	1	1	1	1	1	1	1	1

Enzyme	G755	LV344	LV88	LV58	LV402	LV30	LV31	LV247	LV108	LV414	LV9	M379	M4147
HhaI													
548	0	0	0	0	0	1	1	1	1	1	1	1	0
513	1	1	1	1	1	0	0	0	0	0	0	0	1
450	1	0	0	0	0	0	0	0	0	0	0	0	0
384	1	1	1	1	1	1	1	1	1	1	1	1	1
321	0	1	1	1	1	1	1	1	1	1	1	1	1
115	0	1	1	1	1	1	1	1	1	1	1	1	1
AluI													
462	1	1	1	1	0	0	0	0	1	0	0	0	1
385	0	0	0	0	1	1	1	1	0	1	1	1	0
79	0	0	0	0	1	1	1	1	0	1	1	1	0
77	1	1	1	1	1	1	1	1	1	1	1	1	1
SacI													
560	1	1	1	1	0	0	0	0	1	0	0	0	1
405	0	0	0	0	1	1	1	1	0	1	1	1	0
155	0	0	0	0	1	1	1	1	0	1	1	1	0

Appendix 6.3 Sequence of the SSU rRNA gene of *L. hertigi* (LV42) between bases 938 and 1422 of the *L. donovani* (GB:X07773) sequence

```
  1 GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG CTGTGCGACA
 51 AGCGCTTTCC CATCGCAACT CCGGTTCGGT GTGTGGCGCC TTTGGGGGGT
101 TTAGTGCGCG ATCGGTGCGA GCTCCGGCTC ACCCGGCCGC GTAACGCCTT
151 CCCCAACTCA CGGCCTCTAG GAATGAAGGA GGGTAGTTCG GGGGGGAACG
201 TACTGGGGCG TCAGAGGTGA AATTCTTAGA CCGCACCAAG ACGAACTACA
251 GCGAAGGCAT TCTTCAAGGA TACCTTCCTC AATCAAGAAC CAAAGTGTGG
301 AGATCGAAGA TGATTAGAGA CCATTGTAGT CCATACTGCA AACGATGACA
351 CCCATGAATT GGGGATCTTA TGGGTCGGCA TGCGGCAGGG TTTACCCCTG
401 TGCCAGCACC GCGCCCGCTT TTATAAACTT ACGTATCTTT TCTATCCGGC
451 CTTTACCGGC CACCCACGGG AATATCCTCA GCACGTTTT
```

Appendix 7.1 Sequence of 563bp region of Guatemalan leptomonad G755 between bases 937 and 1450 of the *L. donovani* (GB:X07773) sequence

```
  1  CGGCTTTTGT TGGTTTTAAA GGTCTATTGG AGATTATGGA GCTGTGCGAC
 51  GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG CTGTGCGACA
101  AGCGCTCTCC CATCGCAACC TCGGTTCGGT GTGGTGGCGC CTTTGGGGCG
151  GTTTAGTGCG TTCGTACGGG ACTCCGGTTT CGTGCGGGCG TGTCGCCGTC
201  ACAACTCACA GCCTCTAGGA ATGAAGGAGG GTAGTTCGGG GGAGAACGTA
251  CTGGGGCGTC AGAGGTGAAA TTCTTAGACC GCACCAAGAC GAACTACAGC
301  GAAGGCATTC TTCAAGGATA CCTTCCTCAA TCAAGAACCA AAGTGTGGAG
351  ATCGAAGATG ATTAGAGACC ATTGTAGTCC ACACTGCAAA CGATGACACC
401  CATGAATTGG GGACCTTCTG GGTCGGCTTG CGGCAGGGTT TACCCCTGTG
451  TCAGCACCGT GCCCACTTTT ATCAACCTAC GTATATTTTC CATTCCGGCC
501  TTTACCGGCC ACCCACGGGA TATCCTCAGC ACTTTTCTNT TTTTTCACGC
551  AAAGGCTTGG NGT
```

Appendix 7.2 Alignment of available sequences of Guatemalan leptomonad (G755) and *L. hertigi* (LV42) with representative trypanosomatids. *L.major* (GB:X53915); *L.amazonensis*(GB:X53912); *L.tarentolae* (GB:X53917); *L.donovani* (GB:XO7773); *E.monterogeii* (GB:X53911); *L. herreri* (GB:U50043); *L.guyanensis* (GB:X53911); *C.fasciculata* (GB:X03450); *Leptomonas* (GB:X53914); *T.cruzi* (X) (X53917); *T.cruzi* (M) (GB:M31432); *T.brucei* (GB:M12676).

```
                1                                                                   70
L. major        GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC AAGTGCTT-- ----------
L. amazonensis  GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC AAGTGCTT-- ----------
L. tarentolae   GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC AAGTGCTT-- ----------
L. donovani     GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC AAGTGCTT-- ----------
E. monterogeii  GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC AAGCGCTT-- ----------
L. herreri      GGCTTTTGTT GG--TTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC AAGCGCTT-- ----------
L. hertigi      GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC AAGCGCTT-- ----------
L. guyanensis   GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC AAGTGCTT-- ----------
C. fasciculata  GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC CAGTGCTT-- ----------
Leptomonas      GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC AAGTGCTT-- ----------
G755            GGCTTTTGTT GGTTTTAAAG GTCTATTGGA GATTATGGAG -CTGTGCGAC GGCT--TTTG TTGGTTTT--
T. cruzi (X)    GGCTTTTGTT GGTTTTAAAA GTCCATTGGA GATTATGGGG -CAGTGTGAC AAGCGGCTGG GTGGTTATTC
T. cruzi (M)    GGCTTTTGTT GGTTTTAAAA GTCCATTGGA GATTATGGGG -CAGTGTGAC AAGCGGCTGG GTGGTTATTC
T. brucei       GGCTTTTGTT GGTTTTAGAA GTCCTTGGGA GATTATGGG- ---------- ---------- ----------

                71                                                                 140
L. major        ---------- ---------- ---------- ---------- ---------- -------TCC CATCGCAACT
L. amazonensis  ---------- ---------- ---------- ---------- ---------- -------TCC CATCGCAACT
L. tarentolae   ---------- ---------- ---------- ---------- ---------- -------TCC CATCGCAACT
L. donovani     ---------- ---------- ---------- ---------- ---------- -------TCC CATCGCAACC
E. monterogeii  ---------- ---------- ---------- ---------- ---------- -------TCC CATCGCAACT
L. herreri      ---------- ---------- ---------- ---------- ---------- -------TCC CATCGCAACT
L. hertigi      ---------- ---------- ---------- ---------- ---------- -------TCC CATCGCAACT
L. guyanensis   ---------- ---------- ---------- ---------- ---------- -------TCC CATCGCAACT
C. fasciculata  ---------- ---------- ---------- ---------- ---------- -------TCA CATCG-TACT
Leptomonas      ---------- ---------- ---------- ---------- ---------- -------TCC CATCG-TAC-
G755            ---------- ----AAAGGT CTATT-GGAG ATTATGGAGC -TGTGCGACA AGCGCTCTCC CATCGCAACC
T. cruzi (X)    CACACACATA CACACTCCCT TTTTTTTGGG ACGTGTTTGT ATGTGTGTG- --GCACTCGT CGCCTTTGTG
T. cruzi (M)    CACACACACA CGCACACTCC TTTTTTGGAT GTTGTGTCTT CTGTGTGTGT GCGAACTCGT CGCCTTTGTG
T. brucei       ---------- ---------- ---------- ---------- ---------- ---------- ------GCCG

                141                                                                210
L. major        TCGG--TTCG -GTGTG-TGG CGCCTTT-GA GGGGTTTAGT GCG---TCCG GTGCGAGC-T CCGGTT-CGT
L. amazonensis  TCGG--TTCG -GTGTG-TGG CGCCTTTGGA GGGGTTTAGT GCG---TCCG GTGCGAGC-T CCGGTT-CGT
L. tarentolae   TCGG--TTCG -GTGTG-TGG CGCCTTT-GA GGGGTTTAGT GCG---TCCG GTGCGAGC-T CCGGTT-CGT
L. donovani     TCGG--TTCG -GTGTG-TGG CGCCTTT-GA GGGGTTTAGT GCG---TCCG GTACGAGC-T CCGGTT-CGT
E. monterogeii  CCGG--TTCG -GTGTG-TGG CGCCTTTGG- GTGGTTTAGT GCG---TTCG GTGGGGGC-T CCGGCT-CCC
L. herreri      CCGG--TTCG -GTGTG-TGG CGCCTTTGG- GTGGTTTAGT GCG---TTCG GTGGGG-C-T CCGGCT-CTC
L. hertigi      CCGG--TTCG -GTGTG-TGG CGCCTTTGG- GGGGTTTAGT GCG-CGATCG GTGCGAGC-T CCGGCT-CAC
L. guyanensis   TCGG--TTCG -GTGTG-TGG CGCCTTTGGA GGGGTTTAGT GCG---TCCG GTGCGAGC-T CCGGTT-CGT
C. fasciculata  TTCG--TGCG -GTGTG-TGG TGCCTTTGGA GGGGTTTAGT GCG---TTCG GCTCGGGC-T TCGGTC-CGT
Leptomonas      CTAG--TGCG -GTGTG-TGG CGCCTTTGG- GGGGTTTAGT GCG---TTCG GTTCGGGC-T CCGGTC-CGT
G755            TCGG--TTCG -GTGTGGTGG CGCCTTTGGG GCGGTTTAGT GCG---TTCG -TACGGGACT CCGGTTTCGT
T. cruzi (X)    GGAAATCC-- GTGTGGCACT -GTTTG-TGT TGTTGGCAGA C----TTCGG TCTTACCCTT CGCATCTCAC
T. cruzi (M)    GGAAATCCAG GTGTGGCACT TGTTTGGTGT TGTTGGCAGA C----TTCGG TCTTGCC-TT CCAATGTTTC
T. brucei       CGTGCCTTGG GTCGGTGTTT CGTGTCTCAT TTTTGTGGCG CGCACATTCG GCTCTTCGTG ATGTTTTTTT

                211                                                                280
L. major        CCGGCCG--T AACGCCTT-- TTCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
L. amazonensis  CCGGCCG--T AACGCCTT-- TTCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
L. tarentolae   CCGGCCG--T AACGCCTT-- TTCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
L. donovani     CCGGCCG--T AACGCCTT-- TTCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
E. monterogeii  CCGGTCG--T TACGCCAT-- CCCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
L. herreri      CCGGTCG--T AACGCCAT-- CCCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
L. hertigi      CCGGCCGCGT AACGCCTT-C CCCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
L. guyanensis   CCGGCCG--T AACGCCTT-- TTCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
C. fasciculata  CCGGGCG--T AACGCCTC-- TTCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
Leptomonas      CTGGGCG--T AACGCCTT-- TTCAACTCAC GGCC-----T CT--AGGAAT GAAGGAGGGT AGCTCGGGGG
G755            GCGGGCG--T GTCGCCGT-- CACAACTCAC AGCC-----T CT--AGGAAT GAAGGAGGGT AGTTCGGGGG
T. cruzi (X)    ATGTGTC-AT GC--CTTCCC -TCAACTCAC GGC-----AT CC--AGGAAT GAAGGAGGGT AGTTCGGGGG
T. cruzi (M)    ACATGTG--T CATGCCTTCC CTCAACTCAC GGC-----AT CC--AGGAAT GAAGGAGGGT AGTTCGGGGG
T. brucei       ACA------- ---------- TTCATTGCGA CGCGCGGCTT CC--AGGAAT GAAGGAGGGT AGTTCGGGGG
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                281                                                                 350
L. major        AGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
L. amazonensis  AGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
L. tarentolae   AGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
L. donovani     AGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
E. monterogeii  GGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
L. herreri      GGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
L. hertigi      GGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
L. guyanensis   AGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
C. fasciculata  AGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
Leptomonas      AGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
G755            AGAACGTACT GGGGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
T. cruzi (X)    AGAACGTACT GGTGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
T. cruzi (M)    AGAACGTACT GGTGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT
T. brucei       AGAACGTACT GGTGCGTCAG AGGTGAAATT CTTAGACCGC ACCAAGACGA ACTACAGCGA AGGCATTCTT

                351                                                                 420
L. major        CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
L. amazonensis  CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
L. tarentolae   CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
L. donovani     CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
E. monterogeii  CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
L. herreri      CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
L. hertigi      CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
L. guyanensis   CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
C. fasciculata  CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
Leptomonas      CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
G755            CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-AGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
T. cruzi (X)    CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-GGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
T. cruzi (M)    CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-GGA TCGAAGATGA TTAGAGACCA TTGTAGTCCA
T. brucei       CAAGGATACC TTCCTCAATC AAGAACCAAA GTGTGG-GGA TCAAAGATGA TTAGAGACCA TTGTAGTCCA

                421                                                                 490
L. major        CACTG-CAAA CGATGACACC CATGAATTGG GGATCTTAT- -GGGCCGGCC TGCGGCA-GG GTTT------
L. amazonensis  CACTG-CAAA CGATGACACC CATGAATTGG GGATCTTAT- -GGGCCGGCC TGCGGCA-GG GTTT------
L. tarentolae   CACTG-CAAA CGATGACACC CATGAATTGG GGATCTTAT- -GGGCCGGCC AGCGGCA-GG GTTT------
L. donovani     CACTGCCAAA CGATGACACC CATGAATTGG GGATCTTAT- -GGGCCGGCC TGCGGCA-GG GTTT------
E. monterogeii  TACTG-CAAA CGATGACACC CATGAATTGG GGATCTTAT- -GGGTCGGCT TGCGGCA-GG GTTT------
L. herreri      TACTG-CAAA CGATGACACC CATGAATTGG GGATCTTTT- --GGTCGGCT TGCGGCA-GG GTTT------
L. hertigi      TACTG-CAAA CGATGACACC CATGAATTGG GGATCTTAT- -GGGTCGGCA TGCGGCA-GG GTTT------
L. guyanensis   CACTG-CAAA CGATGACACC CATGAATTGG GGATCTTAT- -GGGCCGGCC TGCGGCA-GG GTTT------
C. fasciculata  CACTG-CAAA CGATGACACC CATGAATTGG GGATCTTTT- -GGGTCGG-C TGCGGCA-GG GTTT------
Leptomonas      CACTG-CAAA CGATGACACC CATGAATTGG GGATCTTTT- -GGGTCGG-T TGCGGCA-GG GTTT------
G755            CACTG-CAAA CGATGACACC CATGAATTGG GGACCTTCT- -GGGTCGGCT TGCGGCA-GG GTTT------
T. cruzi (X)    CACTG-CAAA CGATGACACC CATGAATTGG GGAGTTTTT- --GGTCGTAG GCGTGGTCGG GTTTGAT--T
T. cruzi (M)    CACTG-CAAA CGATGACACC CATGAATTGG GGAGTTTTT- --GGTCGTAG GCGTGGTCGG GCTTGATTAT
T. brucei       CACTG-CAAA CCATGACACC CATGAATTGG GGAACATCAT TGGGTGCCCG TGTGGC-GGC CTTT------

                491                                                                 560
L. major        ---------- -ACCCTGTGT -CAGCACCGC GCCCGCTTTT --ACCAACTT ACGTATCTTT TCTATT----
L. amazonensis  ---------- -ACCCTGTGT -CAGCACCGC GCCCGCTTTT --ACCAACTT ACGTATCTTT TCTATT----
L. tarentolae   ---------- -ACCCTGTGT -CAGCACCGC GCCCGCTTTT --ACCAACTT ACGTATCTTT TCTATT----
L. donovani     ---------- -ACCCTGTGT CCAGCACCGC GCCCGCTTTT --ACCAACTT ACGTATCTTT TCTATT----
E. monterogeii  ---------- -ACCCTGTG- CCAGCACCGC GCCCGCTTTT --ACCAACTT ACGTATCTTT TCTATC----
L. herreri      ---------- -ACCCTGTG- CCAGCACCGC GCCCGCTTTT --ACCAACTT ACGTATCTTT TCTATC----
L. hertigi      ---------- ACCCCTGTG- CCAGCACCGC GCCCGCTTTT --ATAAACTT ACGTATCTTT TCTATC----
L. guyanensis   ---------- -ACCCTGTGT -CAGCACCGC GCCCGCTTTT --ACCAACTT ACGTATCTTT TCTATT----
C. fasciculata  ---------- ACCCCTGTGT CTTGCGCCGT GCCCGCTTTT --ACCAACTT ACGTATCTTT TCCATC----
Leptomonas      ---------- -ACCCTGTGT CTTGCACCGC GCCCGCTTTT --ACCAACTT ACGTATCTTT TCCATT----
G755            ---------- ACCCCTGTGT -CAGCACCGT GCCCACTTTT --ATCAACCT ACGTATATTT TCCATTC---
T. cruzi (X)    ATTTTTTTTC ATCCCGTTCC TCGTCTCGCC AATGAATATT ---AAAATTT ACGTGCATAT TCTTTT----
T. cruzi (M)    ATTATTTTTC ATCCCGTTCC TCGTCTCGCC AATGAATATA T--TAAATTT ACGTGCATAT TCTTTT----
T. brucei       ---------- --TGTGCCGA CCCTC--GGC CCCAATTTAT TTATCAATTT ACGTGCCTAT TCTATCACCC
```

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                561                                                                620
L. major        --CGGCCTT- ---------- ---TACCGGC CACCC-ACGG GAATATCCTC AGCACGTTTT
L. amazonensis  --CGGCCTT- ---------- ---TACCGGC CACCC-ACGG GAATATCCTC AGCACGTTTT
L. tarentolae   --TGGCCTT- ---------- ---TACCGGC TACCC-ACGG GAATATCCTC AGCACGTTTT
L. donovani     --CGGCCTT- ---------- ---TACCGGC CACCC-ACGG GAATATCCTC AGCACGTTTT
E. monterogeii  --TGGCCTT- ---------- ---TACCGGC CACCC-ACGG GAATATCCTC AGCACGTTTT
L. herreri      --TGGCCTT- ---------- ---TACCGGC CACCC-ACGG GAATATCCTC AGCACGTTTT
L. hertigi      --CGGCCTT- ---------- ---TACCGGC CACCC-ACGG GAATATCCTC AGCACGTTTT
L. guyanensis   --CGGCCTT- ---------- ---TACCGGC CACCC-ACGG GAATATCCTC AGCACGTTTT
C. fasciculata  --CGGCCTT- ---------- ---TACCGGC CACCT-ACGG GAATATCCTC AGCACGTTTT
Leptomonas      --CGGCCTT- ---------- ---TACCGGC CACCC-AAGG GAATATCCTC AGCACGTTTT
G755            --CGGCCTT- ---------- ---TACCGGC CACCC-ACGG G-ATATCCTC AGCAC-TTTT
T. cruzi (X)    --TGGTCCTC G-TTTTTTTA CGACGAGGGC CTTTA-ACGG GAATATCCTC AGCACGTTAT
T. cruzi (M)    --TGGTCTTC GTTTTTTTTA CGGCGAGGAC CTTTA-ACGG GAATATCCTC AGCACGTTAT
T. brucei       CCGGTTCCCT CTTTTGAGGT TCTTCCGGGG TTTTTTACGG GAATATCCTC AGCACGTTT-
```

Appendix 8.1
Etymology of selected hosts of *Leishmania*
The names of most *Leishmania* are derived from the names of prominent workers in the field, or of geographical regions in which they are found. The names of the vertebrate hosts have more diverse origins as follows (from Gotch, 1995):-

Class Mammalia
mamma a breast. Animals that produce milk to feed their young.

Subclass Metatheria
meta (Gr) among, between; of place, after; of time, after, later *ther* (Gr) a wild animal. In this case later animals relative to the Prototheria (Monotremata).

Order Marsupalia
marsupium (L) a pouch, a purse.

Family Didelphidae
di- (Gr) prefix meaning two, double *delphus* (Gr) the womb; an allusion to the pouch as a "secondary womb" in which the young develop after birth.

Subclass Eutheria
eu- (Gr) prefix meaning well, nicely; sometimes used to mean the typical animals in a group *ther* (Gr) a wild animal

Order Edentata
e (=ex) (L) prefix meaning out of; can mean without, *dens* (L), genitive *dentis*, a tooth *-atus* (L) suffix meaning provided with; "not provided with teeth". A misnomer, only true of the anteaters, the Giant Armadillo has up to 90.

Family Myrmecophagidae
murmex (Gr) genitive *murmekos*, an ant *phagein* (Gr) to eat. Another misnomer, anteaters eat termites (Isoptera) not ants (Hymenoptera).

Tamandua tetradactyla
tamandua is a Brazilian word for an "ant-trap" *tetras* (Gr) four *daktulos* (Gr) a finger, a toe.

Family Bradypodidae
bradus (Gr) slow *pous* (Gr) genitive *podos*, a foot;

Choloepus didactylus
kholos (Gr) lame, maimed *pous* (Gr) a foot; this name has been given because it has only two digits on its forefeet but three on its hindfeet.

C. hoffmanni
Dr Carl Hoffmann (1823-1859) was in Costa Rica, Central America, from 1854 until his death in 1859.

Family Dasypodidae
dasus (Gr) hairy, rough *pous* (Gr) genitive *podos*, a foot; in this case meaning rough footed.

Dasypus novemcinctus
novem (L) nine *cinctus* (L) a girdle.

Order Rodentia
rodo (L) I gnaw

Suborder Myomorpha
mus (Gr) a mouse *morphe* (Gr) form, resemblance; mouse-like.

Family Cricetidae
cricetus (New L) derived from *criceto* (It) the hamster

Oryzomys palustris Common Rice Rat
oruza (Gr) rice *mus* (Gr) a mouse *palustris* (L) marshy, boggy; can mean living in marshy or boggy places.

Sigmodon hispidus Cotton Rat
sigma (Gr) the letter; *oudos (=oudon)* (Gr) a tooth; an allusion to the sigmoid pattern of the enamel of the molars when the crowns are worn down *hispidus* (L) hairy, shaggy; a reference to the harsh hair.

Psammomys obesus
psammos (Gr) sand *mus* (Gr) a mouse *obesus* (L) fat.

Rhombomys opimus
rhombos (Gr) a rhombus, a reference to the enamel on the upper molars which shows a lozenge shaped pattern *mus* (Gr) a mouse *opimus* rich, fat.

Suborder Hystricomorpha
hustrix (Gr), genitive *hustrikhos*, a hedgehog, a porcupine *morphe* (Gr) form, resemblance;

Family *Erithizontidae*
erithizo (Gr) I rouse to anger, I irritate.

Coendu prehensilis
Coendu is a Brazilian native name for the porcupine *prehenso* (L) I lay hold of *-ilis* (L) adjectival suffix denoting capability; "able to hold".

Family Dasyproctidae
dasus (Gr) hairy *proktos* (Gr) the hindpart, the rump;the hair is not confined to the rump but it is longer there and usually of a different colour which makes it conspicuous.

Dasyprocta agouti
Aguti or *acuti*, is a South American Spanish name.

Family Echimyidae
echi (New L) derived from *ekhinos* (Gr) a hedgehog *mus* (Gr) a mouse; they have sharp bristly hair.

Proechimys spp.
pro (Gr) before, and *echimys*; *pro-* is used here to denote an allied form.

Order Carnivora
caro (L), genitive *carnis,* flesh *voro* (L) I devour

Family Canidae
canis (L) a dog.

Canis familiaris
familiaris (L) domestic, belonging to a household, a servant; can mean a familiar friend.

Family Procyonidae
pro- (Gr) prefix meaning before, in front of *kuon* (Gr) a dog; the raccon family were probably ancestors of the dogs

Nasua nasua Coatimundi
nasua (L) the nose; the nose is exceptionally long, flexible and almost like a trunk. Coatimundi is a Tupi name for this raccoon-like animal.

Order Hyracoidea
hurax (Gr), genitive *hurakos*, a mouse, a shrew-mouse *-oidea* (New L) , from *eidos* (Gr) form, sort, a particular kind.

Family Procaviidae
pro- (Gr) before, hence *protos* (Gr) first *cavia* (New L) form cabiai, a Brazilian word for a rodent, probably a "guinea-pig"; *Procavia* could mean first guinea-pigs, suggesting that other similar animals are descended from these.

Procavia capensis Rock Hyrax or Dassie
-ensis (L) belonging to; it inhabits the Cape Province area amongst others. Dassie is the Afrikaans name for the hyrax.

Heterohyrax brucei Yellow-spotted Hyrax
heteros (Gr) different + hyrax; an allusion to the skull, which is like that of *Dendrohyrax* except that the orbit, or eye-socket, is incomplete behind; James Bruce (1730-1794), a naturalist who was in Ethiopia from 1768-1773 and made some expeditions to discover the source of the Nile.

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