From the Institute for Medical Parasitology, University Clinics of the Rheinische Friedrich-Wilhelm-University Bonn,

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Giessen, Germany.

Evaluation of PCR methods for detection, species identification and determination of genetic variation in *L. infantum*

Inaugural dissertation for the acquisition of the doctoral degree at the Fachbereich Veterinärmedizin of the Justus-Liebig-University Giessen, Giessen, Germany.

Submitted by MARIA KOKOZIDOU
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With permission of the Fachbereich Veterinary Medicine of the Justus-Liebig University, Giessen, Germany.

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Date of the oral examination: 14th April, 2003

Part of this work was presented:

- at the III Status "Eukaryonter Krankheitserreger" Workshop in Bonn, under the title "Identification of *Leishmania* isolates from dogs and humans using ITS-RFLP and PCR-Fingerprinting with single arbitrary primers" and
- as poster at the 2nd Colloquium on *Trypanosoma* and *Leishmania* research in Germany, at the Zentrum für Molekularbiologie Heidelberg. The title was "Identification of *Leishmania* isolates from dogs and humans using ITS-RFLP and PCR-Fingerprinting with single arbitrary primers".

To my family

"…To God be the glory, great things he hath done…."

From the Bible

Contents

ABBREVIATIONS

AP-PCR	arbitrarily primed polymerase chain reaction
bp	base pairs
BSA	bovine serum albumin
CL	cutaneous leishmaniasis
DAF	DNA amplification fingerprinting
DAT	direct agglutination test
dATP	deoxyadenosine triphosphate
DCL	diffuse cutaneous leishmaniasis
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dTTP	deoxythymidine triphosphate
ELISA	enzyme-linked immunosorbent assay
FML	fucose-mannose ligand
GBP	gene B protein
IFAT	indirect immunofluorescent antibody test
kDNA	kinetoplast DNA
LR	leishmaniasis recidivans
ML	mucosal leishmaniasis
PCR	polymerase chain reaction
PCR-SHELA	Polymerase chain reaction solution hybridisation enzyme-linked assay
PKDL	post kala-azar dermal leishmaniasis
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS-PAGE	sodium-dodecyl sulphate-polyacrylamide gel electrophoresis
SSCP	single strand conformation polymorphism
ssDNA	single stranded DNA

ssu rRNA	small subunit ribosomal RNA gene
UV	ultra violet
VL	visceral leishmaniasis
WHO	World Health Organisation

1. Outline and Objectives of this study

Leishmaniasis is a parasitic infectious disease comprised from a variety of syndromes, very different the one with the other in their epidemiology, pathogenesis and clinical picture. According to the World Health Organization (WHO), leishmaniasis is considered as one of the most important parasitic diseases (WHO, 1990). It is endemic in 88 countries in five continents with 12 million people afflicted world wide from it and another 350 million living at risk of infection. About 1-1.5 million new cases of cutaneous leishmaniasis (CL) and 500,000 of visceral leishmaniasis (VL), rise per year (Desjeux, 1996).

The disease is attributed to *Leishmania*, haemoflagellate protozoan parasites of the *Trypanosomatidae* family. In many localities more than one *Leishmania* species co-exist with overlapping animal hosts and vectors. It is a zoonotic disease but it is still not known to what extent that affects the epidemiology, transmission and planning of control measures.

During the recent years parallel to the development of techniques based on the genomic information of organisms, there is an increased effort to apply this knowledge to the applied fields of disease diagnostics. In the course of not many years now, some methods were developed for the diagnosis of leishmaniasis based on the genomic information of the parasite. Some of them could detect the parasite very successfully, some not and some are still standing under question. The aim of this study was the evaluation of PCR methods for detection, species identification and determination of genetic variation in *L. infantum*.

RFLP (restriction fragment length polymorphism) of the ITS (internally transribed spacer), and fingerprinting with single arbitrary primers deriving from the core sequence of the phage M13, from the intergenic tRNA spacers (T3B) and the simple repeat sequences $(GTG)_5$ and $(GACA)_4$ (Schönian *et al*., 1996; Schönian *et al*., 2000), were applied. Those methods have two general goals: first to discriminate if possible between closely related species and complexes and second to establish a degree of evolutionary relationships. This leads to the construction of molecular evolutionary trees that provide a logical framework to view the evolutionary process in these organisms. 36 well characterised *Leishmania* strains, 21 canine isolates from Northern Greece and 9 human isolates went through both those methods.

Those isolates that were identified as *L. infantum,* coming from the whole Mediterranean basin, were processed through a PCR-SSCP (polymerase chain reaction-single stranded conformation polymorphism) with codominant markers, to search levels of genetic variation and heterogeneity among strains of *L. infantum*. For this 8 sets of codominant primer pairs were used and 42 *Leishmania donovani* complex strains (38 *L. infantum*, 2 *L. chagasi* and 2 *L. donovani*).

The 42 *Leishmania donovani* complex strains and 24 clinical canine samples went also through a diagnostic PCR that amplified a 560 bp variable fragment of the ssu rRNA gene (Meredith *et al*., 1993; Osman *et al*., 1998a), in order to evaluate detection efficiencies also in canine samples and clinical speciments.

The results of all procedures combined together exploit and establish even more the situation of whether the *L. infantum* population of the Mediterranean area is a clonal or a recombinant one. Previous studies based on non-molecular approaches and techniques have suggested a clonal population (Jimenez *et al*, 1997) of the same dominant clone (MON1).

This work is part of a greater project that is centered in Berlin at the Institute of Microbiology and Hygiene, Charité, Berlin and is headed by Dr Gabriele Schönian. This project is under work since 1995 and is continued in collaboration with countries where the disease is endemic.

2. Literature Review

2.1 The genus *Leishmania*

Leishmania are haemo-flagellate protozoan parasites that belong to the family of Trypanosomatidae. They are obligate intracellular parasites that are transmitted to the mammalian host by the bites of infected sandflies. On the basis of development in the sandflies, the genus *Leishmania* has been divided into two subgenera. Development of organisms belonging to the subgenus *Leishmania* is restricted to the anterior portion of the alimentary tract of the *Phlebotomus* sandflies (suprapylarian development), whereas organisms belonging to the subgenus *Viannia* develop in the midgut and hindgut of the sandflies *Lutzomyia spp*. (peripylarian development). *Viannia* contains the complex of *L. brasiliensis* (*L. brasiliensis*, *L. guyanensis*, *L. panamensis*), *L. mexicana*, *L. amazonensis* and *L.panamensis*. The subgenus *Leishmania* contains the complex *L. donovani* (*L. infantum*, *L. donovani*, *L. chagashi*), *L. major*, *L. tropica* and *L. aethiopica*. There are in total at least 30 species, of which 12 named and several unnamed infect man (Lainson *et al.*, 1987).

Characterization was originally attempted on geographical and clinical grounds, and later on morphology, culture characteristics, biotypes and life cycles, antibody responses to particular antigens and lately on genetic analysis.

2.1.1 Life cycle of *Leishmania*

Leishmanial parasites are transmitted between long-living (humans, canines) vertebrate hosts by short-living Phlebotominae insects (sandflies) (Lewis 1974; Lane 1993). They have a cycle of development in the vertebrate host and one in the insect. Sandflies ingest amastigotes while sucking blood from the vertebrate host. The amastigotes transform into promastigotes in the alimentary tract of the sandflies (Lane 1993). After 4-7 days they migrate to the foregut where, they develop into infective metacyclic forms. As the female sandfly has its blood meal, necessary before laying on of eggs, the mouthparts of the insect tear tissue and create a tiny pool of blood from which they feed and into which metacyclic promastigotes are deposited.

Metacyclic promastigotes are inocculated with sandfly saliva, which increases infectivity (Titus *et al*., 1988). The host cells are mononucleated cells e.g. macrophages. It is not precisely known how promastigotes enter the macrophages. In the macrophages they transform into amastigotes where they multiply and then they are deposited in different parts of the body, spleen, liver, bone marrow.

Each *Leishmania* species has its own biotope with its own geographical distribution zone and complex of parasite, reservoir and vector and their particular intimate relationship within this setting. In Table 1 a summary of such relationships is presented.

Table 1: Major *Leishmania* species that are of interest to the public health, their reservoir, vector and geographical distribution according to WHO (1996).

***Leishmania* species**	**Vector**	**Reservoir**	**Geographical distribution**
Old World			
L. infantum	*Phlebotomus perniciosus, P. ariasi*	Dogs, foxes, jackals	Mediterranean basin, Middle east, China, Central Asia
L. donovani	*P. argentipes*	Humans	North-east India, Bangladesh, Burma
L. donovani	*P. orientalis, P. martini*	Rodents in Sudan, canines, humans, gerbils	Sudan, Kenya, Horn of Africa
L. major	*P. papatasi, P. duboscqi*	Gerbils (*Rhombomys, Meriones*), Rodents (*Arvicanthus, Tatera*)	Middle East, North India, Pakistan, North Africa, Central Asia, Sub-Saharan savannah, Sudan
L. tropica	*P. sergenti*	Humans	Middle East, Mediterranean basin, Central Asia
L. aethiopica	*P. longipes, P. pedifer*	Hyraxes	Highlands of Kenya, Ethiopia

New World			
L. chagasi	*Lutzomyia longipalpis*	Foxes, dogs, opossums	Central America, Northern South America (Brazil, Venezuela, Yucatan, Belize, Guatemala)
L. brasiliensis	*Lutzomyia spp., Psychodopygus wellcomei*	Forest rodents, peridomestic animals	Tropical forests of South and Central America
L. guyanensis	*Lu. umbratilis*	Sloths (*Choleopus*), arboreal anteaters (*Tamandua*)	Guyana, Surinam, Brazil
L. panamensis	*Lu. trapidoi*	Sloths (*Choleopus*)	Panama, Costa Rica, Colombia
L. mexicana	*Lu.olmeca*	Forest rodents	Yucatan, Belize, Guatemala
L. amazonensis	*Lu. flaviscutellata*	Forest rodents	Tropical forests of South America
L. peruviana	*Lutzomyia spp.*	Dogs	West Andes of Peru, Argentine highlands

Leishmaniases are normally zoonoses infecting wild animals like rodents, edentates and canines. In each transmission cycle there is a restricted number of primary reservoir hosts that maintain the cycle. Some times there are secondary hosts as well, that extend the cycle like the dogs in *L. chagasi* and accidental ones that are not important from the view point of maintaining the cycle like man in the case of *L. major*. It has been suggested that acute human cases of VL in Africa (not in Europe) are also serving as reservoirs. Nevertheless new

epidemiological results on HIV-*Leishmania* co-infected patients in the Medditeranean area and the post-kala-azar dermal leishmaniasis (PKDL) in India and Sudan (WHO, 2000), are overthroughing such suggestions. The PKDL is a condition by which man is a reservoir for *L. donovani* (Osman, 1998b).

In the Old World sandfly vectors belong to the genus *Phlebotomus* and in the New World to the genera *Lutzomyia* and *Psychodopygus*. The breeding sites of many species are unknown. Breeding sites, flying habits, feeding habits, degree of anthropophilia or zoophilia (Lainson, 1983), efficiency of transmission (Dye, 1992), life span and biting during the night, are some of the important determinants of infection, and thus of control measures of the disease as well (Lewis *et al.*, 1987).

When the reservoir and the vector share the same habitat like *P. papatasi* and the gerbil, then the risk of human infection becomes high (*L. major*). Where the habitats are separate but partly overlapping, the risk becomes relatively less, like in the case of *Lu. umbratilis* and *L. guyanensis* (Ashford *et al*., 1987).

Man and/or dog are usually infected by the bite of an infected sandfly. VL has been rarely transmitted by blood transfusion, by sharing needles (drug abusers) (le Fichoux *et al*., 1999), sexual intercourse (Symmers, 1960), accidental or deliberate inoculation in the laboratory (Manson-Bahr *et al*., 1963), or congenitally (Nyakundi *et al*., 1988). Cutaneous leishmaniasis (CL) has been reported to be transmitted by deliberate scarification as a form of immunisation (Gunders, 1987) and through suckling (Marsden *et al.*, 1985).

2.2 The diseases of leishmaniases

Leishmaniasis is not a single disease but a variety of syndromes that differ remarkably with one another. The WHO considers leishmaniasis as one of the most important parasitic diseases (WHO, 1990). Leishmaniases are endemic in 88 countries on the five continents (except Oceania), with a total of 350 million people at risk and 12 million afflicted world-wide. There are 1-1.5 million new cases of CL and 500 000 of VL per year (Desjeux, 1996).

2.2.1 Epidemiology of Leishmaniasis

The epidemiology of leishmaniasis in a given area is directly dependent on the behaviour of the human and/or animal population in relation to the cycle of transmission. There is a variety of factors that influence the transmission of the disease. Some are the following (for review see Kettle, 1995 and Lane, 1993):

- Proximity of residence to sandfly breeding and resting sites.
- Type of housing.
- Occupation.
- Extent of exposure to sandfly bites.
- Natural resistance, genetic or acquired.
- Virulence of the parasite species.
- Zoonotic or anthroponotic reservoirs. It seems that zoonotic reservoirs are particularly stable when wild uncontrolled populations (e.g. rodents) are involved. Up to now it seemed that humans are not a reliable agent because of death and treatments except of the chronic condition of PKDL. Nevertheless recent reports about asymptomatic infections in healthy blood donors in France (le Fichoux, *et al*., 1999) are adding a new parameter to the latter.
- The vectorial capacity, which is defined as the number of infective bites delivered per human per annum (Dye, 1992).
- Density, seasonality, longevity and flight range of sandfly populations.
- Anthropophilia or zoophilia of sandflies and degree of it.

L. infantum causes VL in the Mediterranean basin, Western Asia and Eastern China in a belt between 30° N and 45° N. The infection is enzoonotic in dogs, especially in domestic dogs in South Europe, but feral dogs also may serve as a reservoir in the Middle East and foxes in South Europe and North Africa (Rioux *et al*., 1968). Canine infections in the Mediterranean basin are often like small outbreaks of disease appearing and disappearing. This may reflect the spread of infection by foxes, the availability of breeding sites or reinforcement of the disease from old chronic infections. In any case canine infections do not necessarily lead to human infections (Pozio *et al*., 1981).
In China (Minter, 1987) *L. infantum* was causing VL in man and dogs but it was quickly controlled and the disease was almost eradicated. There are still some infection foci in the hills of east and central China.

L. donovani causes anthroponotic VL, or Kala-azar in the Indian subcontinent and in some parts of China (Ashford *et al*., 1987). Man is the only known reservoir especially in areas where the presence of PKDL is common. Nevertheless destabilisation of the disease has come through famine, malaria and influenza (Dye *et al*., 1988) and mass spraying of DDT to control malaria that effected the sandfly vector. The vector *P. argentipes* rests in cattle sheds that are often closely attached to houses, breeds in organic detritus on the ground and a subpopulation of it is anthropophilic (Thakur *et al*., 1981).

L. donovani causes enzoonotic VL in Sub-Saharan Africa, especially in southern Sudan, Ethiopia, Somalia and northern Kenya. In Sudan *P. orientalis* is the vector and certain rodents transmit the infection. In Kenya *P. martini* has been found carrying the parasite. Infected dogs are a rare phenomenon and are not considered to be reservoirs in this case (Mutinga *et al*., 1980; Mansour *et al*., 1970). Rare cases have been reported from Niger, Chad, Central African Republic, Zambia, Malawi, Zaire and Angola without much information along with it.

L. chagasi causes VL in the New World (Shaw *et al*., 1987). The main endemic area is Northeast Brazil where the vector is *Lutzomyia longipalpis* and foxes, dogs and opossum were found serving as reservoirs. Sporadic cases have been reported from Mexico, Honduras, El Salvador, Colombia, Venezuela and Bolivia.

L. major is responsible for most zoonotic CL of the Old World. It is endemic in the hot semideserts and dry silt valleys of North Africa, Middle East, the Arabian Peninsula, Rajasthan in India, Turkmenia, Uzbekistan, Tadjikistan, Kazakhstan, across the west of sub-Saharan Africa, central Sudan and Northern Kenya. The reservoirs are gerbils, girds and fat rats (*Arvicanthus, Tatera*). Lesions are localized, mainly in harless skin, especially ears, and persist throughout the animal's life. *P. papatasi* is the main vector bur also *P. sergenti* plays a role. Transmission is greatest between April and June. People are at risk in expanding towns, new settlements, when entering the desert as hunters, soldiers or tourists (WHO 1990, 1996). Epidemics often occur after years of quiescence (Belazzoug, 1982).

L. tropica causes anthroponotic CL in the Old World. It has been reported round the Mediterranean basin from Greece eastwards, from Northern Serbia and Romania through Turkey, Middle East, West Asia in Afghanistan, Pakistan and India up to New Delhi and on the whole of the northern African littoral. Man is the principal reservoir but the parasite has been isolated from *Rattus rattus* in Iraq, and the skin of dogs in India, Russia and Morocco. *P. sergenti* is the main vector and the *P. papatasi* secondary. Transmission peaks late summer. There have been a few cases of VL by *L. tropica* in India, Kenya and Saudi Arabia (Mebrahtu et al., 1989) and a few of mucosal leishmaniasis (ML) (Lanotte *et al*., 1981)

L. aethiopica is responsible for cutaneous leishmaniasis in the highlands of Ethiopia, western Kenya and eastern Uganda. The vectors are *P. longipes* and *P. pedifer* and the reservoirs are the hyraxes *Procavia habessinica* and *Heterohyrax brucei* (Ashford *et al*., 1973). This parasite is able to suppress the human immune response and produce the diffuse cutaneous leishmaniasis (DCL) and lepromatous cutaneous leishmaniasis (LCL) (Bryceson, 1970).

L. brasiliensis is the most common agent that causes CL and ML (espundia), in Central and South America. It is found in Belize, Guatemala, Honduras, Costa Rica, Panama, Peru, Argentina, Bolivia, Paraguay, Colombia, Venezuela, throughout the Amazonian forest below heights of 2000 meters, and in hot forests of the pacific coast of Colombia and Central America. Zymodeme analyses showed great heterogeneity of *L. brasiliensis.* It possibly went through changes in reservoirs and vectors as a natural result of adaptation to all the different settings (Oliveira-Neto *et al*., 1988). Incidental infections have been found in many genera like dogs, equines in the suburban areas (Aguilar *et al*., 1987), while the natural forest

reservoirs have not been yet identified. Some of the sandfly vector species are *Ps. welcomei*, *Lu. whitmani* and *Lu. intermedia,* all of them are anthropophilic (Aguilar *et al*., 1987).

L. panamensis is responsible for leishmaniasis in Costa Rica, Honduras, Nicaragua, Panama, Colombia and the pacific coast of Ecuador. Its natural host is the sloth *Cleopus hoffmanni* (Herrer *et al*., 1980) and accidental infections were reported in wild animal species and in dogs. Infection rates in humans are also high in the areas mentioned above (Sanchez *et al*., 1992).

L. guyanensis is restricted to the Amazonian forests of Brazil, Colombia, French Guyana, Guyana and Surinam. Its natural hosts are the arboreal sloth *Choleopus didactylus*, the anteater *Tamandra tetradactyla* and *Choleopus marsupialis*. The main vectors are *Lu. umbratilis* and *Lu. Anduzei* (Dedet *et al*., 1989).

L. peruviana is responsible for CL in the high valleys of the Peruvian Andes and the Argentinean highlands. By isoenzyme analysis it can not be distinguished from *L. brasiliensis*. Dog is considered the urban reservoir but there is no information for a wild one. *Lu. peruenis* and *Lu. verrucarum* are the vectors (Llanos-Cuentas *et al*., 1999).

L. brasiliensis, L. peruviana, L. panamensis and *L. guyanensi*, are the main causative agents of mucocutaneous leishmaniasis in central and South America.

L. mexicana is prevalent in the Yucatan peninsula of Mexico, through Guatemala, Honduras, Panama and Colombia. It causes CL and DCL. Various forest rodents are the reservoir. Almost 90 % of Yucatan males that work in the forests are leishmanin positive (Andrade-Narvaez *et al*., 1990).

L. amazonensis infections are reported from the Amazon forests of Brazil, Bolivia, Colombia, Ecuador, Peru, French Guyana and Venezuela. Forest rodents carry skin infections and the vector *Lu. flaviscullеta* is widespread but not anthropophilic. Thus human infections in the area are relatively rare but there is a high rate of DCL cases and some VL cases (Barral *et al*., 1991).

There may be many more species as it has been mentioned before but the information is still scarce. Leishmanial parasites closely related to *L. mexicana* have been reported from Texas and the Dominican Republic (Schnur *et al*., 1983). Two unnamed species have been found in Namibia (Grove, 1989), Angola, Zaire and Tanzania, one in man and one in the rock hyrax *Procavia capeses*, but fundamental understanding of the life cycle and epidemiology is still needed. A rarer species of *Leishmania*, *Leishmania donovani archibaldi*, has infrequently been reported to cause leishmaniasis in the Horn of Africa (Lainson *et al*., 1987).

The geographical distributions are greatly overlapping making it very difficult many times to distinguish the one from the other. Worldwide, the disease is common and grossly unreported. Zoonotic and human infections are often associated with environmental changes and the transmission cycles adapt (Shaw *et al*., 1987; Lainson, 1983) to new conditions.

2.2.2 Pathogenesis and clinical features of leishmaniasis

Leishmaniasis is a variable disease with a variety of syndromes that are manifested alone or in combinations (Garnham, 1987).

The incubation period ranges from a few days to several months. The sandflies are biting humans and animals in the uncovered and hairless areas of the body. At the inoculation site an erythematous nodule appears. The nodule grows to an ulcer with a raised edge. This sore remains often in that stage without further development and when it heals it leaves scar tissue. Scars can even disable if they are on the face or over a joint.

After inoculation of the parasite through the sandfly bite, *Leishmania* promastigotes are phagocytosed in the skin by activated macrophages. Patients with acute leishmaniasis fail to produce T helper cell 1 (Th1) cytokines and the parasite interferes with the killing mechanism of the macrophages (Bogdan *et al*., 1990; Russo *et al*., 1992). The parasites transform into amastigotes and start to divide. Amastigotes have an affinity for macrophages and endothelial

cells of arterioles and capillaries, leading to tissue lysis and necrolysis. Then one of the following events finds place:

- The immune system kills the parasites and the person becomes immune to reinfection by that species.
- A local infection develops until either the immune system of the host eradicates it or is defeated by it permitting dissemination.
- The infection disseminates to the viscera (*L. infantum, L. chagasi*), oronasal mucosa (*L. brasiliensis*) or skin (*L. aethiopica*, *L. mexicana*).

Parasites multiply in the cells of the mononuclear phagocyte system like blood monocytes, macrophages, histiocytes, epithelioid cells, Kupffer cells of the liver, reticuloendothelial cells in spleen and lymphoid tissue.

Clinical pictures (based on Bowman, 1995; Cook, 1996 and Garcia *et al*., 1993)

2.2.2.1 **Cutaneous leishmaniasis (CL)** due to *L. major* has an incubation period of 1 week to 2 months. Lesions develop to necrotic foci rapidly and they become inflamed and exudative wet sores. Lesions do not spread to the mucosa and are often located on the limps (Nadim *et al*., 1968). During epidemics the disease is quite severe, with multiple lesions deeply ulcerated, and all ages are affected.

CL due to *L. tropica* is slower in evolution and affects more the children. The initial nodule may develop a few satellite nodules but in is crusting slowly and heals.

CL due to *L. infantum* and *L. chagasi* is even less aggressive. The lesions are nodular, they never ulcerate and last 1-3 years.

CL due to *L. aethiopica* causes solitary lesions centrally on the face. Many times a spreading nodule, tumour or plaque develop and if the lesion reaches the border of the nose or mouth, the infection may spread along the mucocutaneous margins but does not spread into the oronasal cavities. Sores heal slowly over 2-5 years.

CL due to *L. brasiliensis* causes single, deep, fast developing ulcers. 80 % of the sores heal within one year.

CL due to *L. guyanensis* is often presented with multiple lesions and up to half of the cases have lymphatic spread and even oedema.

CL due to *L. panamensis* is also associated with lymphatic and lymph node involvement and sores persist for years.

CL due to *L. mexicana* is often known by the "chiclero ulcer", which is CL eroding the cartilage of the pinna of the ear.

CL due to *L. amazonensis* produces solitary lesions but not much is known about their evolvement.

2.2.2.2 **Diffuse cutaneous leishmaniasis** (DCL) occurs in about 1 per 10 000 infections with *L. aethiopica* (Bryceson, 1970). The primary lesion does not ulcerate but the infection spreads slowly through the bloodstream and relocates. The clinical picture often resembles to lepromatous leprosy and cause grotesque deformity. Spontaneous healing is rare in this case.

2.2.2.3 **Leishmaniasis recidivans (LR)** is a condition that can result in infection with *L.tropica*. It has the clinical picture of Lupus vulgaris and represents persistence of the infection in the face of a vigorous immune response (Pettit, 1962).

2.2.2.4 **Visceral leishmaniasis (VL)** is due to *L.donovani* in India and West Africa, *L.infantum* in the Mediterranean basin and *L.chagasi* in Central and South America. Incubation period ranges from 3 weeks over 2 years (Jopling, 1955). It is also known as kala-azar, which is the most severe form of the disease which if left untreated, has a mortality rate of 100 %. It is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anaemia. Globulins are over-produced in the beginning but immune complex-mediated disease is rare in humans (Pearson *et al*., 1983), while it is quite common in dogs (Koutinas *et al*., 1995). In Sudan and in the Mediterranean area the disease may be present as afebrile lymphadenopathy. Abdominal pain is due to the enlarged spleen. Epistaxis is common but usually no other form of haemorrhage. The differential diagnosis stands amongst leishmaniasis and malaria, brucellosis, bacterial endocarditis, typhoid, miliary tuberculosis and haemopoietic malignancy. Very often patients in an endemic area may not seek attention for up to a year (Cole, 1944; Maru, 1979). In the Mediterranean VL skin lesions in human are uncommon (Sciliro *et al*., 1978). Still the clinical picture in the dogs (Kontos *et al*., 1993, Koutinas *et al*., 1999) is involving variable cutaneous lesion (exfoliate dermatitis, skin ulcerations) ocular lesions (conjunctivitis, keratoconjuctivitis sicca, blepharitis, uveitis), poor body condition (Koutinas *et al*., 1992), chronic renal failure, peripheral lymphadenopathy or lymph node hypoplasia and masticatory muscle atrophy (Vamvakidis *et al*., 1999),

2.2.2.5 **Post-kala-azar dermal leishmaniasis (PKDL)** is a sequel to the infection with *L.donovani*. Most patients have a history of previous treatment for VL, self-healing illness resembling VL, or no history. PKDL in India resembles lepromatous leprosy with verrucous, papilomatous, xanthomathous and gigantic nodular forms (Morgan, 1962), while in East Africa it resembles more to sarcoidosis and tuberculosis with papular rash over face or well-defined rounded papules (Rashid *et al*., 1986).

2.2.2.6 **American mucosal leishmaniasis** (Llanos-Cuentas *et al*., 1984; Walton, 1973; Marsden, 1986). Almost 40% of the patients with cutaneous ulcers are infected by *L. brasiliensis* and a smaller portion with *L. guyanensis* and *L. panamensis*. The initial lesion is a nodule, usually on the anterior septum or inferior turbinate and the initial symptom is nasal obstruction or epistaxis. The infection spreads from the mucosa to the mucocutaneous junctions of the lips and nose and sometimes on to the surrounding facial skin and/or conjunctiva. Complications that often show up are nasal, pharyngeal and laryngeal obstruction from exuberant growth or fibrotic stenosis. Death may also ensure from secondary sepsis, pneumonia or starvation while spontaneous healing is unusual.

2.3 Leishmaniasis in immunosuppressed patients

In 1986 a new dimension of VL leishmaniasis was recognised when the first reports of VL as a complication to the HIV infection arrived (Altes *et al*., 1991; Alvar *et al*., 1992). Some of those cases are considered to be reactivation of latent infections and some new infections where normal defence mechanisms are impaired (AIDS), absent (congenital diseases), or bypassed (penetration of the skin barrier), and finally the cellular immunity is failing to respond. Most reports are coming from South Europe where both VL due to *L. infantum* and HIV are endemic, and some from Brazil. In Spain it is estimated that 50 % of adults with VL are HIV positive, and that 3 % of HIV-infected individuals will acquire VL (Pintado *et al*., 2001). The disease responds slowly to treatment and relapse is very common. AIDS and VL are locked in a vicious circle, since the one reinforces the other. VL quickly accelerates the onset of AIDS (with opportunistic diseases as tuberculosis or pneumonia) and shortens the life expectancy of HIV-infected people. The coinfected people often exhibit unusual clinical symptoms, which can make diagnosis difficult (WHO, 2000).

AIDS increases the risk of VL by 100-1000 times in endemic areas. Both the HIV virus and *Leishmania* parasites, destroy the same cells and increase the disease severity and consequences (WHO, 2000). The evolution of Leishmania/HIV co-infection is being closely monitored by extending the geographic coverage of the surveillance network and by improving case reporting. WHO encourages active medical surveillance of HIV patients and intravenous drug users, main population at risk. Also case notification is compulsory in only 40 of the 88 endemic countries. Thus WHO strongly suggests the remaining 48 endemic countries to follow suit. A graphic example is given in the WHO map that follows (Figure 1).

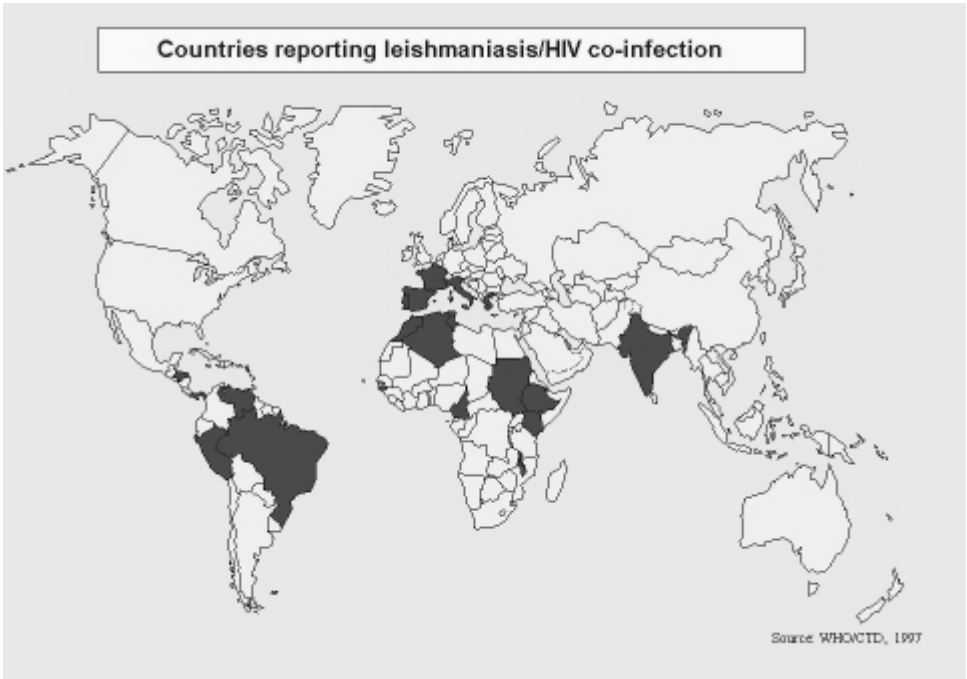


Figure 1: Countries reporting leishmaniasis/HIV co-infections (taken from the WHO at http://www.who.int/emc/diseases/leish/leisgeo1.html)

2.4 Treatment

The intracellular site of development of the parasite in leishmaniasis patients makes particular pharmacokinetic demands upon drugs.

2.4.1 Pentavalent antimonials (sodium stibogluconate, meglumine antimoniate) remain the drugs of choice for reasons of cost, availability and efficacy together. However, cases of antimony resistance were reported from epidemics in India and Sudan (Thakur *et al*., 1988; Seaman *et al*., 1993), and in the case of HIV coinfected patients, relapse is the common follow-up. Also dose schemes in excess of 20 mg Sb/kg BW (Body Weight) should be monitored daily because of the side effects.

2.4.2 Aminosidine (paromomycin) is an aminoglycoside antibiotic, non-toxic in conventional doses in patients with normal renal function but because of its aminoglycoside properties, has all the potential for renal and ototoxicity. Given with antimonials it has a unique synergistic effect than either drug alone (Seaman *et al*. , 1993; Scott *et al*., 1992; Chunge *et al*.,1990). Aminosidine ointment (15 % with 15 % urea in white shoft parafin) cures 80 % of the Old World sores if applied for up to 12 weeks (Bryceson *et al*., 1994).

2.4.3 Pentamidine has been a traditional drug for leishmaniasis as well, administered for 15 days on a dose 2-4mg/Kg . It is also an effective drug but its potential side effects overlap its effectiveness, including hypoglycaemia followed by diabetes mellitus, hypotention (if administered rapidly), nausea, vomiting, abdominal pain and headache (Pearson *et al.,* 1996).

2.4.4 Amphotericin B has proved to be 400 times more potent than antimonials against *Leishmania* because it binds to ergosterol, the major cell membrane sterol of amastigotes (Davidson *et al*., 1993), causing an impairment of barrier function that results in the loss of protons and cations from the cell (Warnock, 1991). Nevertheless, despite its effective properties (Davidson *et al*., 1993) the conventional Amphotericin B desoxycholate is little employed because of the side reactions of fever and phlebitis during infusion, anaemia, kidney dysfunction, and hypocalaemia (Utz, 1964).

2.4.5 Liposomal Amphotericin B . The formulation of AmBisome (Vestar, San Dimas, CA, USA) has been licensed as antileishmanial drug. The size of the vesicles in which Amphotericin B is encapsulated is 80 nm. Once intravenously injected liposomes escape to a very small extent from the intravascular space. Vesicles of an average size 100 nm, can pass through the fenestrae in the liver to reach the hepatic parenchyma cells, the fixed macrophages and the monocytes, by which they are endocytosed (Gregoriadis, 1991). After that, they end up in the lysosomal apparatus of the cells, the vesicles are disrupted by phospholipases and freed drug can diffuse through the lysosomal membranes to reach other cell compartments (the

parasitophorus vacuole and the parasite). All the above, very well explain the high efficacy of the drug in treatment of visceral leishmaniasis (Croft *et al*., 1991; Berman *et al*., 1986), the high value of the drug half life remaining detectable for long time after treatment (Gangneux *et al*., 1996), the administration of lower doses in short courses (Castagnola *et al*., 1996) and its one-tenth less toxicity comparing to conventional Amphotericin B (Proffitt, 1991).

The economical condition of the endemic areas is very poor, and for reason of meeting effectivity with low cost of treatment, pentavalent antimonials are the drugs of choice in those areas. For the same reason they are the ones suggested from the WHO.

In the cases of canine leishmaniasis long term therapy courses are recommented with Allopurinol 20 mg/kg/day per os for months to years, which manifests a clinical healing but doesn't eliminate the parasite. Combinations of Allopurinol 100 mg/kg/day and megluminantimonate 30 mg/kg/day until healing of the clinical symptoms and then long-term courses of 1 weekly therapy per month with 20mg/kg Allopurinol. Antimonials of course are in use, Megluminantimonate (1^{st} and 2^{nd} day 100 mg/kg, 3-10 days 200-300 mg/kg and 2x in time distance of 14 days s.c. in the chest area or very slow i.v.), Natrium-Stibogluconat (10-20 mg/kg dayly for 2x10 days with a 10 days interval with a lot of liquids i.v.) and Diamidine Pentamidin (2-4 mg/kg util 5x with 3-3 days time distance between them i.m. or 2 mg/kg dayly for 14 days given in 500 ml liquid i.v. slowly). All three give a clinical healing that rarely lasts. Dogs with nephritis following antimonial treatment are a phenomenon often observed. Also treatment with Paromomycin, a liposomal formulation of Amphotericin B (0.5-0.8 mg/kg 2x per week i.v. until an accumulative dosis of 8-16 mg/kg is reached), gave mostly a temporary result (Rommel *et al*., 2000). Along with the systemic treatment the skin ulcers should be at the same time treated with the appropriate antibacterials. The development of a vaccine is in embryonic stages and tourists should seriously consider whether they should let their dogs escort them while travelling in the Medditeranean. In such cases dogs can be treated with Permethrin (10 mg Permethrin pro kg) washings or baths every 1-2 weeks during the exposition time, and/or carry Permethrin collars.

Vector control methods are hardly established in the case of the sandfly vectors. Details in their life cycle are often not known. The breeding places in the various areas are still not clearly defined in detail. Conventionaly impregnated bed nets are inefficient because of the small size of the sandflies. Nevertheless there is evidence that deltamethrin-impregnated

collars protect domestic dogs from sandfly bites. Dogs wearing the collars were bitten by approximately 80 % fewer sandflies than before collars were fitted (Halbing *et al*., 2000).

2.5 Diagnosis of leishmaniasis

2.5.1. Parasitological diagnosis

In VL and CL, parasites may be isolated from 80 % of the sores during approximately the first half of their natural course (Cuba *et al*., 1984). Tissue juice, not blood is scraped with a scalpel blade from a nodule. The nodule is previously grasped firmly between finger and thumb to exclude blood and an incision of a few mm long is made into the dermis. The material obtained can be used to prepare a smear that will be stained with Giemsa, Wright's or Leishman's stain, or inoculate culture media for the isolation and the culture of the parasite. Biopsy smears may be used for culture, inoculation into hamsters, impression smears or immunohistology in tissue sections (Sells *et al*., 1981). Lesions smears and culture are best in cases with cutaneous lesions and biopsy and hamster innoculation when mucosal lessions are predominant.

In VL splenic aspiration is the most sensitive method (Chulay *et al*., 1983). The obtained material is used to inoculate culture tubes and make smears. Same procedure is followed for patients with AIDS (del Mar Sanz *et al*., 1991). Bone marrow and lymph node aspirations have proved to be very useful as well. Lymph node aspirations are especially useful in routine field diagnosis in both human and dogs.

2.5.2 Serological diagnosis of leishmaniasis

2.5.2.1 **Leishmanin (Montenegro) test:** The test measures delayed type hypersensitivity to *Leishmania* antigens. Leishmanin is a suspension of washed promastigotes in a solution of 0.5 % phenol in saline. The antigen must be standardised against cases and controlled in the endemic area (Leeuwenburg *et al*., 1983). 0.1 ml solution is inoculated into the volar surface of the forearm. The area is measured 48-72 h later. The test comes out positive in over 90 % of the CL and ML cases, less frequent in *L. aethiopica* infections and in

ML with multiple sites of disease (Cuba *et al*., 1984). In active VL it is negative but within several months to a year after recovery, individuals elicit a positive response. Overall it is a good test for epidemiological surveys of a population to identify groups at risk of infection from CL (Weigle *et al*., 1991).

2.5.2.2 **Indirect Immunofluorescent Antibody Test (IFAT):** The procedure of the IFAT test uses as antigen whole *Leishmania* promastigotes. Dried antigen slides are stored at -70°C until use. A two-fold serial dilution of the test serum in phosphate buffered saline (PBS) is placed on 12 spots slides. The dilution may vary from 1:1 up to 1:1040 or further. Fluorescein-conjugated goat anti-human IgG at an optimal dilution is added to each spot. The slides are considered to be positive when more than 50 % of the parasites show complete peripheral flourescence (Pappas *et al*., 1985). In different studies a reasonable sensitivity and specificity ranging from 80 % to almost 100 % was reported but cross reactions were observed with sera obtained from malaria, American trypanosomiasis, schistosomiasis, leprosy and syphilis patients (Latif *et al*., 1979). Researchers tried to overcome some of this problem by first absorbing the sera with a *Trypanosoma cruzi* lysate (Camargo *et al*., 1969).

L. brasiliensis promastigotes were unsuccessful as antigen for IFAT, while amastigotes gave good results (Walton *et al*., 1972).

IFAT has proven to be very suitable for the detection of reservoir hosts of leishmaniasis like dogs, foxes and rodents in the Mediterranean area (Manciati *et al*., 1986; Sideris *et al*., 1999), in Brasil (Courtenay *et al*., 1994) and in Iran (Zovein *et al*., 1984).

In western Turkey 490 dogs, were examined using either IFAT or direct agglutination test DAT. Anti-*Leishmania* antibodies were found by at least one test in 5.3% (26/490) of the dogs. Infections were confirmed by parasitological examination of or polymerase chain reaction (PCR) on lymph node aspirates in 65% and 76.4% of the seropositive dogs tested, respectively. The confirmation rate was 85% by combining the results of PCR and microscopy (Ozbel *et al*., 2000).

2.5.2.3 **Enzyme-linked immunosorbent assay (ELISA):** In this method the antigen is being absorbed on the surface of a well or a microtiter plate, then the patients serum is added and the antibodies bind to the antigen forming the antibody-antigen complex. Non reacting

molecules are washed away and an enzyme-linked anti-IgG is added, followed by the substrate. The enzyme is detected by the amount of colour produced and is relevant to the amount of antibodies present in the patient's serum.

Engvall and Perlmann (1972) and Schnur and Zuckerman (1977) developed the first ELISAs for antibody detection. Variations evolved from that.

A modified DOT-ELISA was developed (Pappas *et al*., 1984; 1985) where formalin fixed *L. donovani* promastigotes were fixed on filter discs, placed in a microtiter plate. The sensitivity of this method was 98 %, however there was high cross reactivity with sera from patients suffering from African trypanosomiasis, Chagas' disease and lupus erythematosus. Adhya *et al*. (1995) found anti-*Leishmania* antibodies in the blood of 23 out of 39 early VL patients, using immobilised crude antigen of *L. donovani*, that captured antibody in serum 1:500 (Jaffe *et al*., 1987).

Today the standard micro-ELISA using intact promastogotes obtained from *in vitro* culture as antigen, is in practice in cross-sectional and longitudinal studies of leishmaniasis giving good results but still cross reacting in the co-endemic areas with sera from patients with African trypanosomiasis (El Amin *et al*., 1986).

To eliminate cross-reactions different sets of chemically defined peptides are used, which are conjugated to a protein carrier such as bovine serum albumin (BSA) (Hommel *et al*., 1997). Application of different sets of synthetic peptides, such as a set of five peptides derived from the amino acid sequence of a gp63-like protein (Fargeas *et al*., 1996), lead to lower sensitivity (71 %) but increased specificity (93 %) compared to the crude antigen ELISA (80 % and 79 %, respectively). Other sets of peptides used in other studies were the fucose-mannose ligand (FML) (Palatnik-de-Sousa *et al*., 1995) and a glycoprotein present on the promastigotes and amastigotes of *L. donovani.* Also the C-terminal region of the 70 kilodalton (kD) heat shock protein of *L. brasiliensis* (Amorim *et al*., 1996), a 28 amino acid sequence derived from the repetitive element of gene B protein (GBP) of *L.major* (Jensen *et al*., 1996). The rK39, a recombinant product consisting of the 39 amino acid repeat which is part of a 230-kD protein predominant in the *L. chagasi* amastigotes (Burns *et al*., 1993) was tryed as well. They all gave variable sensitivity and specificity numbers dependent on the *Leishmania* species

infecting the patients, but no cross reactivity and none gave a catholic sensitivity and specificity result for all different kinds of leishmaniasis.
A purified 200-kDa antigenic fraction from *L. donovani* axenic amastigotes was diagnostically evaluated by ELISA for the detection of antibody response in VL, PKDL and control patients. It seemed to have a potential prognostic significance and may be able to differentiate between VL and PKDL (Kaul *et al*., 2000).

The main problem with ELISA is, that like other serological tests it can not distinguish between current, clinical and past infection (Hommel *et al*., 1997).

2.5.2.4 **Western Blotting:** For western blotting, proteins are originally separated by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitro-cellulose or nylon membrane. If there are antibodies present in the patient sample, they react with the components of the membrane and the antibody-antigen complex can be detected as in ELISA.
Jaffe *et al*., (1984), using western blot and/or radioimmunoprecipitation analyses, recovered five distinct groups of indicabble *Leishmania* antigens comprising of 20 different molecules, and ranging from 18 to 84 kDa. All of them were recognised by the monoclonal antibodies D-2, D-10, and D-13 and from antibodies present in the sera of VL patients. Evans *et al*., (1989), found that the most frequent bands are at 116 kDa, 70 kDa, and 26 kDa; less frequent at 93 kDa, 74 kDa, 62 kDa, 46 kDa and 32 kDa. The patterns were very distinct for VL and could be used to differentiate patients with VL from those with Chagas' disease or CL.
De Colmenares *et al*. (1995) suggested a non-invasive method for VL diagnosis. He detected fractions of 72-75 kDa in the urine of 14 out of 15 VL patients and a fraction of 123 kDa in the urine of 10 of the 15 patients.
Mary *et al*. (1992) studied the sera of 37 VL patients, 11 of them HIV/VL patients. He found that antigens of 14 kDa were detectable in 92 % of the VL patients and of 16 kDa in 95 % of the sera. Same resulted from the HIV/VL patients; the 14 kDa band was not present in some samples while the 16 kDa one was constantly there.

Using immunoblot analysis in India in 35 kala-azar patients and 67 controls, the antibody response to five antigens (40kDa, 55 kDa, 65 kDa, 70 kDa and 82 kDa) of *L. donovani* was examined. The majority (83%) of kala-azar cases recognised at least four of these five parasite antigens (Salotra *et al*., 1999).

2.5.2.5 **Direct Agglutination test (DAT):** In this test the antigen preparation consists of whole organisms and a serological response (mainly IgG) recognises surface-born antigens of the parasite. DAT is a relatively fast and simple technique, with high sensitivity and specificity (Hommel *et al*., 1997; Meredith *et al*., 1995), thus one of the most widely used immunological tests that has been applied in diagnostic and epidemiological studies.
The main disadvantages of it are cross-reactivity (Hommel *et al*., 1997), the persistence of the antibodies after apparent cure (Hommel *et al*. 1997, Zijlstra *et al*., 1991), and the thermal instability of the aqueous antigen (Zijlstra *et al*., 1997).
An improved DAT based on stable, freeze-dried antigen, has been developed for use on canine serum samples (Dog-DAT) which showed a sensitivity of 100 % and specificity of 98.8 % (Oskam *et al*., 1996). DAT showed its value in large-scale sero-epidemiological surveys in eastern Sudan (Zijlstra *et al*., 1991), the Mymensingh district of Bangladesh (Chowdhury *et al*., 1993) and in the Himalayas (Rab *et al*., 1995).

DAT was used in 2 villages in Sudan, screening domestic animals (donkeys, cows, sheep, goats, camels and dogs in a search for the *L. donovani* transmission cycle. DAT detected reaction rates above the cut-off titres in donkeys (68.7%), cows (21.4%) and goats (8.5%), and in wild rats (5.5%) while ELISA showed reaction rates above the cut-off optical density in cows (47.6%), goats (13.6%), and in rats (4.1%) (Mukhtar *et al*., 2000)

2.5.3 Molecular diagnosis of leishmaniasis

2.5.3.1 **Polymerase chain reaction (PCR):** PCR is the amplification of a known specific nucleic acid sequence, using oligonucleotide primers (around 20-mers), which specifically bind to the DNA flanking the region of interest. The amplification is achieved by using a heat-stable DNA polymerase isolated from *Thermus aquaticus* (Saiki *et al*., 1988). The target DNA is denatured at 94°C and the double strands (ds) become single strands (ss). Then the primers are allowed to anneal at a temperature specific for each set (somewhere between 50-65°C). So after each amplification cycle, each double stranded DNA molecule gives rise to two ds copies of target DNA. PCR products are visualised after gel electrophoresis and Southern blotting.

A variety of clinical materials have been used for detection of *Leishmania*, like bone marrow, lymph node aspirates and peripheral blood, skin scrapings or sand flies. A variety of target sequences has been used as well, but maximum sensitivity has been achieved by using multi-copy sequences like ribosomal RNA genes, kinetoplast DNA, mini-exon-derived RNA genes and genomic repeats. The specificity of the PCR can be adapted to specific needs by targeting conserved or variable regions (Weiss, 1995).

In bone marrow taken from parasitologically confirmed VL patients, parasite DNA was amplified by PCR in all samples by Andresen *et al*. (1997), all by Mathis *et al*. (1995) and all samples by Osman *et al*. (1997). In a comparative study, Piarroux *et al.* (1994) showed that the sensitivity of PCR was 82 %, of microscopy 55 % and of culture 55 %; using bone marrow aspirates through all diagnostic approaches.

In human lymph node aspirates *Leishmania* DNA was detected in all 6 samples by Andresen *et al.* (1996) and in all 33 of 38 sample by Osman *et al*. (1997). In peripheral blood preserved in filter papers, from parasitologically confirmed VL patients, sensitivity was 63 % (Meredith *et al*. 1993), and 70 % (Osman *et al.* 1997). Andresen *et al*. (1996) used venous blood and the sensitivity was 92.5 %. Adhya *et al*., (1995) found that PCR was able to detect *Leishmania* DNA in 77 % of Indian VL patients at an early stage of the disease. Mathis *et al* (1995) demonstrated *Leishmania* DNA in leukocytes from the Ficoll-Plaque, in 64 % of blood samples from HIV/Leishmania co-infected patients, both in PCR and in culture.

In 60 % of skin biopsies (Mathis *et al*., 1995), from patients with CL, PCR detected parasite DNA, while in a comparative study by Andresen *et al.*, (1996), 86 % of the samples taken from CL patients were positive whereas microscopy detected only 55 % of the samples. In the cases of PKDL, Osman *et al.* (1998), using slit skit, demonstrated a 83 % sensitivity by PCR and only a 30 % sensitivity by microscopy).

In the case of epidemiological studies PCR has been used in a limited extent but with very impressive results though. El-Hassan *et al*. (1993), reported the presence of *Leishmania* in *Arvicanthis niloticus*, a vector of *L. donovani* in eastern Sudan, while Mathis *et al*. (1995), using canine samples, had a 100 % sensitivity in lymph node aspirates but 38.5 % in blood samples. Carreira *et al*., (1995) in Central America showed that PCR provided rapid diagnosis

with a sensitivity of 60 % and with a degree of concordance of 87 % to the different molecular techniques used.

2.5.3.2 Polymerase chain reaction solution hybridisation enzyme-linked assay (PCR-SHELA): In the case of *L. donovani* complex, a 60 bp repetitive degenerate sequence (Lmet2) was found (Howard *et al*., 1991a). Qiao *et al*., (1995) developed an assay which targets the this sequence combined with post-PCR hybridisation and colorimetric detection in microtiter plates coated with avidin. One of the primers was labelled with digoxigenin and a biotinylated probe was used for hybridisation that was binding to avidin and to the labelled primer. Substrate addition produced a colorimetric reaction and absorbency was measured at 405 nm after 1 h using an ELISA reader. The sensitivity of this PCR-SHELA was found to be very high, as few as 5 parasites could be reliably detected, and the assay appeared to be semi-quantitive. A main problem with this technique was that when PCR products were checked on an agarose gel, the resulting bands were very smeary, most possibly because a tandemly repetitive sequence was used (Kokozidou, 1996).

2.6 Species and strain identification

2.6.1 **Enzymatic identification (isoenzyme analysis):** Isoenzymes are variant enzymes with identical functions. Each of them in sample may appear as one of several alternative bands after electrophoretical separation. A pattern is defined as a unique arrangement of one or more bands displayed by one or more enzymes. The series of bands (one per enzyme), produced by a given isolate is its enzyme profile. Groups of isolates having the same isoenzyme profile are called zymodemes (Rioux *et al.*, 1990).

Following the recommendations of an expert committee, each zymodeme is labelled with either London School of Hygiene and Tropical Medicine or University of Montpellier designation, e.g. LON-1 or MON-1 (Piarroux *et al*., 1994).

Isoenzyme analysis is time consuming and tedious (Andresen *et al*., 1996), requires prior cultivation of the parasites and is performed in specialised laboratories only today. The main drawback of isoenzyme analysis is that only those mutations in proteins are detected that lead to an altered charge of the molecule. Furthermore, selection may act on the enzymes used (Taylor *et al*., 1999)

2.6.2 **Southern blotting using DNA probes:** Van Eys *et al*., (1989) developed the method first, using two recombinant probes pDK10 and pDK20 derived from nuclear DNA. The pDK10 probe could differentiate the Old World CL-causing species from the *L.donovani* complex and the pDK20 probe was able to distinguish between all Old World *Leishmania* species (Van Eys *et al*., 1991).

Most researchers that moved that direction targeted mainly kinetoplast DNA (kDNA), because it molecules exist in 10 000 copies and have variable regions that differ amongst minicircle classes in the same network (Simpson, 1987; Barker, 1989). Smith *et al*., (1989) developed kDNA minicircle sequence probes for *L. major*, but they cross reacted with *L. infantum*. Gramiccia *et al.*, (1992) developed a probe from a minicircle fragment from a dermatotropic *L. infantum* strain which showed only week hybridisation to *L. donovani* and *L. chagasi* but equally detected dermatotropic and viscerotropic strains. An *L. aethiopica* specific sequence was reported by Laskay *et al*., (1991).

Lee *et al*., (1995) found an A+T rich repeat DNA sequence from the divergent region of the maxicircle DNA of *L. amazonensis*, which was conserved only to these species which were infective to man and not to the non-infective ones. Howard *et al*., (1991) recovered the Lmet2 sequence, a 60 bp repetitive degenerate sequence from *L. donovani*, that specifically hybridise only to the *L. donovani* complex.

2.6.3 Use of species specific primers in PCR: The PCR can be adapted to specific needs by targeting conserved or variable regions and thus it is possible to characterise the parasite present in the specimen to the genus complex or species level.

Primers were designed to amplify a *L. donovani* minicircle sequence. Cross-reactions were present with other *Leishmania* spp. (Smyth *et al*., 1992). Because of the great variability within the kDNA the use of probes is limited to the geographical region from which the isolate comes from (Meredith *et al*., 1993). Bhattacharyya *et al*., (1993), were able to detect CL species using primers derived from the conserved and variable regions of a kDNA minicircles.

Ramos *et al*., (1996) could distinguish the *L. brasiliensis*, *L. donovani* and *L. mexicana* complexes, the *L. major* and *L. aethiopica* species, and couldn't distinguish between *L. tropica* and *L. aethiopica* species. They used primer pairs generating mini-exon intergenic regions and repeats.

The internal transcribed spacer (ITS), located between the small subunit rRNA and the large subunit rRNA genes and include the 5.8S rRNA gene region, was amplified by Schönian *et al*., (2000). Consistent size variations were observed among various *Leishmania* species (Schönian, personal communication, unpublished results).

2.6.4 Restriction fragment length polymorphism (RFLP) of an amplified region: Restriction enzyme digestion of PCR products allowed further differentiation. Van Eys *et al*., (1992) could distinguish *L. donovani* and *L. brasiliensis* from the other species by their characteristic restriction pattern after digesting the PCR product (small subunit ribosomal RNA genes) with restriction enzymes such as *Rsal* and *Hhal*. Cupolillo *et al*., (1995), digested the ITS region with 10 different restriction enzymes, demonstrating a variety of intra- and inter- specific variations, in a number of New World *Leishmania* isolates. Espinoza *et al*., (1995), amplified the Gp63 region in 58 *L. peruviana* isolates and exposed the amplicons

EcoRI and *SalI*, demonstrating 19 and 16 distinct RFLP patterns respectively. Also digestion of the ITS region was used by Schönian G *et al*., (2000), in accordance to a PCR with single primers fingerprinting technique, to reveal genetic heterogeneity among 10 *L. aethiopica* isolates examined. In principle this method can be used for direct detection of *Leishmania* in clinical samples (without cultivation, for the identification of Old World and New World species of *Leishmania* (RFLP) but also for strain typing (RFLP and/or SSCP).

2.6.5 AP-PCR (Arbitrarily Primed PCR) and RAPDs (random amplified polymorphic DNAs) as fingerprinting methods.

Three PCR- based methods for DNA fingerprinting were developed in the early 1990s. Arbitrarily primed PCR (AP-PCR) by Welsh and McClelland (1990), random amplified polymorphic DNA (RAPD) assay by Williams *et al*., (1990) and DNA amplification fingerprinting (DAF) by Caetano-Anollés *et al.*, (1991). The common strategy that underlies them all is that they are all based on the use of arbitrary primers that perform the PCR amplification of random genomic DNA fragments. Each primer or combination of them generates a characteristic pattern of amplification products, which is visualised by either radionuclide incorporation, ethidium bromide or silver staining. Polymorphisms between individuals or strains are detected as differences between the patterns of DNA fragments from different DNAs using a given primer or set of primers. This strategy provides a number of advantages over the classic DNA fingerprinting through RFLP because it permits easy and rapid generation of polymorphic markers since it uses very small amounts of DNA and does not require any knowledge of target DNA sequence.

The above three methods basically differ from one another in the length of primers used, amplification conditions, separation, visualisation of amplified DNA fragments and the fingerprinting patterns produced. The latter are varying from quite simple (RAPD) to highly complex (DAF).

RAPD-PCR amplifies genomic DNA using short primers (9-10 bases) of amplicons separation takes place on agarose gels and detection is performed after ethidium bromide staining. It is an easier, faster and less expensive method compared with AP-PCR and DAF (agarose gels vs. polyacrylamide gels and ethidium bromide staining vs. radionuclide incorporation or silver staining). It allows the detection of polymorphisms in closely related organisms and therefore, provides a powerful tool for gene mapping, marker-assisted selection in breeding programmes,

population and pedigree analysis, phylogenetic studies and individual and strain identification. Polymorphisms between individuals or strains result from sequence differences which inhibit primer binding or otherwise interfere with amplification and can be simply detected as DNA fragments that are amplified from the individual or strain but not from another. Two basic criteria must be met when RAPD primers are chosen: a minimum of 40 % G+C content (50-80 % G+C content is generally used) and the absence of palindromic sequences (Williams *et al*., 1993, 1990).

Noyes *et al*., in 1996, evaluated 28 different RAPD primers. 13 of them yielded patterns of taxonomic value when DNA was amplified from 4 different, closely related *Leishmania* of the *Viannia* group. When kDNA was amplified with RAPD primers it was possible to differentiate the cutaneous species and when genomic DNA was used as template different *L. donovani* isolates could be distinguished (Bhattacharyya *et al*., 1993). Pogue *et al*., in 1995 used successfully arbitrary primed AP-PCR to identify intra- and interspecific *Leishmania* genetic polymorphisms and polymorphic DNA to identify genetic polymorphisms between species and isolates. A combination of kDNA-PCR fingerprinting and hybridisation with kDNA probes, was found to be useful for both sensitive detection and direct identification of *Leishmania* species (Breniere *et al*., 1999).

Based upon the same principals a system for the identification and determination of the relationships of species and strains within the genus *Leishmania* was developed using single primers in the polymerase chain reaction (Schönian *et al*., 1996). They demonstrated that species-specific PCR profiles were obtained by amplifying the genomic DNA of the Old World and New World *Leishmania* species with different single primers. The PCR profiles produced provided a simple way of identification of *Leishmania* isolates at species level and the information from these amplification patterns was used to construct phylogenetic trees and to measure the genetic relationship of *Leishmania* species. The primers that were used were annealing to mini- and microsatellite DNA sequences (M13 core sequence), simple repeat sequences (GTG_5 and $GACA_4$), or derived from an intergenic spacer for tRNA genes (T3B). With these primers stringent conditions (high annealing temperatures) could be used for PCR which significantly improved the reproducibility of the technique (in contrast to RAPD where very low annealing temperatures can be used with the short primers).

2.6.6 Single strand conformation polymorphism (SSCP) analysis.

SSCP analysis represents a comparatively new technique (Orita *et al*., 1989), which is easily implemented and generates useful markers. SSCP analysis is based upon the principle that electrophoretic mobility of a single-strand DNA molecule in a non-denaturing gel is dependent upon both its size and its shape. A number of stable shapes and conformations are formed when secondary base pairing occurs among nucleotides on a single DNA strand (ssDNA). The length, location and number of intra-strand base pairs determine secondary and tertiary structure of a conformation. Point mutations that affect intra-strand interactions may therefore change the shape of a molecule and alter its mobility during electrophoresis.

In principle, the mobility of a denatured single-strand DNA molecule should be sensitive to point mutations. Orita *et al*., (1989) developed SSCP analysis to test this principle and showed that intra-strand interactions are highly sensitive to the primary sequence of the molecule. Later on, Hayashi (1991), proved in an extensive study that SSCP detects 99% of point mutations in DNA molecules 100-300 bp in length and 89% of mutations in molecules of 300-450 bp in length.

SSCP analysis is methodologically and technically simple. Double-stranded DNA (dsDNA) molecules (e.g. PCR product) are denatured to single strands with heat and then plunged into ice-water (4°C) to promote the formation of intra-strand complexes while reducing the renaturation of the complementary strands. Those products are electrophoresed on polyacrylamide gels at 4°C, in cool room, to ensure that intra-strand conformations are not disturbed. The gel is then silver stained to detect the mobility of the different DNA conformations. Others alternatively have labelled one strand and visualised its conformations with autoradiography or enzyme detection. Different researchers used different sizes of gels, which all allowed to visualise band shifts (Hiss *et al*., 1994; Axton *et al*., 1998).

PCR-SSCP has been successfully used to recognise medically important opportunistic fungi (Walsh *et al*., 1995), to search genetic divergence among species within the parasite *Fasciola hepatica* (Itagaki *et al*., 1995), to recognise which parasite population of *Plasmodium vivax* causes relapsed infections (Craig *et al*., 1996), to detect mutations in bilharziasis-associated bladder cancer (Tamimi *et al*., 1996), to search genetic variations of the midkine (MK) gene in human sporadic colorectal and gastric cancers (Ahmed *et al*., 2000) and to analyse mutation and expression of the p27KIP1 and p57KIP2 genes in human gastric cancer (Shin *et al*., 2000).

SSCP has been widely used in biomedicine but so far has not been exploited extensively in Parasitology.

2.7 Leishmaniases in the Mediterranean

The Mediterranean basin is what geographically stands between three very different continents, very different to their natural and geographical status. There is an interactive relationship between them and they also individually receive influences from the further part of each continent that stands behind each coast. The *Leishmania* species that mainly exist there are *L. infantum*, *L. major* and *L. tropica*. *L. infantum* is responsible for the VL, is distributed throughout the Mediterranean and infects humans and dogs. *L. tropica* is restricted to Middle East, Tunisia and parts of Southern Greece and is responsible for anthroponotic CL. *L. major* causes rural VL and is widespread throughout North Africa and Middle East. Some countries like Tunisia and Israel are suffering from leishmaniases caused by all three species (Gradoni *et al*., 1984).

Leishmaniases are common in the Mediterranean basin, known from ancient years. The first reports of kala-azar, called "Ponos" (pain), are coming from the island of Spetses, Greece (Karamitsas, 1879; Yiannacopoulos, 1879). In Greece there are two forms of the disease: visceral (VL) and cutaneous (CL) leishmaniasis. VL is present in all Greece, continental and insular, and is caused by *Leishmania infantum*. It is a zoonosis with the domestic dog as its reservoir (Garifallou *et al*., 1989; Léger *et al*., 1988). CL is endemic in the Ionian Islands, Crete, Southern Peloponnese and Central Greece, is caused by *L. tropica* and is anthropoonotic without any known animal reservoir host (Igoumenakis, 1930; Malamos, 1947; Nicolis e*t al*., 1978; Garifallou *et al*., 1984). Leishmaniasis has been reported to 5-22.5 % of the dogs in the districts of Crete, Athens and the island of Hydra (Desjeux, 1991). In the decade of '40 the reports were of about 160 human cases per year. In the '50 about 32 per year, in the '60 about 60 cases per year, during 1979-1981 a total of 153 cases, in the '80 about 50 cases per year and during 1990-1992 89 cases of VL (Desjeux, 1991; WHO 1993) were reported.

There is very little information from the Balkan countries of Albania, ex-republics of Yugoslavia and Bulgaria. A report exists about a 25-year-old woman of Yugoslavian origin, that was diagnosed for CL after a long incubation period of two years (Matzdorff *et al*., 1997), and an other one of a 52 year old janitor from Graz, that was diagnosed for VL after a two week vacation in Croatia (Wenzl *et al*., 1992). There is also a case of VL reported for a girl from Albania that was diagnosed in Florence (Calabri *et al*., 1997). A case from Bulgaria is of a 24-year old German man suffering from painful ulcers, that were mimicking a mycotic infection. *Leishmania major* could be detected by PCR investigation (Linss *et al*., 1998). The above mentioned Balkan countries have a lack of medical data not because the disease is not endemic there but because of political and geographical changes along with the low level of the health system make it impossible to have any kind of epidemiological data at the moment.

Concerning the middle East countries of Turkey, Syria, Lebanon and Israel it is most definite that they are strokes from the disease, in different clinical forms. In western Turkey, district of Manisa from June 1993 to August 1997, 37 human VL cases were reported and 5.3% (26/490) of the dogs, both from *L. infantum* (Ozbel Y *et al*., 2000). Infantile Mediterranean VL (*L. infantum*) and anthropoonotic CL (*L. major*) have long been known to exist in the western and south-eastern Turkey, respectively (Ozensoy *et al*., 1998).
In Syria 1035 dogs were tested and there were 70 % positive when material from lymph nodes was cultured for *Leishmania infantum* (Dereure *et al*., 1998). Aleppo is a district endemic for *L. tropica* (Tayeh *et al*., 1997), while the oriental sore due to *L. major* is prevalent in the area of Damascus (Khiami *et al*., 1991).
In Lebanon a total of 81 000 people were examined and 0.18 % of the rural versus 0.41 % of the urban population was suffering from CL while VL was practically non-existent in either environment (Nuwayri-Salti *et al*., 2000a, Nuwayri-Salti *et al*., 2000b).
Reports from Israel, from an area between Tel-Aviv and Jerusalem, are discriminating *L. infantum* for VL of canines and humans, for the cases of five dogs and one child (Baneth *et al*., 2000). CL is mostly due to *L. major* and is mainly endemic in the Jordan and the Rift valleys, while CL due to *L. tropica* is much less common, almost sporadic (Klaus *et al*., 1994). Again very little is known about the Palestinian areas for the same reasons as with the balcan countries.

Egypt is a country that because of its geographical location on the crossroad of the Asian and the African continents supplies with reports that discriminate different *Leishmania* species.

Therefore CL was attributed to *L. major* (Morsy, 1996), VL to *L. infantum* (Morsy, 1997a), while the import of *L. tropica* from Saudi Arabia is being discussed (Mohareb *et al*., 1996). There is also an interesting case report on DCL (diffuse cutaneous leishmaniasis) not due to *L. aethiopica* as usual, but due to *L. major* (Morsy *et al*., 1997b).

CL is also reported from Libya, 151 cases from the endemic area Al-Badarna in Jabal Nafusa, during the time between October 1991 to September 1992 (El-Buni *et al.*, 1996).

There are recent reports from Tunisia on sporadic VL due to *L. infantum* MON-1 and MON-24 isolated from 24 patients (Aoun *et al*., 2000; Belhadj *et al*., 2000) which are the main responsible agents of the disease, but there are still some old foci of CL caused by *L. major* (Sassi *et al*., 1999). Some sporadic cases of CL in North Tunisia were identified as caused by *L. infantum*, by the use of a deoxyribonucleic acid probe (Ben-Ismail *et al*., 1992).

L. infantum MON-1 and MON-24 have been reported from Algeria, being responsible for canine leishmaniasis (Marty *et al*., 1998). Harrat *et al*., (1996) note that the human cases of leishmaniasis are gradually rising since 1980, reaching the numbers of 1121 cases of VL per year and 2000 cases of CL per year. They also isolated *L. infantum,* strains (MON-1) and *L. major* (MON-25) from humans, other mammals and sandflies.

In the emerging epidemic focus of Taza, north Morocco, the isoenzyme characterisation revealed the presence of *L. tropica* (Bichichi *et al*., 1999). In the same area from October 1995 to November 1996, 132 human cases were reported due to *L. tropica* with peculiar clinical manifestations as impetiginized, ulcerocrusted and noduloulcerative forms that were predominant (61 %) (Chiheb *et al*., 1999). A seroprevalence study of canine leishmaniasis was carried out in five provinces in northern Morocco: Taounate, Al Hoceima, Zouagha Moulay Yacoub, Chefchaouen and Ouezzane. A total of 1 013 dogs were screened with IFAT and 87 showed antibody titre > or = 100, 83 of them asymptomatic and 4 symptomatic (Nejjar *et al*., 1998). The organism responsible was *L. infantum* (MON-1).

Going back to the European continent, Spain, Portugal and France are countries presenting more or less a similar picture. Both human and canine populations are suffering from *Leishmania infantum* while leishmaniasis is increasing in immunocompetent and AIDS patients.

A survey in red wild foxes in Guadalajara, Spain revealed prevalences of 74 % of leishmaniasis (Criado-Fornelio *et al*., 2000). On an other survey in the Balearic islands (Mallorca) out of a total of 112 dogs, 77 % presented immune responses against *Leishmania*, either humoral or cellular (Solano-Gallego *et al*., 2000). AIDS patients (Reus *et al*., 1999) and

intra venous drug users are suggested as new groups in Spain at high risk (Chicharro *et al*, 1999).

In France the co-infection rates of HIV-*Leishmania*, rises gradually (Rosenthal E *et al*., 2000), and cutaneous infantile leishmaniasis is no more an unusual phenomena (del Giudice *et al*., 1998). In Southern France *Leishmania* infections were reported in asymptomatic blood donors, 76 individuals out of 565 were found seropositive and 9 of these were positive when parasite minicircle kinetoplast DNA was amplified via PCR (Le Fichoux *et al*., 1999). Even a unique case of disseminated feline leishmaniasis has been reported from France (Ozon et al., 1998). In an interesting survey from 1992 (Marty *et al*.,) in an endemic focus of canine leishmaniasis in Alpes-Maritimes, a highly endemic focus of canine leishmaniasis (17 % of dogs seropositive in 1985), a total of 237 humans tested for reaction against leishmanin. A total of 30 % rected positively. A higher proportion of positive skin tests was obtained in the 61-70 years age group (62%) than in the younger groups. The proportion of positive tests also increased with the duration of living in this locality (22% for less than 5 years; 66% for more than 21 years). These data confirm the continuing occurrence of transmission of leishmaniasis to human beings in this focus.

In Portugal leishmaniasis is a zoonosis in most regions where it occurs, with dogs as the main reservoirs of the disease, usually suffering from a viscerocutaneous, chronic infection (Abranches *et al*., 1998). From Portugal comes also a recent report of a congenital infection of a child from its asymptomatic mother (Meinecke *et al*., 1999). Pratlong *et al*., (1995), characterised 100 *Leishmania* isolates by isoenzyme analysis. Many of them were of the MON-1 zymodeme, while they all were of *L. infantum.* Even the dermotropic strains had caused VL to the AIDS patients.

In Italy an epidemiological survey was carried out on the distribution of canine leishmaniasis in Western Liguria (Northern Italy). Blood sera collected at different times from dogs were subjected to IFAT and ranges of 22.1-30.3 % were positive (Zaffaroni *et al*., 1999). The disease extends to the whole length of the country to the South (Ciaramella *et al*., 1997). Also the infection amongst the HIV patients increases (Gradoni *et al*., 1996).

Interestingly in a survey that was done in Germany within January 1993 and September 1995, revealed that a total of 132 dogs diseased from leishmaniasis. Of those 35 had travelled to the endemic areas and 97 were brought to Germany by those endemic countries (Spain, France, Portugal, Italy and Turkey) (Gothe *et al*., 1997). Bogdan (2001) refers to the case of a German 15 month old child that suffered from leishmaniasis whithout ever entering an endemic area.

Meanwhile cutaneous leishmaniasis caused by *L. infantum* in a horse was reported from South Germany (Koehler et al., 2002).

Reviewing the above the *Leishmania* species that mainly exist in the Mediterranean basin are *L. infantum*, *L. major* and *L. tropica*. *L. infantum* is responsible for the VL, is distributed throughout the area and infects humans and dogs. *L. tropica* is restricted to Middle East, Tunisia and parts of Southern Greece and is responsible for anthroponotic CL. *L. major* causes rural VL and is widespread throughout North Africa and Middle East. Some countries like Tunisia and Israel are suffering from leishmaniases caused by all three species (Gradoni *et al*., 1984).

3. Materials and methods

3.1 Parasites

The parasites that are isolated from tissue material of a patient (human or canine) after growing in grouth medium, are called an isolate. When isolates are further characterised (based on molecular or isoenzyme analysis) and certain properties of them are defined are called strains. Therefore many different isolates after characterisation may be revealed to belong to the same strain or not (WHO, 1990).

A total of 90 isolates and *Leishmania* samples were used in this study.

36 DNA samples derived from isolates of human cases and were well characterised by isoenzyme analysis (Table 2). They were obtained from the Institute for Microbiology and Hygiene, Charité Hospital, Humboldt University, Berlin, from the Royal Tropical Institute Amsterdam and from the Kuvin Centre for the Study of Infectious and Tropical Diseases, Hadassah Medical School, Hebrew University Jerusalem.

Another 30 *Leishmania* isolates derived from the collection of the Institute for Medical Parasitology, Bonn, Germany. 21 were of canine origin, obtained from a previous sample collection project between the Institute for Medical Parasitology, Bonn, Germany and the Veterinary Faculty, Aristotelian University, Thessaloniki, Greece. They were isolated from dogs in Northern Greece but no clinical history was available.

Another 9 isolates were obtained from human patients who visited the Institute for Medican Parasitology in Bonn within the last 10 years. The patients had previously travelled to Ethiopia, Iran, India, Sudan, Brazil, Mexico and Spain. Inially 16 isolates were available but only the above mentioned 9 of them were successfully recovered in cultured since most of them were kept in liquid nitrogene for long time without intermediate passage.

Table 2. Characterised human *Leishmania* strains used in this study.

Isolate Code	**WHO Code (based on isoenzyme analysis)**	**Taxon**	**Origin**	**Patient's clinical picture**
INF-01	MHOM/TN/80/IPT1[a]	infantum	Tunisia	VL
INF-02	MHOM/FR/62/LRC-L47	*infantum*	France	VL
INF-03	MHOM/ES/87/Lombardi	*infantum*	Spain	CL
INF-04	MHOM/CN/78/D2	*infantum*	China	VL
INF-05	MHOM/FR/80/189	*infantum*	France	VL
INF-06	MHOM/FR/80/189	*infantum*	France	VL
INF-07	MHOM/IL/89/LRC-L571	*infantum*	Israel	NK
INF-08	Human patient isolate, identified at the Charitè.	*infantum*	possibly Turkey	NK
INF-09	MCAN/PT/94/IMF193	*infantum*	Portugal	NK
INF-10	MCAN/TR/96/EP16	*infantum*	Turkey	NK
INF-11	MHOM/TR/94/EP3	*infantum*	Turkey	NK
INF-12	MCAN/IL/94/Robi	*infantum*	Israel	NK
INF-13	MCAN/IL/96/Skidro	*infantum*	Israel	NK
INF-14	MCAN/IL/96/LRC-L695	*infantum*	Israel	NK
INF-15	MCAN/IL/96/LRC-L709	*infantum*	Israel	NK
INF-16	MCAN/IL/97/LRC-L716	*infantum*	Israel	NK

INF-17	MCAN/IL/97/LRC-L717	*infantum*	Israel	NK
INF-18	MCAN/IL/97/LRC-L718	*infantum*	Israel	NK
INF-19	MCAN/ES/??/Whiskey	*infantum*	Spain	NK
CHA-01	MHOM/BR/74/PP75 [a]	*chagashi*	Brazil	NK
DON-01	MHOM/IN/80/DD8[a]	*donovani*	India	NK
DON-02	MHOM/KE/83/NLB189	*donovani*	Kenya	PK
DON-04	MHOM/KE/85/NLB323	*donovani*	Kenya	NK
DON-06	MHOM/SD/75/LV139	*donovani*	Sudan	CL
DON-08	MHOM/SD/68/1S	*donovani*	Sudan	VL
DON-10	MHOM/IN/71/LRC-L51[a]	*donovani*	India	VL
AET-03	MHOM/ET/94/Abauye	*aethiopica*	Ethiopia	DCL
TRO-02	MHOM/SU/74/SAF-K27 [a]	*tropica*	Sudan	CL
TRO-22	MHOM/TR/95/URFA7	*tropica*	Turkey	CL
MAJ-01	MHOM/SU/73/5ASKH [a]	*major*	USSR	CL
MAJ-03	MHOM/SD/90/Sudan3	*major*	Sudan	NK
MEX-03	Isolate from a Dutch patients identified at the Charitè	*mexicana*	?	NK
AMA-01	MHOM/BZ/73/M2269 [a]	*amazonensis*	Belize	NC
BRA-01	MHOM/BR/75/M2903 [a]	*brasiliensis*	Brazil	NC
GUA-01	MHOM/BR/75/M4147 [a]	*guyanensis*	Brazil	NC
PAN-01	MHOM/CR/87/NEL3	*panamensis*	Costa Rica Comanne	NC

VL: visceral leishmaniasis; CL: Old World cutaneous leishmaniasis; NC: New World cutaneous leishmaniasis; MCL: mucocutaneous leishmaniasis; PK: Post-Kala Azar; NK: not known [a] :WHO reference strains; where nothing is filled in the patient's clinical picture means that there is no data about it.

All unidentified isolates were given a code for facilitation purposes. So the 21 canine isolates from northern Greece were coded as S(d)1 (sample from dog 1) from S(d)1 to S(d)21. The 9 human ones were coded from S(h)22 to S(h)30 (as sample from human).

In addition 24 DNA samples from German dogs were included. The dogs were infected with *Leishmania* while travelling in South Europe (mostly Italy and Spain). Dr S. Steuber, Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, kindly provided DNA samples that were extracted from infected bone marrow (21), lymph nodes (1) and blood (2) of these dogs. Those samples were coded as BS1-BS24 (BS: biological sample).

Epigrammatically, the total number of samples used for this study was 66 various *Leishmania* isolates of canine and human origin and 24 total DNA samples of *Leishmania* infected tissue material.

3.2 Parasite culture

The 21 dog isolates from Northern Greece and 16 human isolates from the collection of the Institute for Medical Parasitology in Bonn were cultured in RPMI-1640 medium (Sigma No. R 6504 with L-glutamine). 2.0 g/l of sodium bicarbonate or 26.7 ml/l of sodium bicarbonate solution (7.5% w/v) were added to the prepared medium. Finally, the medium was supplemented with 15% foetal calf serum (Seromed) and according to Howard *et al*., 1991b, 5% human urine from a volunteer and incubated at 26°C after inoculation with parasites.

For a rapid and reproducible growth in culture, a ratio of no more than 1 vol. of inoculum to 4 vol. of fresh medium was required. Most rapid growth in new medium was observed when sluggish or largely immotile promastigotes were innoculated.

The cultures were harvested at an approximate density of $2x10^6$ parasites/ml. They were washed twice in phosphate buffered saline (PBS) and processed for the DNA extraction. Parasites were successfully cultured from all 21 canine isolates but only from 9 of the 16 human cases (see above).

3.3 Parasite preservation

The parasites were mixed with medium (RPMI, 10 % FKS), sterile glycerine (Roth, 7530.1) and subjected to a programmable freezing uni, freezing at about 1°C/min to -70°C. The parasites were held in that temperature overnight and then taken to –70°C in liquid nitrogen, where they were kept permanently.

An alternative method that was used and worked very well was keeping the parasites with the mixed glycerine at 4°C for 2-6 h, then transferring them to –20 or –30 °C for 24 h subsequently to –80°C for 36 h and finally to liquid nitrogen. To recover the organisms prior to transfer into fresh medium, the cryotube was plunged into a water bath at 25°C until the parasites were thawed.

3.4 DNA extraction (according to Schönian *et al*., 1996)

The parasites (300 µl) were resuspended in lysis buffer (50mM NaCl, 10mM EDTA, 50mM Tris-HCl, pH 7.4). Sodium dodecylsulphate was added to a final concentration of 0.5 % w/v and the solution was shaked well until it became viscous. Proteinase K and ribonuclease were added to final concentrations of 100 µg/ml each and the mixture was then incubated overnight at 60°C.

The DNA was extracted using an equal volume of buffered phenol, followed by an extraction with phenol-chlorophorm-isoamyl alcohol mix (v/v 25:24:1). One volume of chlorophorm-isoamyl alcohol was added, and the mixture was vortexed and centrifuged at 3000 rpm

(Heraeus Sepatech Biofuge 13R) for 10 min. Adding 1/10 of the volume of 3 M sodium acetate, pH 5.2, and one volume of isopropanol precipitated the DNA.

The mixture was left for at least 60 min at 4°C and centrifuged at 8000 rpm for 15 min. The supernatant was rejected and an equal volume of 70 % ethanol was added. The mixture was again centrifuged and the supernatant was discarded. The resulting DNA was dried in a vacuum dessicator and resuspended in 200-500 µl TE buffer [10mM Tris (pH 7.5), 1mM EDTA (pH 7.2)].

Extraction efficiencies and qualities were checked on 1 % agarose gel and DNA concentration and purity were estimated by measuring the optical densities at 260 and 280 nm.

3.5 Diagnosis and differentiation

The following techniques were employed for diagnosis and differentiation of the different isolates and samples.

3.5.1 Diagnosis of visceral leishmaniasis using PCR

In the current study a PCR technique was employed which was originally developed by Meredith *et al.* (1993) for amplification of *Leishmania* DNA directly in biological materials. A 560 bp variable fragment of the ssu rRNA gene of *Leishmania* spp. is amplified in this assay. The protocol used in this study is based on Omran et al. (1997, 1998) and has been applied after personal communication with O. F. Omran.

Five microliters of isolated DNA were added to 45 µl of a PCR mixture containing 20 mM Tris-HCl; 50 mM KCl; 4mM $MgCl_2$; 250 mM of each deoxynucleoside triphosphate; 0.5 U of Taq polymerase and 100 pmol of both primers 174 (5'-GGTTCCTTTCCTGATTTACG-3'), and 798 (5'GGCCGGTAAAGGCCGAATAG-3'). Samples were initially denatured at 94° C for 10 min and 38 cycles followed consisting of denaturation at 94° C for 75 sec, annealing at

60° C for 1 min, and extension at 72° C for 2 min. A final extension in 72° C for 3 min followed.

Amplification reactions were visualised on a 2 % agarose gel, and a 100 bp DNA ladder was used as molecular weight standard. Samples were scored positive when the PCR product of 560 bp could be detected.

The 24 total DNA samples (BS1-BS24) of *Leishmania* infected tissue material provided by Dr. S. Steuber (Bundesinstitut für Gesundheitlichen Verbraucherschutz und Vterinärmedizin, Berlin), were processed through that PCR.

3.5.2. Amplification and restriction enzyme digestion of the ITS region

The following specific oligonucleotide primers were used to amplify the Internal Transcribed Spacer (ITS) region which is lying between the small subunit rRNA and the large subunit rRNA genes and includes the 5.8S RNA gene (Innis *et al*., 1990; Schönian *et al*., 2000): LITSR (5'-GTG GAT CAT TTT CCG ATG) and LITSV (5'-ACA CTC AGG TCT GTA AAC).

Amplification reactions were performed in volumes of 50 µl containing 20 ng template DNA, 1.5 mM Mg^{++}; 20 mM Tris/HCl pH 8 and 50 mM KCl_2, 200 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia Biotech), 25 pmol of each primer and 2U *Taq* DNA polymerase (Gibco BRL).

Samples were overlaid with sterile, light mineral oil and amplified as follows: initial denaturation, 2 min at 95°C; denaturation, 20 sec at 95°C; annealing, 60 sec at 51°C; and extension, 60 sec at 72°C; 35 cycles were run. This was followed by a final extension cycle of 6 min at 72°C. PCR products were checked on a 1 % agarose gel and kept at 4°C until processed.

Restriction fragment length polymorphism (RFLP) patterns were produced when the PCR products were digested with restriction enzymes *Hae*III and *Cfo*I. Each reaction was loaded with 17µl PCR product, 10 U *Hae*III (Boehringer Mannheim) or 10 U *Cfo*I (Boehringer Mannheim) and 2 µl 10x Buffer Blue or 10x SuRE/Cut buffer L (Boehringer Mannheim) respectively. The mixtures were vortexed and incubated for 2 h at 37°C. Then they were

stored at 4°C until run in 1 % agarose gels for 2 h at 3 V/cm in 0.5 x TBE buffer (0.045 M Tris-borate, 1 μM EDTA). Digestion products were detected after staining the gels with ethidium bromide.

The 30 *Leishmania* [S(d)1-S(d)21 and S(h)1-S(h)9] isolates from the collection of the Institute for Medical Parasitology, Bonn, Germany were processed through the above. Isolates from the 36 well characterised isolates were used as standard controls.

3.5.3 PCR fingerprinting with single arbitrary primers

The following oligonucleotides were used as single primers in the PCR experiments: the simple repeat sequences $(GTG)_5$-(5'-GTG GTG GTG GTG GTG), $(GACA)_4$-(5'-GAC AGA CAG ACA GAC A) (Ali *et al*., 1986) the core sequence of phage M13 (5'-GAG GGT GGC GGT TCT) (Huey *et al*., 1989), and the T3B oligonucleotide which was derived from intergenic tRNA genes' spacers (5'-AGG TCG CGG GTT CGA ATC C) (McClelland *et al*., 1992.

Amplification reactions were performed in volumes of 50μl containing: 10-50 ng template DNA, 20mM Tris/HCl, pH 8.0, 50 mM KCl_2, 4.5 mM Mg^{++}, 200 mM each of dATP, dCTP, dGTP and dTTP and 1.5U *Taq* DNA polymerase. The primers $(GACA)_4$ and M13 core were added at a final concentration of 25 pmol per assay and the $(GTG)_5$ and T3B were added at concentrations of 10 and 5 pmol per assay, respectively.

Samples were overlaid with sterile, light mineral oil and amplified as follows: initial denaturation, 2min at 95°C; denaturation, 20 s at 95°C; annealing, 30 s at 50°C for the $(GTG)_5$ primer, 60 s at 50°C for the $(GACA)_4$ primer, 60 sec at 50°C for the M13 core primer, 32 s at 52°C for the T3B primer; extension, 80 s at 72°C for the $(GTG)_5$ and T3B primers and 20 sec at 72°C for the $(GACA)_4$ and M13 core primers; a total of 32 cycles was run for the $(GTG)_4$ and T3B primers whilst 35and 27 cycles were run for the $(GACA)_4$ and M13 core the primers, respectively. A final extension for 6 min at 72°C followed and the reaction tubes were held at 4°C prior to analysis.

The samples were concentrated in a Speed Vac to an approximate volume of 20 µl and subjected to electrophoresis in 1.2 % agarose gels for 5 h at 3 V/cm in 0.5 x TBE buffer. The amplification products were visualised under UV light after staining the gels with ethidium bromide.

The 30 *Leishmania* isolates [S(d)1-S(d)21 and S(h)1-S(h)9] from the collection of the Institute for Medical Parasitology, Bonn, Germany were processed through the above. Isolates from the 36 well characterised isolates were used as standard controls.

3.5.3.1 Computer-assisted data analysis

After staining the gels were photographed, and the DNA fragments were sized and compared with the use of scanner-associated computer hardware and software (RFLPscan, version 2.01, Scanalytics CSP Inc., Bilerica, MA, USA). The similarity indices representing the ratio of shared bands over total bands within two lanes being compared during the matching operation were estimated for the different *Leishmania* species tested as well as for isolates belonging to the same species. Distance matrices based on N (N - 1)/2 pairwise comparisons between N data sets were calculated and evolutionary trees were constructed by UPGMA (unweighted pair group method using arithmetic averages) which is a cluster based analysis (Sneath *et al*., 1973; Saitou *et al*., 1987) using the Treecon programme (Van de Peer *et al*., 1993). The genetic distance (Gdxy) was computed according to Nei *et al*., 1979, as following:

$$Gdxy = 1 - \frac{2Nxy}{Nx+Ny}$$

Nxy stands for the number of bands shared in lines x and y, Nx for the number of fragments in line x and Ny for the number of fragments in line y.

Bootstrap analysis was also used to place confidence intervals on phylogenies. This is a kind of statistical analysis to test the reliability of certain branches in the evolutionary tree (Efron *et al*., 1983; Felsenstein, 1985; Swofford *et al*., 1996). It involves resampling one's own data, with replacement, to create a series of bootstrap samples of the same size as the

original data. Bootstrap values at a branching point denote the number of bootstrap trees comprising a cluster of the same composition. In our case a sufficient number of 100 replicas performed bootstrap analysis and bootstrap values of 75 minimum were concidered well supported for the respective branch.

3.6 PCR-SSCP

SSCP was used to screen for polymorphisms in ITS1 products as well as in anonymous PCR markers.

All PCR reactions were first optimized using the DNA from the WHO reference strains and from some of the *L. infantum* and *L. donovani* strains identified earlier by Schönian *et al*., (1996). Afterwards a total of 45 samples were processed through it. The PCR products were going through the SSCP at the Hoefer SE400 system vertical electrophoresis chamber system (Hiss *et al*., 1994) in a cold room. When the electrophoresis was finished the gels were silver stained and fixed between sheets of foil.

3.6.1 PCR of the ITS1 region

The ITS1 region is lying between the small subunit rRNA and the 5.8S RNA genes. For the amplification of this region the primer pairs LITSR (5´- CAG GAT CAT TTT CCG ATG) and L5.8S (5'- TGA TAC CAC TTA TCG CAC TT) (Schönian, personal communication) were used. Each 50 µl assay contained 20 ng DNA, 25 pmol of each primer, 200 µMol of each dNTP, 1.5 Mol Mg^{++} and 2 U Taq DNA polymerase. A total of 32 cycles followed; denaturation for 20 sec at 95°C, annealing for 30 s at 53°C and extension at 72°C for 60 s. The initial denaturation was at 95°C for 2 min and the final extension at 72°C for 6 min. The product was kept at 4°C until run at the SSCP.

3.6.2 PCR reactions of the codominant markers

Schönian and co-workers at the Institute for Microbiology and Hygiene, Charité Hospital, Humboldt University, Berlin, Germany, have designed codominant PCR markers able to detect both alleles in a diploid organism, able to dectect intraspecies variations in *Leishmania* spp. from randomly amplified monomorphic DNA (Schönian *et al*., 2000). For the current experiments 9 primer pairs annealing to anonymous DNA sequences of the *L. donovani* complex (*L. donovani*, *L. infantum*, and *L. chagasi*), were used.
The primer pairs that were used for the reactions are listed in Table 3.

Table 3: Primer sequences used for the amplification of codominant anonymous markers for the *L. donovani* complex.

Marker	F: 5'-3' forward primer	R: 5'3' reverse primer	Expected product (bp)
L1012	ACA CAC AGG CAT GTG GGT ACG	TAC ACT CCG TTT GGT TTC CG	200
L0720	CAA CGT ATG CGG TTT GTC TC	GAG TGC GCA TCT ACT AAT CG	300
L0510	ATA GGT TAA CGG CAA CGC AC	TGA CAG AGA CAC ACA ACG AC	250
L0114	CTA CCA AGA AGG GTG GCA AG	GGT GCA GTA CTC GTA CCT AC	200
L1112	TGC CGA GAG GAG GGA AAG	GAT ATG CAC ACG CAC AAA GC	350
L0413	CTC ACG CTT TGT GCT TGT GT	CAA CAA GGC GTA TTT CCA CG	300
L0110	GGC AAA GAA AAA GAG CaG CG	CCT GTC GTG CGT TGA ATA TC	550
L1119	CCT CTA TTC CAC ATA TTT CT	AAT CAG CAA GGA CAC CA	400

Amplification reactions were performed in volumes of 50 µl containing 20 mM Tris-HCl, 50 mM KCl, 1U *Taq* DNA polymerase, 50-200 mM each of dATP, dCTP, dGTP and dTTP, 1-1.5mM Mg^{++}, 10-60 pmol/reaction of each primer and 6-20 ng template DNA (details listed in Table 4).

Table 4: Amplification reactions with the codominant markers for *L. donovani* complex (technical data).

Primers	dNTP's (mM of each)	Mg^{++} (mM)	Primers (pmol of each)	DNA (ng)	Annealing temperatures	No of cycles
L1012	150	1.5	30	15	50	34
L0720	200	4	30	20	52	34
L0510	75	1.5	30	10	54	34
L0114	200	1.5	60	8	51	34
L1112	50	1.5	15	6	55	32
L0413	50	1	10	6	54	30
L0110	50	1.2	13	6	51	32
L1119	100	3	50	10	47	34

Samples were overlaid with sterile, light mineral oil and amplified as follows: initial denaturation, 3 min at 95°C; denaturation, 1 min at 94°C; annealing, 50-55°C for 30 s; extension, 1 min at 72°C; a total of 32-34 cycles were run. A final extension for 6 min at 72°C followed and the reaction tubes were held at 4°C prior to analysis (details in the table 4).

Amplification products were subjected to electrophoresis in 1.5 % agarose gels for 2 h at 5 V/cm in 0.5 x TBE buffer and visualized under UV light after staining the gels with ethidium bromide. The PCR products were 200-600 bp in size (Table 3).

3.6.3 Preparation of the SSCP gel

The gel matrix for the SSCP (MDE-gel, FMC-Bioproducts) was 0.8 µm thick and was prepared the night before the run and was left to stand overnight. Both glass plates were first washed twice with distilled water and once with ethanol. The plate which was to be removed leaving the gel to be stained on the other plate, was sprayed with Acrylease (Stratagene). A

total of 100 ml gel consisted of 25 ml MDE-gel, 6 ml 10XTBE, 69 ml bidest, 40 µl TEMED and 400 Ml 10 % APS.

Each slott was loaded with 2 µl stop solution, 2 µl SDS, 10% EDTA and at least 10-20 µl PCR product. The mixture was previously denatured at 98°C for 15 min and immediately transferred into ice water (0°C) for at least 10 min.

Expected fragments of 200-400 bp in size were electrophoresed for 3 h at 30 mA, while bigger fragments were separated overnight at 6 mA using the Hoeffer 600 system (Pharmacia) in a cool room (4°C). Sandwich gels were prepared as well using a divider plate to run a double number of samples. Consequently the sandwich gels were electrophoresed at doubling current compared to the respective single ones but for the same time.

3.6.4 Silver staining of the SSCP gel (Budowle *et al*., 1996)

First the gel was fixed in 1 % nitric acid for 10-15 min. Then it was briefly rinsed with double distilled water and stained for 20-25 min in 0.2% Silver nitrate solution. After washing for 5-10 min with double distilled water the gel was developed in 0.28M sodium carbonate plus formaldehyde (37 %) (fresh solutions always). That was followed by another washing in double distilled water and by fixation in 10% acetic acid for 5 min. After washing in double distilled water the gel was neutralised for 30 min in a solution containing 20 % ethanol and 10 % glycerol, and brought between two cellophane replacement sheets (Roth). It was left to dry on air, while held by plastic frames.

The *Leishmania* isolates from the collection of the Institute for Medical Parasitology, Bonn, Germany that were identified via PCR-RFLP and PCR fingerprinting with single arbitrary primers, belonging to the *L.donovani* complex [S(d)1-S(d)21 S(h)27 and S(h)29], along with the standard isolated of *L.donovani* complex, were processed through the PCR-SSCP.

3.7. Index of reagents, disposables, machinery, buffers and solutions.

3.7.1 Index of reagents

Company	Reagents	Catalogue number
Boehringer Mannheim	Cfo I	693 936
	Hae III	693 936
Fluka	TEMED	386451190
FMC-Bioproducts	MDE Mutation Detection Gel Solution	50621
Gibco BRL	Ammonium peroxisulfate (APS)	5523UA
	DNA marker 100 bp	15615-016
	DNA marker 123 bp	15613-011
	DNA marker 1 kb	15628-050
	PCR kit	18038-026
Merck	Acetic acid	634
	Chloroform	1.02431
	Ethanol	1.00983.2511
	Formaldehyde	1.04003
	Isoamyl alcohol	977.1000
	Silver nitrate	101510.0050
	Sodium acetate	15.0100
	Sodium carbonate	1.06392.1000
	Sodium chloride	6404.1000
	Sodium dodecyl sulfate (SDS)	1.00983.2511
Peqlab	MoSieve^{TM}-Agarose MS500	35-3010
	peqGOLD Universal agarose	35-1020

Pharmacia Biotech	dNTP set, ultrapure	27-2035-01
Qiagen	PCR purification kit	28104
Roth	Boric acid	69.43
	Glycerine	7530.1
	Phenol (buffered)	0038.1
Seromed	Foetal calf serum	S0115
Sigma	EDTA	ED-255
	Ethidium bromide	E-1510
	Penicillin/Streptomycin solution	P-0906
	Proteinase K	P-6556
	Ribonuclease A	R-6513
	RPMI-1640	R-6504
	Sodium carbonate	S-8875
	Trizma base	T-8524
Stratagene	Acrylease nonstick plate coating	300132
TIB MOLBIOL Syntheselabor, Berlin	Primers	

3.7.2 Index of disposables

Company	Product	Catalogue number
Eppendorf	Safe lock tubes 2 µl	0030120.094
Millipore	0.22 µm filter units	5VGSB1010
Nunc	Tubes fur cell culture	156758
Peqlab	Flat cap PCR tubes 0.5 ml	82-0350
Pharmacia Biotech	Glass plates for Hoefer 600, 18x16	80-6178-99
	Glass plate divider	80-6490-20
Polaroid (Balmes & Gondorf)	Polaroid film, 665 PIN	
Roth	Cellophan Ersatzfolien	K423.1
Schleicher & Schüll	Sterile filter holders 0.2 µm	FP030135
Sigma	Microcapillary round tips	T2531
	Tips 0.5-10 µl aerosol stop	P8312
Central supply department of the university Clinics of the Reinische Friedrich-Wilhelm University in Bonn,	Tips 0.5-10 µl	32353379
	Tips 10-100 µl	
	Tips 100-1000µl	

3.7.3 Index of machinery

Machinery	Company
Camera	Polaroid MP-4 Lnd camera
Centrifuge	Heraeus Sepatech Biofuge 13R
Centrifuge	Heraeus Varifuge 3.0R
Electrophoresis chambers	Renner
Electrophoresis chamber	OWL model A1
Gel drying frames, 24x24 cm	Roth
Pipette 0.1-1 µl	Eppendorf
Pipette 1-10 µl	Eppendorf
Pipette 10-100 µl	Eppendorf
Pipette 100-1000 µl	Eppendorf
Power supply	Biometra standard Power pack P250
Power supply	Pharmacia biotech EPS 3500
Programmable freezing unit	REVCO, ultra low temperature
UV transilluminator	Biometra
Speed vac	Christ RVC 2-18
Thermocycler	T3 Thermocycler-Biometra
Vertical slab gel unit, Höfer Model SE400	Pharmacia Biotech
Water bath	Memmert

3.7.4 Solutions and buffers

Ethanol-Glycerol solution	**Ethanol 20%, Glycerol 10% in aqua bidest**
Ethidium bromide solution	stock solution 10 mg/ml; working solution 0.5 µg/ml
Leishmania lysis buffer	50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.4
$NaCO_2$-Formaldehyde	sodium carbonate 29.6 g/l; 0.5 ml formaldehyde
Phenol/Chloroform/Isoamyl alcohol	phenol/chloroform/isoamyl alcohol in volumes 25:24:1
RPMI-1640 medium	RPMI 1640; 2 gr sodium bicarbonate; 15% foetal calf serum
Sodium acetate	Sodium acetate 3M; autoclave
Stop solution (loading buffer)	30 % glycerol; 0.25 % bromophenol blue; 0.25 % xylene cyanol FF; 10 mM EDTA
TBE buffer	trizma base 108 g/l; boric acid 55 g/l; EDTA 20 mM (pH 8)
TE buffer	trizma-base (pH 7.5) 10 mM; EDTA (pH 7.2) 1mM

4. Results

4.1 Parasites

All 21 canine isolates collected in Northern Greece (S(d)1-S(d)21) could be successfully cultured. However living parasites could be collected only from 9 (S(h)22-S(h)30) of the 16 human isolates which were under cryopreservation for the last 7-12 years without subsequent passage. Motile parasites could be seen in the canine isolate cultures after 3-4 days and they could be harvested after 7 days, while in the human isolates, it would last much longer. In one of the human isolates, living parasites were first observed after two months. In general the cultures were left in the incubator until parasites or bacterial and/or fungal contamination were seen in the microscope, trying to exhaust all time limit.

The long term maintenance of leishmanial parasites by serial subculture *in vitro* is problematic since promastigotes tend to lose their ability to transform into metacyclic forms when cultured for a long time (Evans *et al*., 1989).

4.2 Diagnosis of visceral leishmaniasis using PCR

This diagnostic PCR was described in §3.5.1. The isolates that were scored as positive, yielded a product of 560 bp and were all the *L. infantum*, *L. chagasi* from Table 2, the isolates S(d)1-S(d)21, S(h)27, S(h)29 and the 24 (BS1-BS24) samples from German dogs that were infected with *Leishmania* while travelling in South Europe. The latter 24 were clinical samples from dogs, that had travelled to South Europe and they were diagnosed with leishmaniasis using another PCR method at the Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin in Berlin.

4.3 RFLP analysis of the ITS region

All the 21 canine (S(d)1-S(d)21)and 9 human isolates (S(h)22-S(h)30) were processed through the PCR amplifying the ITS region (see § 3.5.2). After diggestion with the restriction enzymes *Hae*III and *Cfo*I, the patterns were checked after gel electrophoresis. Reference strains listed in Table 2 (§3.1) were always used as controls.

The 21 canine isolates after the digestion with *Hae*III gave a pattern of three bands corresponding to an average molecular weight 89 bp, 236 bp and 690 bp (examples in Figure 2). That was exactly the RFLP pattern that all the characterised *L. infantum*, *L. donovani* and *L. chagasi* listed in Table 2 gave.

Similarly homogeneous results were produced after the digestion of the above mentined isolates with *Cfo*I. The resulting pattern was comprised of five bands of average sizes of 345 bp, 305 bp, 158 bp, 94 bp and 71 bp (examples in Figure 3).

Comparing the patterns of the isolates S(h)22-S(h)30 with patterns of different reference strains it was possible to identify them at the species or complex level listed in Table 5.

Table 5: Identification of S(h)22-S(h)30 at the species/complex level by RFLP analysis of the ITS region in comparison to reference strains.

Isolate codes	Corresponding reference complex or strain
S(h)22	TRO-02, TRO-22
S(h)23	AET-03
S(h)24	TRO-02, TRO-22
S(h)25	TRO-02, TRO-22
S(h)26	*L.brasiliensis* complex
S(h)27	*L.donovani* complex
S(h)28	*L.brasiliensis* complex
S(h)29	*L.donovani* complex
S(h)30	*L.brasiliensis* complex

4.4 PCR fingerprinting with single arbitrary primers

All the isolates listed in Table 2, the 21 canine isolates (S(d)1-S(d)21) from Northern Greece and the 9 human isolates (S(h)22-S(h)30) were processed through fingerprinting PCR (§ 3.5.2), using all primers [T_3B, $(GACA)_4$, M_{13}, $(GTG)_5$]. Figures 4 to 7 show selected examples of fingerprinting patterns using the above mentioned primers.

All *Leishmania* isolates were repeatedly tested. The polymorphic fragment patterns were reproducible with slight variations in the intensity and occasionally in the banding pattern. The latter was observed in one or two bands, usually the very large (>3 kb) and very small (< 250 bp) ones. Those isolates which were prone to variation were excluded from the analysis. Faint bands were considered only if they were consistently found in different experiments. Bands of higher intensities, might be due to the amplification of repetitive sequences, the influence of neighbouring sequences on annealing to the target sequence or might have been generated because of a lower degree of mismatch between primer and target sequence (Welsh *et al*., 1991; Godwin *et al*., 1991).

Distinctive sets of amplification products were observed for each taxon and for every sample depending on the taxon it belonged to. The discriminating capacity of the primers were similar. With the primer M_{13} core sequence, each isolate yielded 11 to 31 bands ranging between 3300 bp and 300 bp; with the primer T3B 9 to 26 bands of 3390 bp to 445 bp; with the primer $(GACA)_4$ 11 to 25 bands ranging between 3600 bp and 360 bp and with the primer $(GTG)_5$, 16 to 28 bands of a molecular weight 3550 bp to 400 bp. The main grouping was made under each primer so that information could be collected for each isolate prone to variation. Therefore under each primer all amplification products were scored for each isolate according to molecular weight. In the end for each group of amplification products a matrix was produced comprised not anymore from particular weight corresponding to the produced bands but from the two numbers 1 or 0 corresponding to scoring positive or negative the production or not of each possible band for each isolate.

4.4.1 Computer assisted analysis with the TREECON for Windows v.1.3b

The results went through the computer-assisted analysis (§ 3.5.2.1) and the produced matrices were analysed by TREECON for Windows (version 1.3b, 1998) whereof evolutionary trees - dendrogrammes- were constructed and produced (Van de Peer *et al*., 1994; http://bioc-www.uia.ac.be/u/yvdp).

4.4.1.1. Dendrogramme produced after PCR with the primer $(GACA)_4$

The tree produced by this primer (Dendrogramme 1) is very well supported by bootstraping to the clades of the species identification. Especially good are the bootstraping values within the *L. donovani* complex (bootstraping values of 97-99) and the *L. brasiliensis* complex (bootstraping values of 93-100). Within the *L. infantum* isolates there is one distinct grouping of the Greek isolates while the rest of the *L. infantum* isolates are constituting the second group without any particular subgrouping. The later is very well supported by bootstrap value (99).

4.4.1.2. Dendrogramme produced after PCR with the primer $(GTG)_5$

The $(GTG)_5$ primer once again gives identical patterns for all the greek isolates. The Dendrogramme 2 shows very similar results to Dendrogramme 1, with bootstrap values supporting very well the complex clades (bootstraping values of 89-100) and the *L.infantum* species clade (bootstraping value of 93). Unfortunately the PCR with this primer could not be applied to all samples because of technical reasons. Therefore it was not taken into accont when the matrices were combined to get more general results. Nevertheless the results that were obtained in Berlin where the method is routinely established are reported and discussed.

4.4.1.3. Dendrogramme produced after PCR with the primer *M13*

The M13 primer is supported very well by the bootstraping the species identification. According to Dendrogramme 3 there is a grouping of the Greek isolates, and altogether they are according with some of the *L.infantum* isolates coming from Spain, Tunisia, Portugal, France, Turkey and China. There is also a major grouping of the Israeli isolates together with isolates from Turkey, Spain and Portugal.

4.4.1.4. Dendrogramme produced after PCR with the primer *T3B*

According to Dendrogramme 4, with the primer T3B the Greek *L.infantum* isolates had identical patterns when using primer T3B. Patterns are supported with high bootstrap values (more that 50). The rest of the Mediterranean isolates represents the majority of the second grouping. Within the second grouping there are the isolates coming from China, France and Spain belonging to one subgroup and those from Israel, Turkey and Portugal belonging to another. The Greek isolates were homogenous while others showed some differences.

Nevertheless the group of INF-15 and INF-17, the group of INF-11, INF-13, INF-14, INF-09 and INF-10 and the group CHA-01 and S(h)29, produced identical patterns within the group.

4.4.1.5. Dendrogramme produced after combination of the matrices produced through primers M13, T3B and *(GACA)*$_4$

The results from the three primers M13, T3B and (GACA) $_4$ were combined to the construction of the evolutionary tree 5 (Dendrogramme 5), where the results are a combination of what was shown above. The three complexes of *L. donovani*, *L. brasiliensis* and *L. mexicana* are clearly distinct. Samples S(d)1-S(d)21 belong to *L .infantum*, S(h)22-L(h)25 belong to *L. tropica* or *L. aethiopica*, S(h)26 and S(h)28 to *L. guyanensis*, S(h)27 to *L. donovani*, S(h)29 to *L. chagasi* and S(h)30 to *L. brasiliensis*. The Greek *L. infantum* isolates are grouping together. Israelian isolates are also forming a cluster, including the Turkish isolates in their group. The bootstrap values are very high to the clades of the complex, species and also the grouping of the *L. infantum* strains. There are three clusters of *L. infantum* formed with excellent statistical support (bootstrap values 99-100), one with the Greek canine isolates, one with the isolates from Turkey and Israel and a third with the rest of the *L. infantum* isolates.

4.4.1.6. Dendrogramme produced for the *L.donovani* complex, combining the matrices produced from the primers *M13, T3B and (GACA)* $_4$

Dendrogramme 6 was made combining the PCR results from the primers M13, T3B and (GACA) $_4$, for the *L. donovani* complex (*L. infantum*, *L. chagasi* and *L. donovani*) reference strains and the samples S(d)1-S(d)21 that were identified as *L.infantum*. The bootstrap values of the clades that lead to species are high (72-95) as well as those of the intaspecies grouping of the *L. infantum* (99-100). *L. infantum* and *L. donovani* are clearly seperable with very good statistical support. The Greek isolates on the one hand are grouping together and so do those from Israel. The other isolates are distributed between those two groups. The two isolates from Turkey, one canine and one human (INF-10 and INF-11), and the geographically heterogenous isolates INF-05, INF-04, INF-01 and INF-03 that are coming from France, China, Tunisia and Spain, respectively, are grouping together.

4.4.2. Taxonomic identification of the tested samples

Substantial polymorphic patterns were revealed only when isolates of different species were compared. There were no such observed for *L. infantum* and *L. chagasi*, for *L. mexicana* and *L. amazonensis* and for *L. guyanensis* and *L. panamensis,* respectively. *L. major*, *L. tropica* and the members of the *L. mexicana*, *L. brasiliensis* and *L. donovani* complexes were clearly differentiated from each other by their varying amplification product patterns. Within those

complexes similar PCR profiles were found and several common DNA fragments were amplified. Small differences resemble rather those strains belonging to the same species than different species.

Overall the unidentified isolates that were processed through fingerprinting with single arbitrary primers gave the following results; Isolates S(d)1-S(d)21 were clearly identified as *L. infantum* according to all single primers. Data obtained with the primer M13 suggest that isolates S(h)22-S(h)25 belonged to *L. aethiopica* and *L. tropica* species (Figure 8). Using the primer T_3B they were more similar to *L. aethiopica*, while with the $(GACA)_4$ S(h)22, S(h)24 and S(h)25 were similar to *L. tropica* and S(h)23 to *L. aethiopica*. S(h)26 and S(h)28 had similar patterns to *L. brasiliensis* complex when they were primed with the primers M_{13} (Figure 8) and T_3B, while when primed with $(GACA)_4$ they were more similar to *L. guyanensis* which is part of the complex. S(h)27 was identified to be *L. donovani* according to all primers, S(h)29 *L. chagasi* and S(h)30 *L. brasiliensis.*

4.5 Fingerprinting using the primer pair L1119

Trying to optimize the PCR reactions with the codominant markers (see §3.2.2) different to expectations, the primer pair L1119 did not produce a single band. However, it produced interesting fingerprinting patterns that after optimisation gave very similar results with the other four single primers (Figure 7).

All reference strains, isolates S(d)1-S(d)21 and S(h)22-S(h)30 were processed through it and each isolate yielded 11 to 22 bands ranging between 1800 bp and 300 bp. The results from the L1119 were processed through the same computer assisted analysis like the other primers and the evolutionary tree 7 (Dendrogramme 7) was constructed.

The complexes *L. donovani*, *L. mexicana* and *L. brasiliensis* are distinct while *L. aethiopica*, *L. tropica* and *L. major* are relatively standing on their own. Within the *L. brasiliensis* complex *L. guyanensis* and *L. panamensis* grouped very close together and the *Viannia* group is very well statistically supported. The Greek *L. infantum* isolates are found in three different subgroups. In general the isolates of the *L. infantum* complex are distributed homogeneously within the complex without comprising any groups that indicate major geographical separations. That may be due to the fact that in this case the primer pair that was used, was

randomly chosen. It may be amplifing on chromosomal DNA i.e the multiple bands, indicate a repeated sequence of different size in the chromosomal genome.

4.6 Analysis of codominant anonymous PCR markers

The results from the PCR-SSCP with ITS1 amplifying primer set and the codominant markers are summarised in Table 7. The isolates that were processed with the codominant markers were all *L.infantum* isolates, including the Greek canine isolates S1-S21, the DON-01 and DON-04, the *L.chagasi* reference strain and the LS29 that was identified as *L.chagasi* (Table 2).

The underlying principle of the SSCP method is that the electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is dependent on its size and structure (= conformation). Since conformations are highly dependent on primary sequence, each ssDNA molecule will adopt a tertiary conformation, based on nucleotide sequence, which results in differences in relative mobility. Examples of that can be seen in Figures 12 compared to figures 10 and 11. Figure 10 shows that the pattern of the given isolates is identical for all given isolates when the PCR product of the primer pair L0110 is subjected to SSCP. This means that there is no single nucleotide mutation in the sequence of this amplified product for the given isolates. The same result was obtained when the primer pair L0720 was used for the isolates S(d)1-S(d)11 (figure 11). Nevertheless the results are different when the primer pair L1112 was used (figure 12). Differences are observed in the reference strains MHOM/CN/78/D2, MHOM/FR/80/189, MHOM/IL/89/LRC-L571 and the rest of the subjected isolates mainly because of single nucleotide mutations.

To summarise the results of this experiment the Table 7 was prepared. Each column represents a primer pair and each line a tested isolate. Different signs stand for each primer for each different pattern.

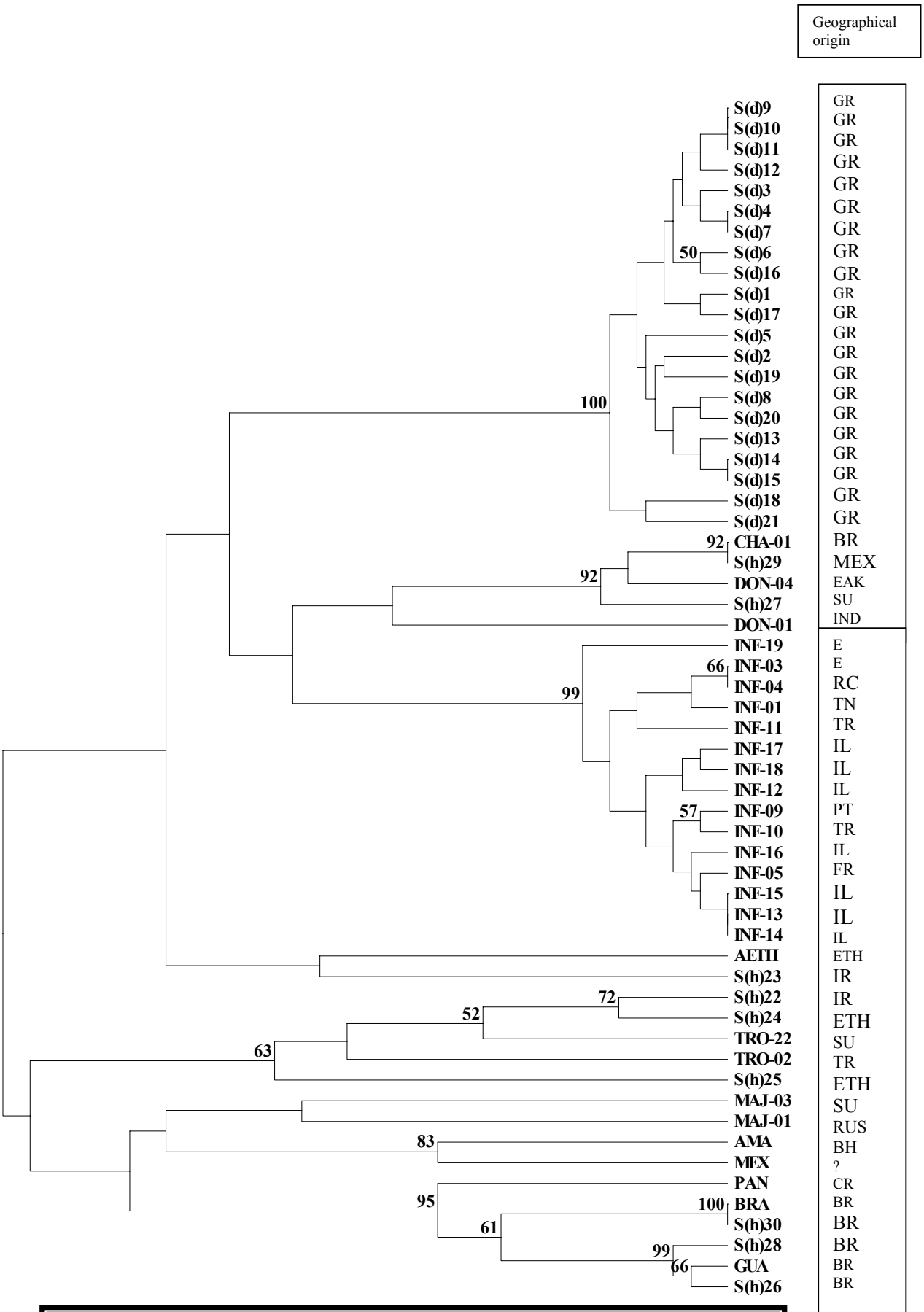
Thus the SSCP assay of the ITS1, L01012, L0720, L0510, L0114, L0413 and L0110 amplicons yielded no variation, while 5 different groups were observed when the L1112 primer was used. The chinese isolate INF-04 gave a unique pattern, the two french isolates INF-05 and INF-06 gave another, the spanish INF-19 a third, the CHA-01 and S(h)29 a

fourth. In all other cases a fifth common pattern was obtained. It is important to note that L1112 is the only primer which seperates *L. chagasi* (CHA-01) and the S(h)29 from others of the *L. donovani* complex. S(h)29 was identified as *L. chagasi* also with the fingerprinting with the single arbitrary primers. It is intersting to see that the two *L. donovani* strains that were used (DON-01 and DON-04) do not yield any different patterns. When the primer pair L1012 was employed in PCR with *L. major* DNA, a band of about 600 bp was produced while the product of the *L. infantum* and *L. chagasi* was only 200 bp in size (not shown).

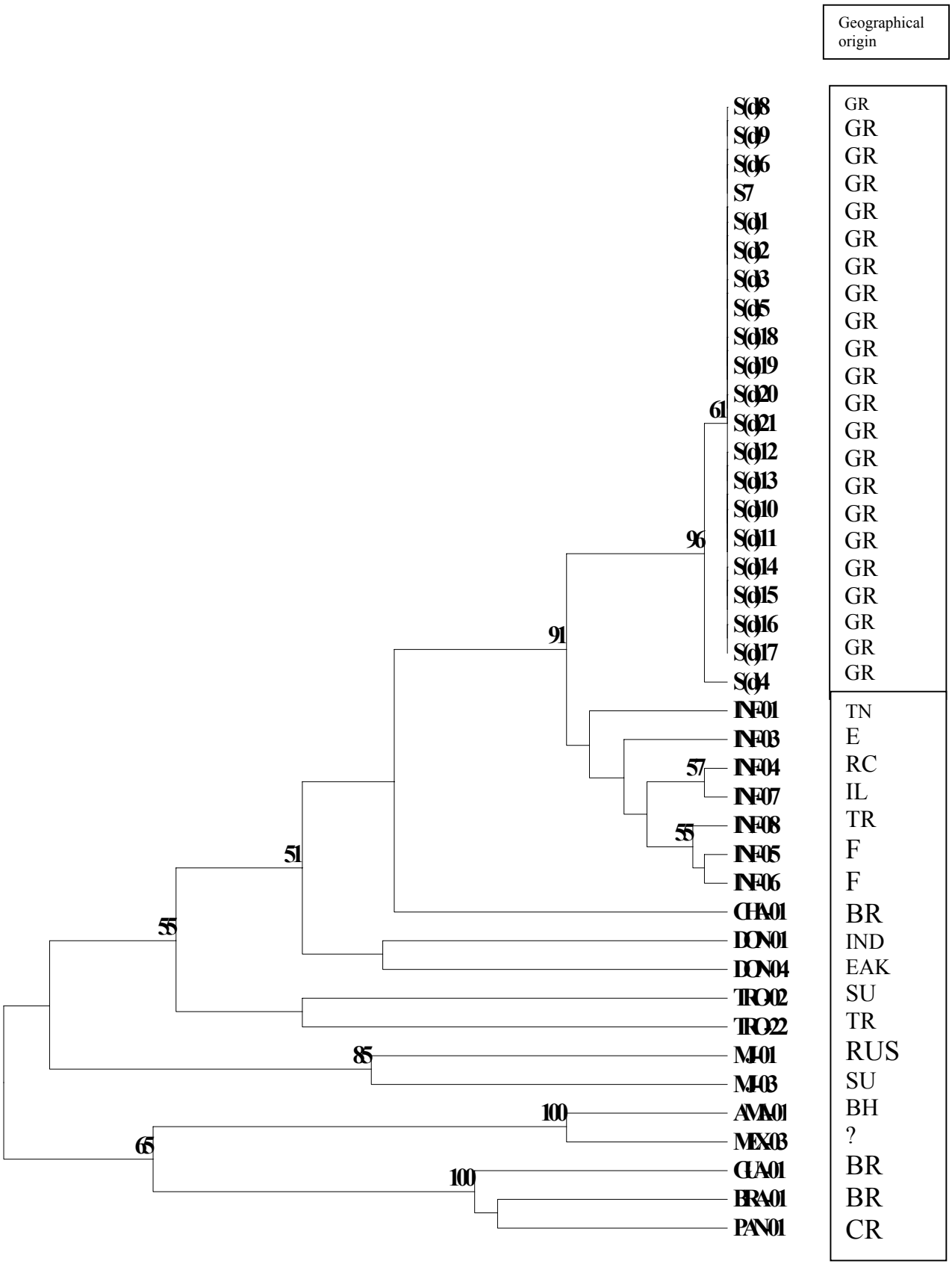
Table 7: Results of the PCR-SSCP of anonymous markers. In each row, one symbol has been chosen representing the pattern yielded from the specific primer. When a different symbol appears in the given row, is because a different pattern is yielded from the specific primer pair, when it amplifies in the specific isolate.

Sample No	ITS1	L1012	L0720	L0510	L0114	L1112	L0413	L0110
S(d)1	•	∇	*	◊	□	♠	ℵ	°
S(d)2	•	∇	*	◊	□	♠	ℵ	°
S(d)3	•	∇	*	◊	□	♠	ℵ	°
S(d)4	•	∇	*	◊	□	♠	ℵ	°
S(d)5	•	∇	*	◊	□	♠	ℵ	°
S(d)6	•	∇	*	◊	□	♠	ℵ	°
S(d)7	•	∇	*	◊	□	♠	ℵ	°
S(d)8	•	∇	*	◊	□	♠	ℵ	°
S(d)9	•	∇	*	◊	□	♠	ℵ	°
S(d)10	•	∇	*	◊	□	♠	ℵ	°
S(d)11	•	∇	*	◊	□	♠	ℵ	°
S(d)12	•	∇	*	◊	□	♠	ℵ	°
S(d)13	•	∇	*	◊	□	♠	ℵ	°
S(d)14	•	∇	*	◊	□	♠	ℵ	°
S(d)15	•	∇	*	◊	□	♠	ℵ	°
S(d)16	•	∇	*	◊	□	♠	ℵ	°
S(d)17	•	∇	*	◊	□	♠	ℵ	°
S(d)18	•	∇	*	◊	□	♠	ℵ	°

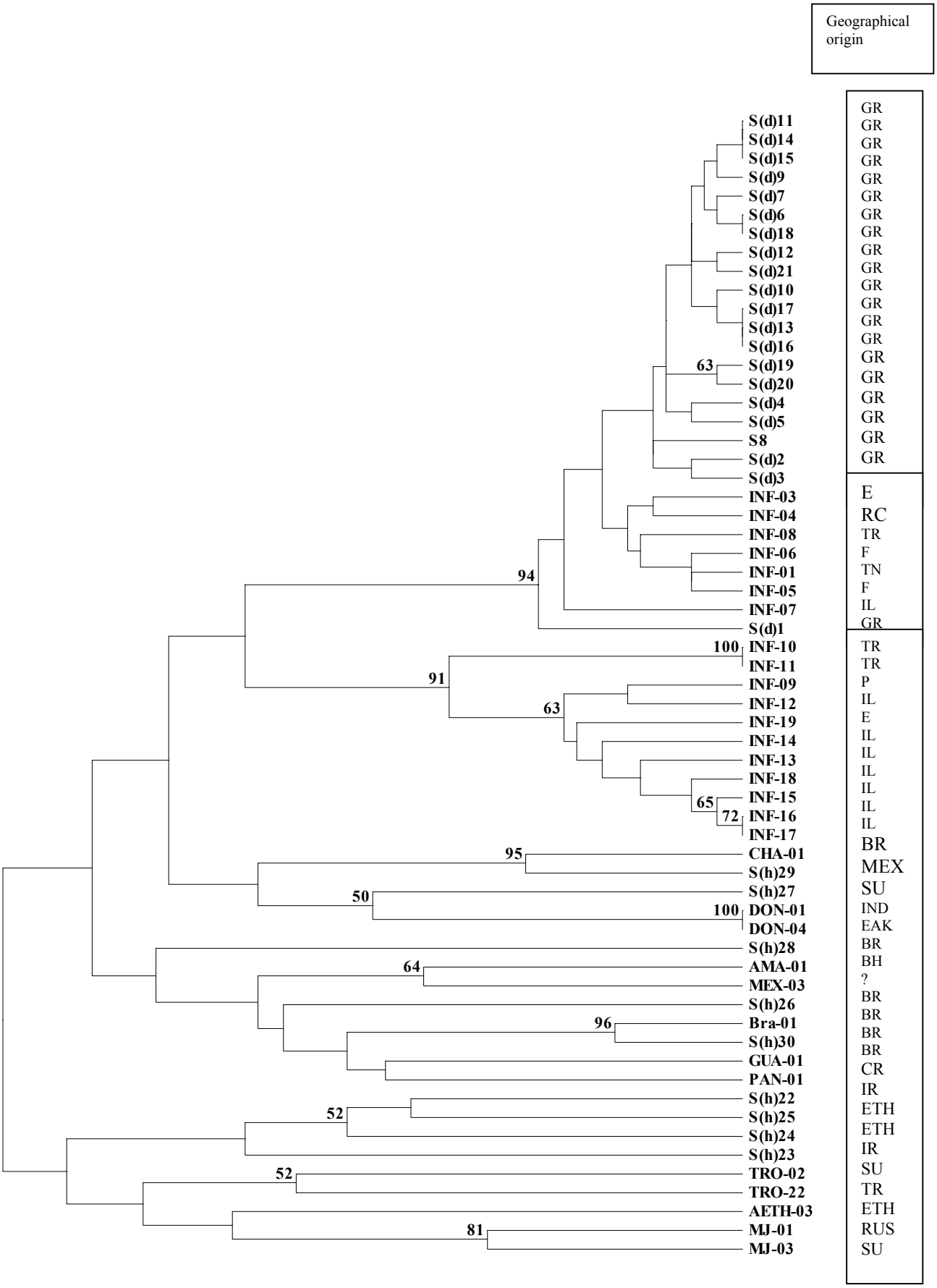
S(d)19	•	∇	*	◊	□	♠	ℵ	°
S(d)20	•	∇	*	◊	□	♠	ℵ	°
S(d)21	•	∇	*	◊	□	♠	ℵ	°
INF-01	•	∇	*	◊	□	♠	ℵ	°
INF-03	•	∇	*	◊	□	♠	ℵ	°
INF-04	•	∇	*	◊	□	∠	ℵ	°
INF-05	•	∇	*	◊	□	∧	ℵ	°
INF-06	•	∇	*	◊	□	∧	ℵ	°
INF-07	•	∇	*	◊	□	♠	ℵ	°
INF-09	•	∇	*	◊	□	♠	ℵ	°
INF-10	•	∇	*	◊	□	♠	ℵ	°
INF-11	•	∇	*	◊	□	♠	ℵ	°
INF-12	•	∇	*	◊	□	♠	ℵ	°
INF-13	•	∇	*	◊	□	♠	ℵ	°
INF-14	•	∇	*	◊	□	♠	ℵ	°
INF-15	•	∇	*	◊	□	♠	ℵ	°
INF-16	•	∇	*	◊	□	♠	ℵ	°
INF-17	•	∇	*	◊	□	♠	ℵ	°
INF-18	•	∇	*	◊	□	♠	ℵ	°
INF-19	•	∇	*	◊	□	ℑ	ℵ	°
CHA-01	•	∇	*	◊	□	∅	ℵ	°
S(h)29	•	∇	*	◊	□	∅	ℵ	°
DON-01	•	∇	*	◊	□	♠	ℵ	°
DON-04	•	∇	*	◊	□	♠	ℵ	°
S(h)27	•	∇	*	◊	□	♠	ℵ	°



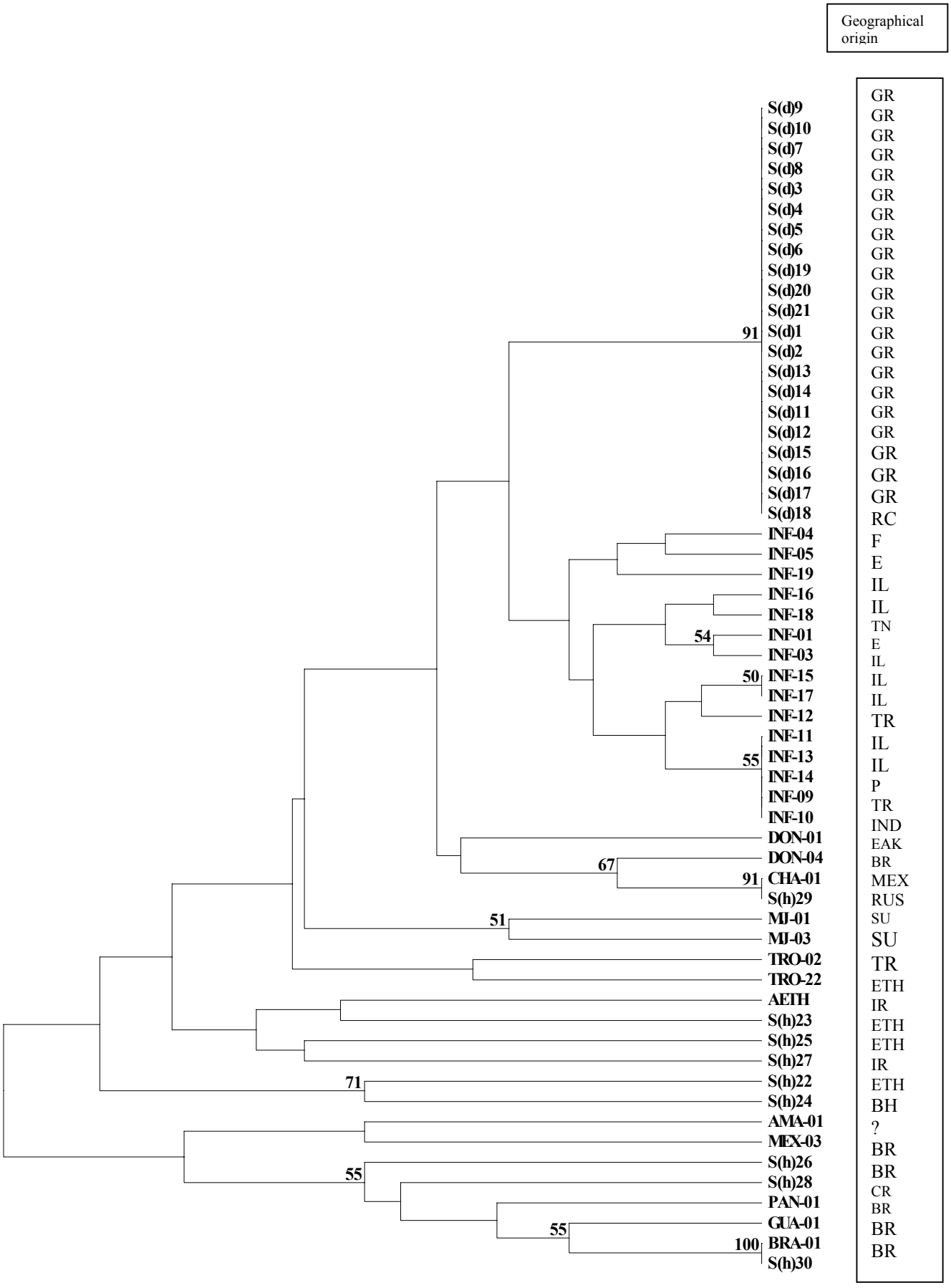
Dendrogramme 1: Evolutionary tree produced by the results of the fingerprinting PCR with the single arbitrary primer $(GACA)_4$.



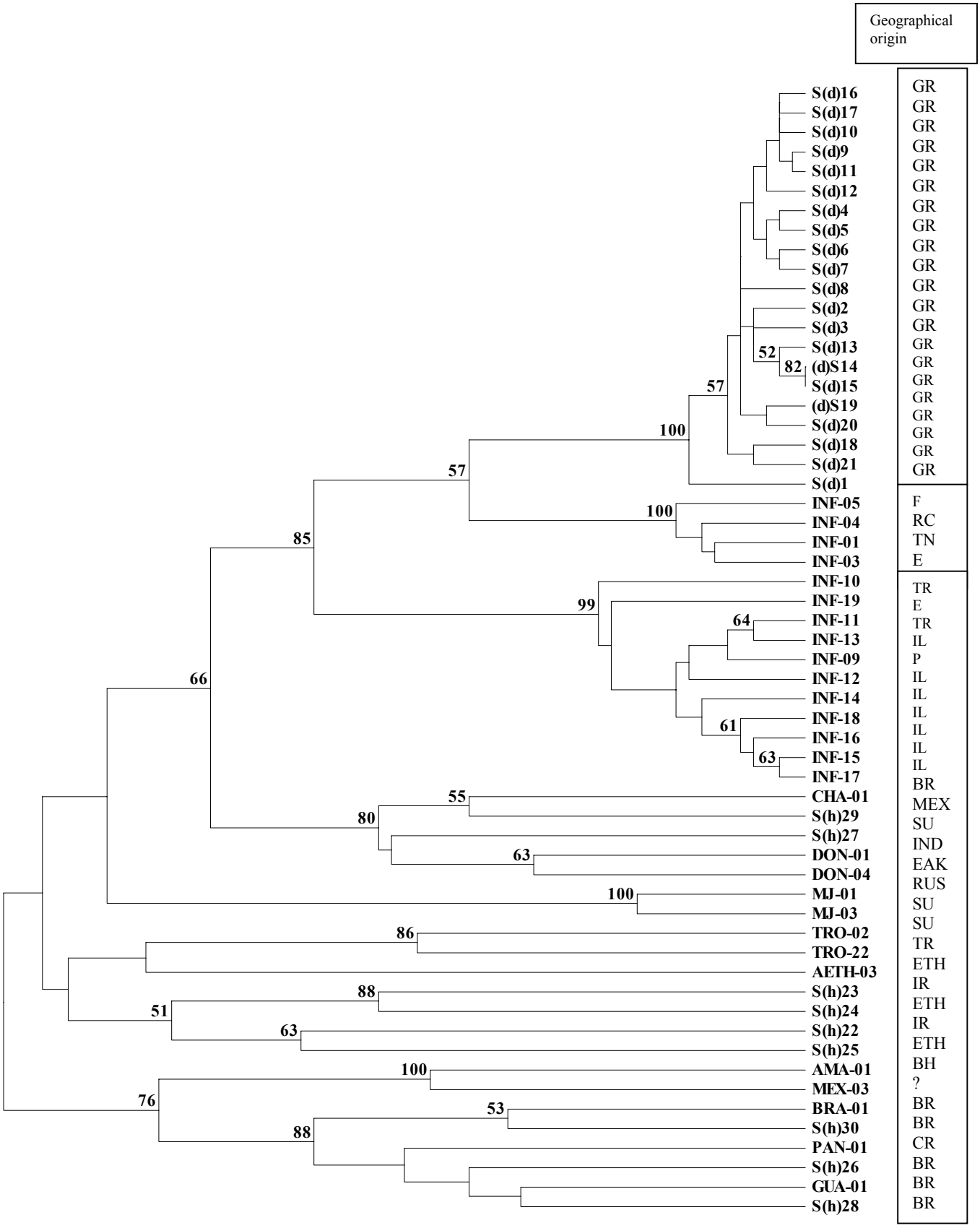
Dendrogramme 2: Evolutionary tree produced by the results of the fingerprinting PCR with the single arbitrary primer $(GTG)_5$.



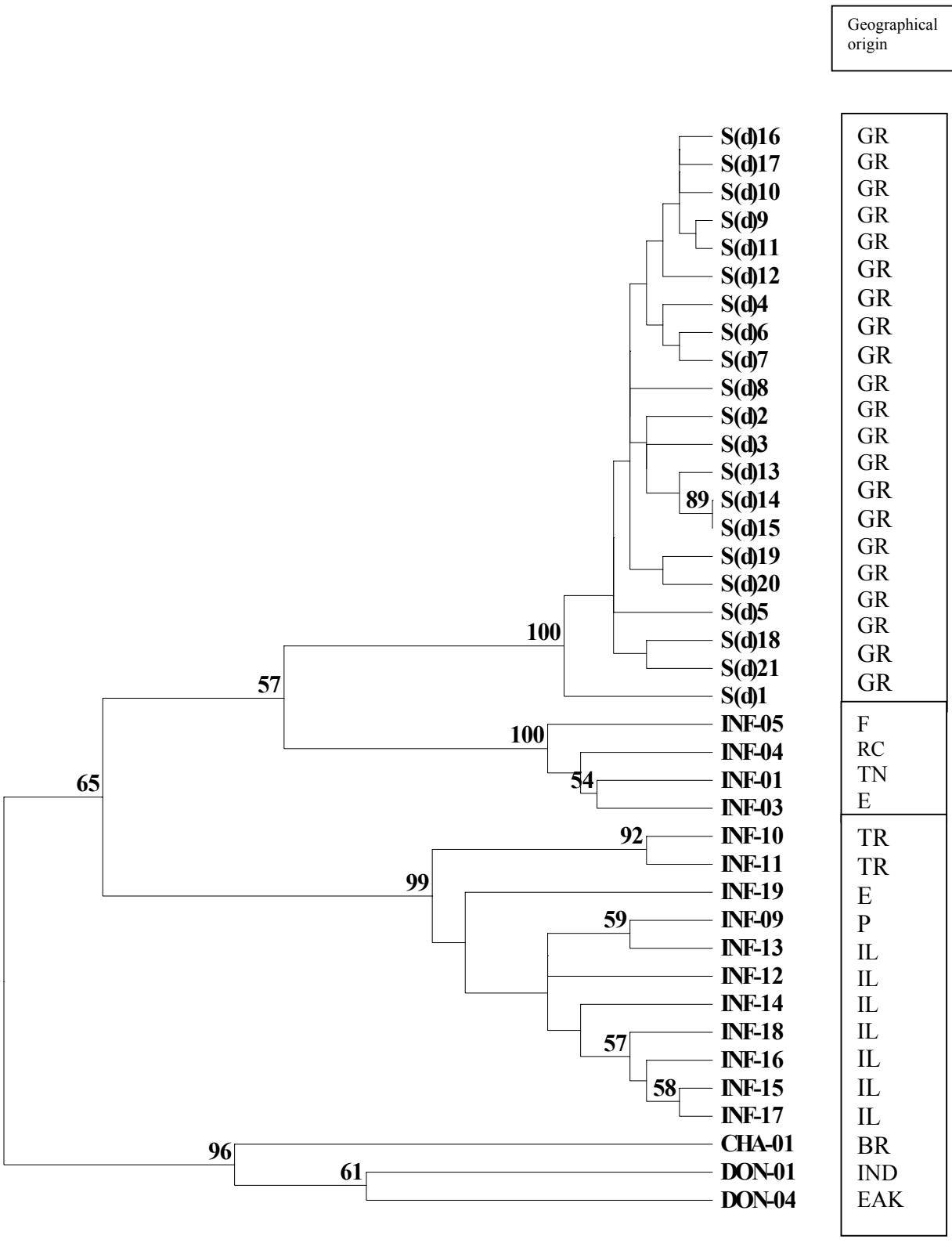
Dendrogramme 3: Evolutionary tree produced by the results of the fingerprinting PCR with the single arbitrary primer M13.



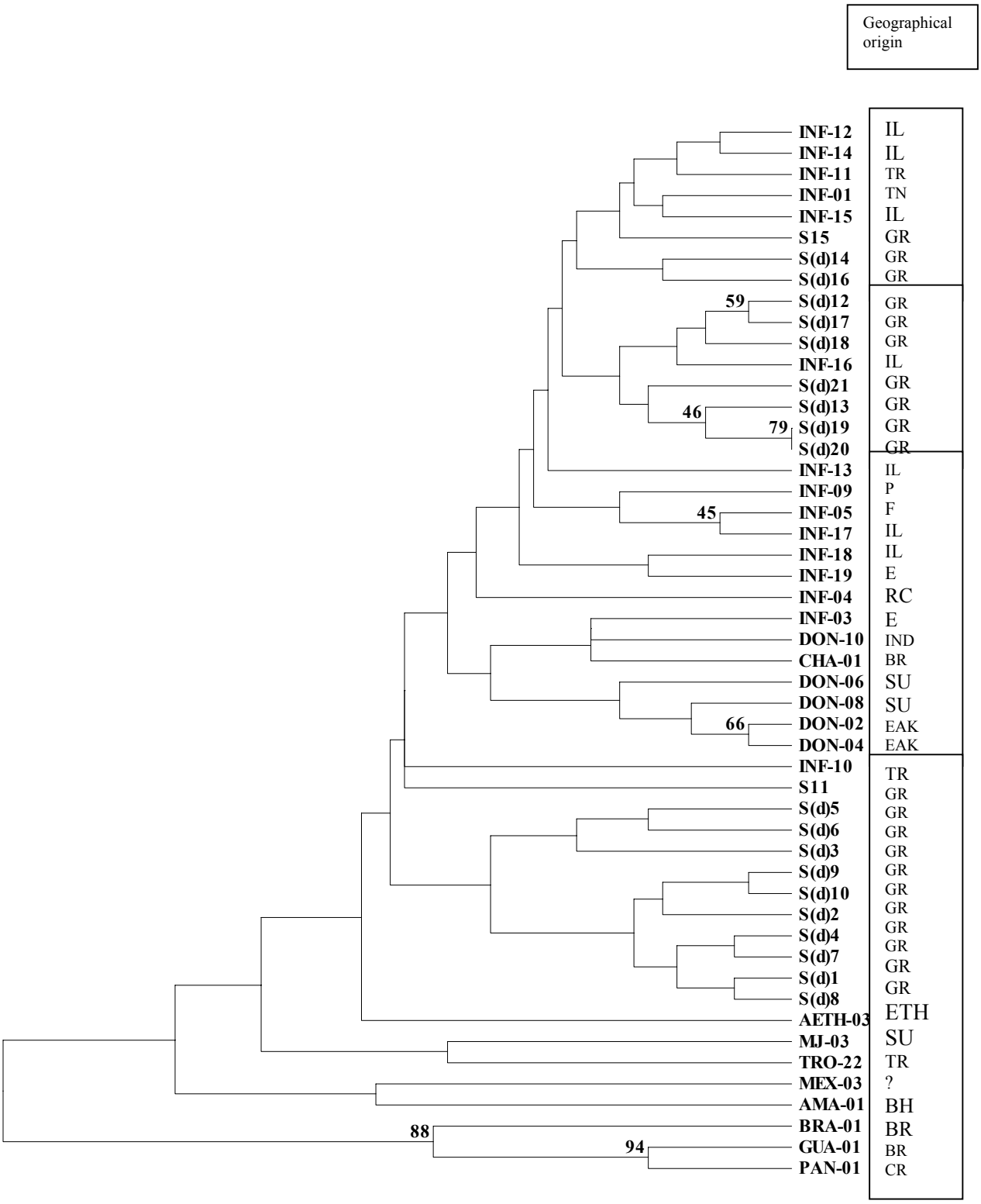
Dendrogramme 4: Evolutionary tree produced by the results of the fingerprinting PCR with the single arbitrary primer T3B.



Dendrogramme 5: Evolutionary tree produced by the results of the fingerprinting PCR with the single arbitrary primers M13, $(GACA)_4$ and T3B.



Dendrogramme 6: Evolutionary tree produced for the *L.donovani* complex with the results of the fingerprinting PCRs with the single arbitrary primers M13, T3B and $(GACA)_4$.



Dendrogramme 7: Evolutionary tree produced by the results of the fingerprinting PCR with the primers pair L1119.

The country of origin contained in the brackets next to each isolate, in the right side of each dendrogramme is signed according to the internationaly used abbreviations as following:

BH: Belise

BR: Brasil

CR: Costa Rica

E: Spain

EAk: Kenya

ETH: Ethiopia

F: France

GR: Greece

IL: Israel

IND: India

IR: Iran

MEX: Mexico

P : Portugal

RC : China

RUS: Russia

TN: Tunesia

TR: Turkey

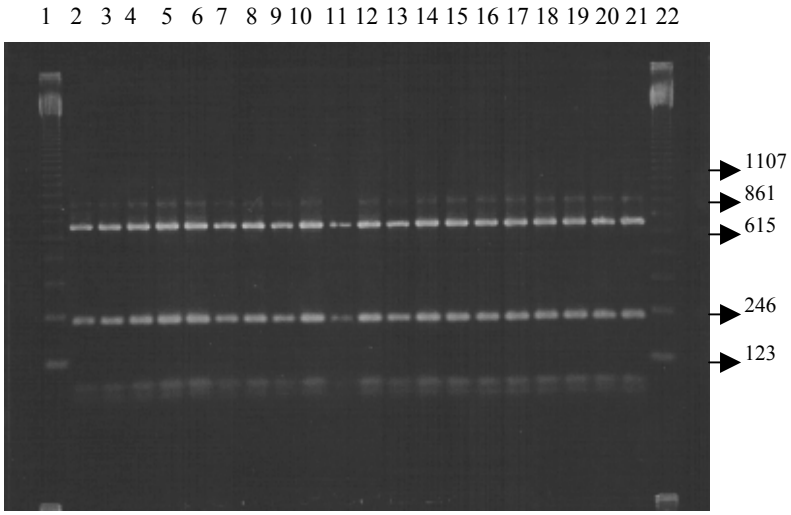


Figure 2: RFLP analysis patterns of the ITS region after digestion with HaeIII. Lanes 1 and 22: molecular size markers in bp; lanes 2-21: Leishmania isolates S(d)1-S(d)20 from dogs from Northern Greece.

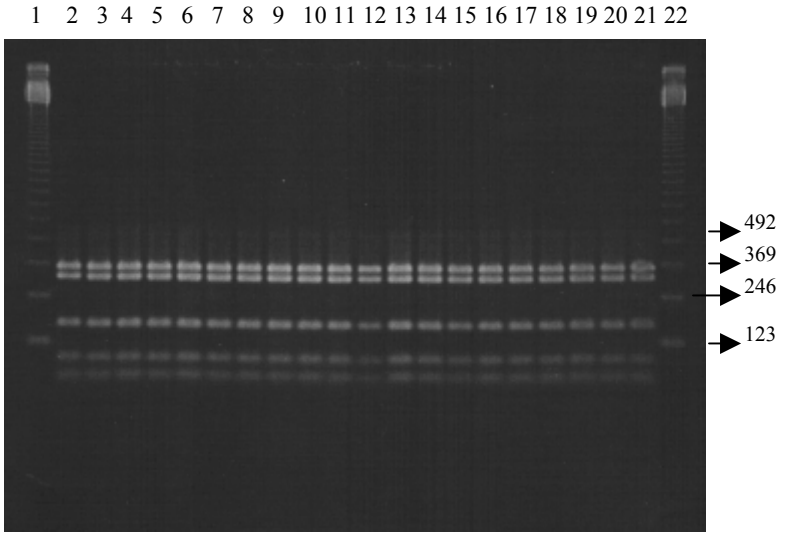


Figure 3: RFLP analysis of the ITS region after digestion with CfoI. Lanes 1 and 22: molecular size markers in bp; lanes 2-21: *Leishmania* isolates S(d)1-S(d)20 from dogs.

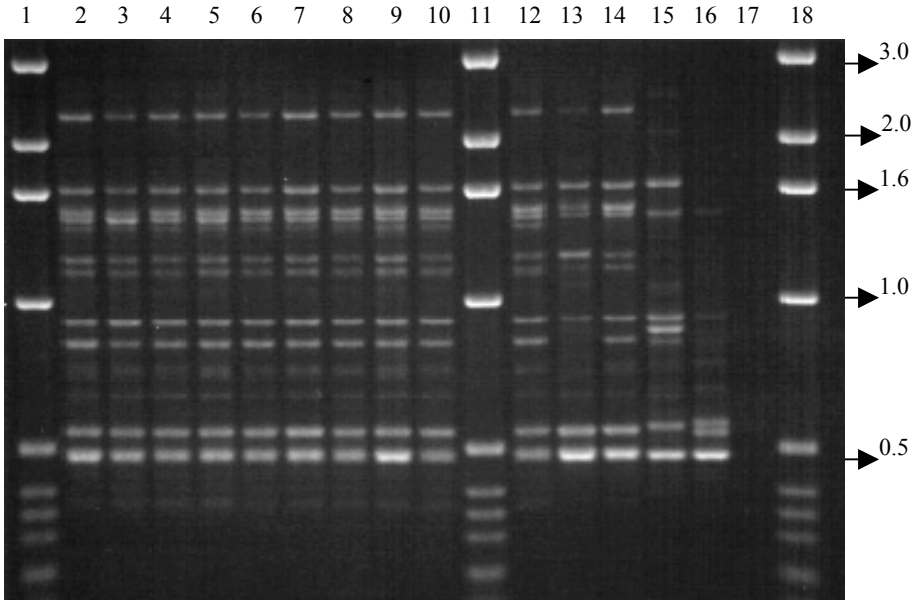


Figure 4: PCR fingerprinting profile obtained with the T3B primer. Lanes 1, 11 and 18: molecular size markers in kb; lanes 2-10 and 12: *Leishmania* isolates from dogs; lane 13: *L.donovani* (MHOM/IN/89/DD8); lane 14: *L.infantum* (MHOM/TN/80/IPT1); lane 15: *L.major* (MHOM/SU/73/5ASKH); lane 16: *L.tropica* (MHOM/SU/79/LRC-L39); lane 17: control sample without DNA.

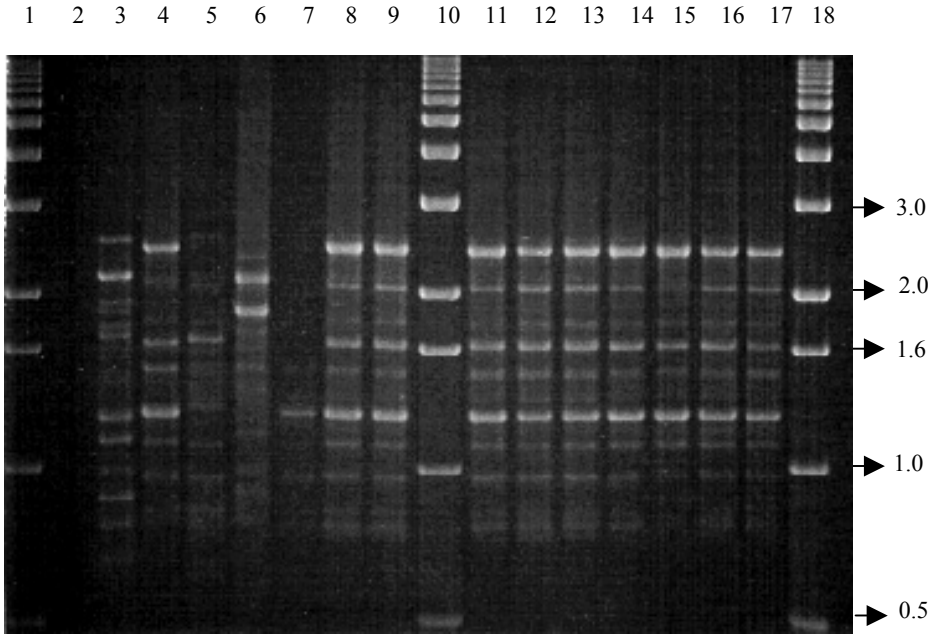


Figure 5: PCR fingerprinting profile obtained with the $(GACA)_4$ primer. Lanes 1, 10 and 18: molecular size markers in kb; lane 2: control sample without DNA; lane 3: *L.major* (MHOM/SU/73/5ASKH); lane 4: *L. infantum* (MHOM/TN/80/IPT1); lane 5: *L.donovani* (MHOM/IN/80/DD8); lane 6: *L.tropica* (MHOM/SU/79/LRC_L39); lanes 7-9 and 11-17: *Leishmania* isolates from dogs.

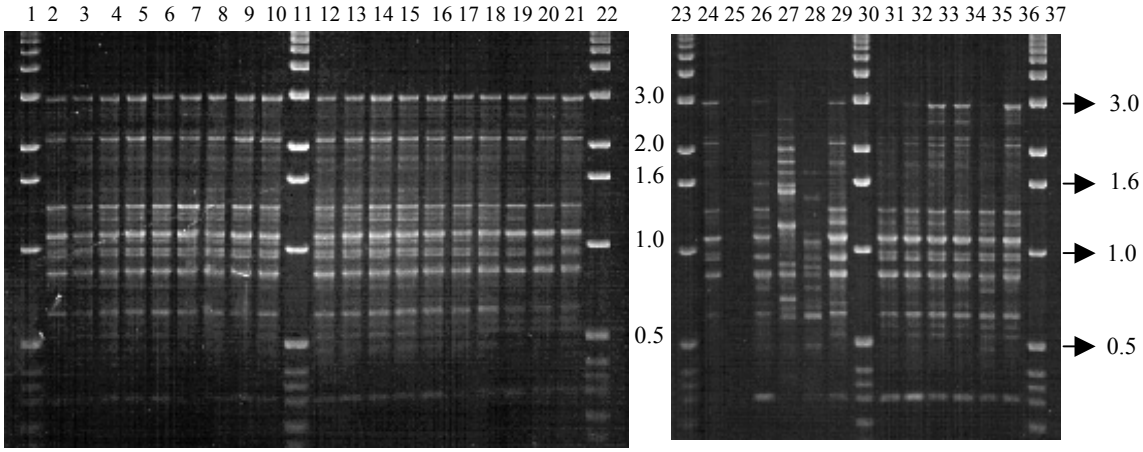


Figure 6: PCR fingerprinting profile obtained with the M_{13} core sequence primer. Lanes 1,11,22,23,30 and 37: molecular size markers in kb; lanes 2-10, 12-21 and 24: *Leishmania* isolates from dogs; lane 25: control sample without DNA; lane 26: *L.donovani* (MHOM/IN/80/DD8); lane 27: *L.tropica* (MHOM/SU/79/LRC-L39),; lane 28: *L.major* (MHOM/SU/73/5ASKH); lanes 29 and 31-36: *L.infantum* (MHOM/TN/80/IPT1, MHOM/FR/62/LRC-L47,MHOM/ES/87/Lombardi,MHOM/CN/78/D2,MHOM/FR/80/189, MCAN/IL/96/LRC-L709, MHOM/TR/96/EP16).

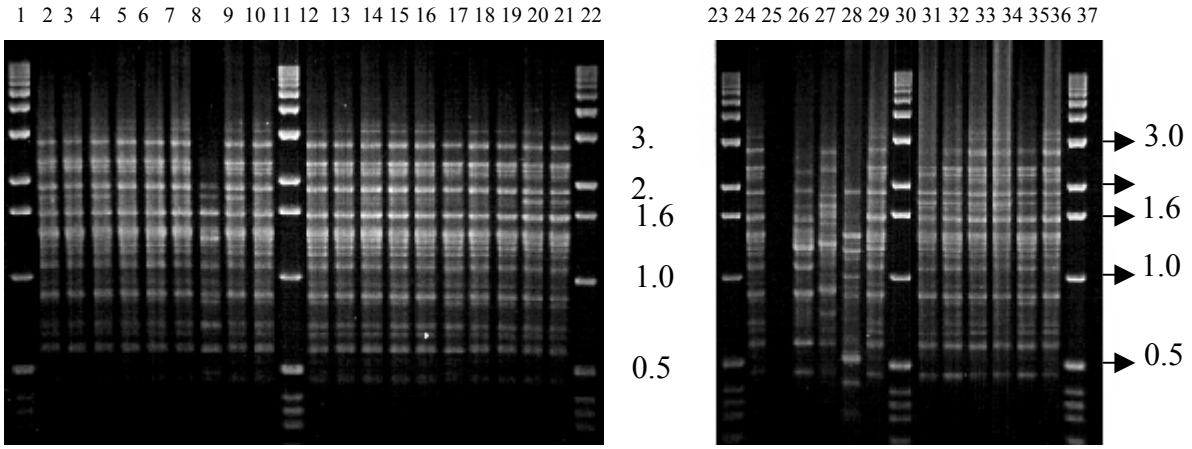


Figure 7: PCR fingerprinting profiles with the $(GTG)_5$ primer. Lanes 1,11,22,23,30 and 37: molecular size markers in kb; lanes 2-10, 12-21 and 24: *Leishmania* isolates from dogs; lane 25: control sample without DNA; lane 26: *L.donovani* (MHOM/IN/80/DD8); lane 27: *L.tropica* (MHOM/SU/79/LRC-L39); lane 28: *L.major* (MHOM/SU/73/5ASKH); lanes 29 and 31-36: *L.infantum* (MHOM/TN/80/IPT1, MHOM/FR/62/LRC-L47,MHOM/ES/87/Lombardi,MHOM/CN/78/D2,MHOM/FR/80/189, MCAN/IL/96/LRC-L709, MHOM/TR/96/EP16).

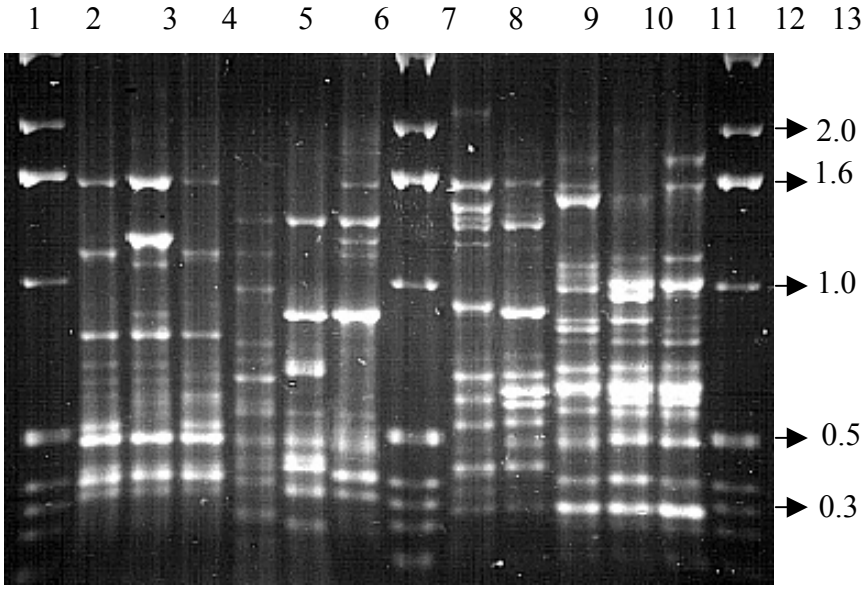


Figure 8: PCR fingerprinting profile with the L1119 primer pair. Lanes 1, 8 and 14: molecular size markers in kb; lane 2: *L.infantum* (MHOM/TN/80/IPT1); lane 3: *L.donovani* (MHOM/KE/85/NLB323); lane 4: *L. chagashi* (MHOM/BR/74/PP75; lane 5: *L.major* ((MHOM/SD/90/Sudan3); lane 6: *L.tropica* (MHOM/TR/95/URFA7); lane 7: *L.aethiopica* (MHOM/ET/94/Abauye); lane 9: *L.mexicana* (patients isolate); lane 10: *L.amazonensis* (MHOM/BZ/73/M2269); lane 11: *L.brasiliensis* (MHOM/BR/75/M2903); lane 12: *L.guyianensis* (MHOM/BR/75/M4147; lane 13: *L.panamensis* (MHOM/CR/87/NEL3.

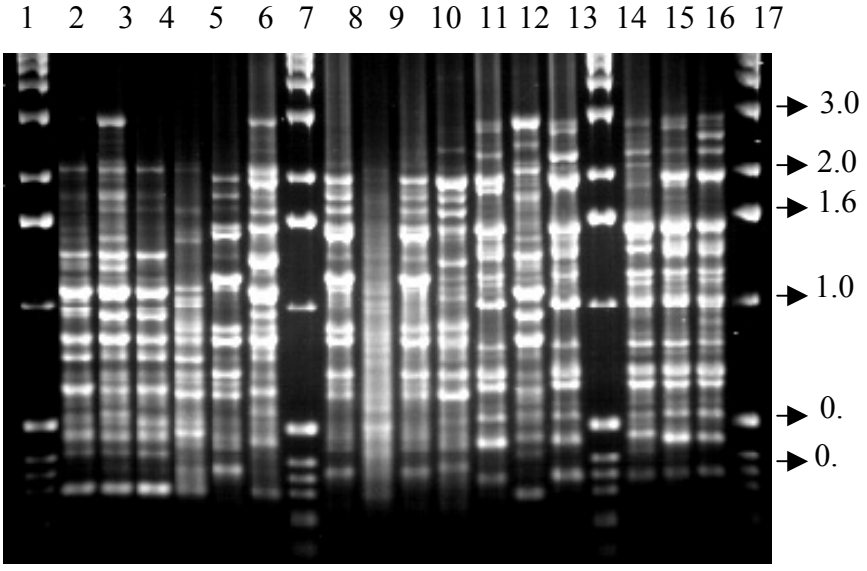


Figure 9: PCR fingerprinting profile with the M13 core sequence. Lanes 1, 8, 16 and 20: molecular size markers in kb; lane 2: *L.infantum* (MHOM/TN/80/IPT1; Lane 3: *L.donovani* (MHOM/KE/85/NL/B323); Lane 4: *L.chagasi* (MHOM/BR/74/PP75); Lane 5: *L.major* (MHOM/SU/73/5ASKH); lane 6: *L.tropica* (MHOM/SU/74/SAF-K27); lane 7: *L.aethiopica* (MHOM/ET/94/Abauye; Lane 9: S(h)22; Lane 10: S(h)23; Lane 11: S(h)24; Lane 12: S(h)25; Lane 13: S(h)26; Lane 14: S(h)27; Lane 15: S(h)28; Lane 17: *L. brasiliensis* (MHOM/BR/75/M2903); Lane 18: *L.guyianensis* (MHOM/BR/75/M4147); Lane 19: *L.panamensis* (MHOM/CR/87/NEL3).

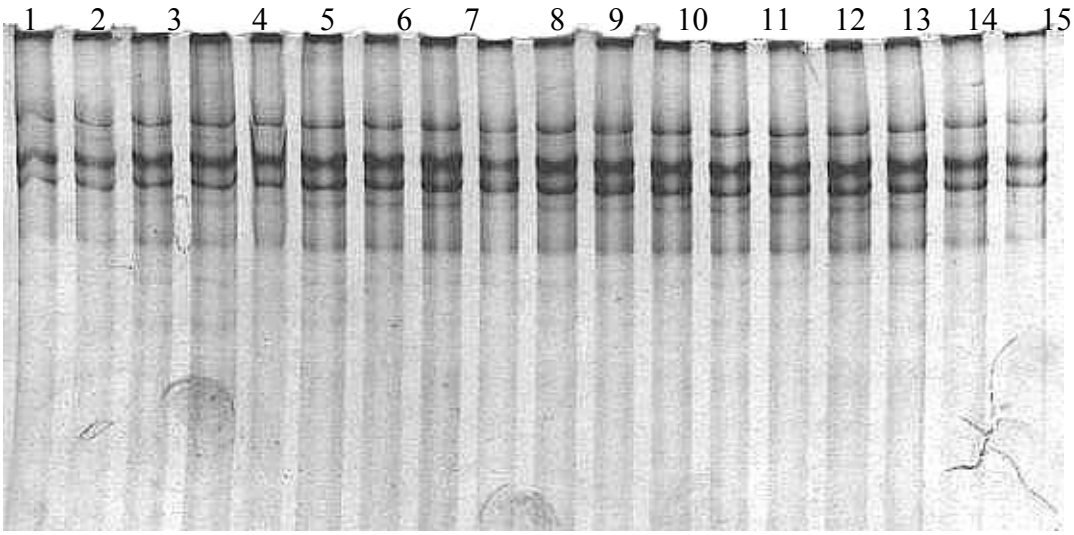


Figure 10: SSCP pattern of the PCR product of the primer pair L0110. Lanes 1-18 contain amplicons of canine isolates S(d)1-S(d)18 that were identified to belong in the *L.infantum* by PCR fingerprinting with arbitrary primers.

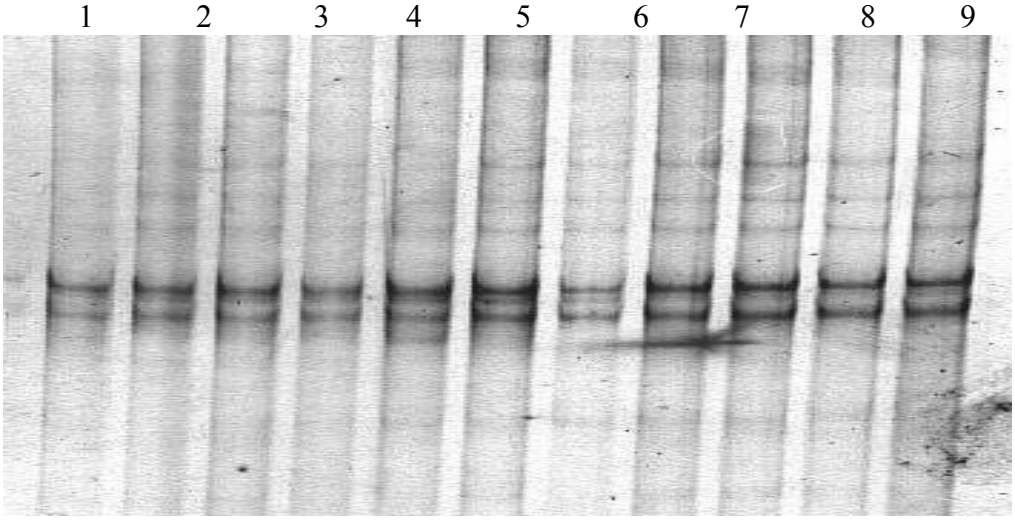


Figure 11: SSCP pattern of the PCR product of the primer pair L0720. Lanes 1-11 contain amplicons of canine isolates S(d)1-S(d)11 that were identified to belong in the *L.infantum* by PCR fingerprinting with arbitrary primers.

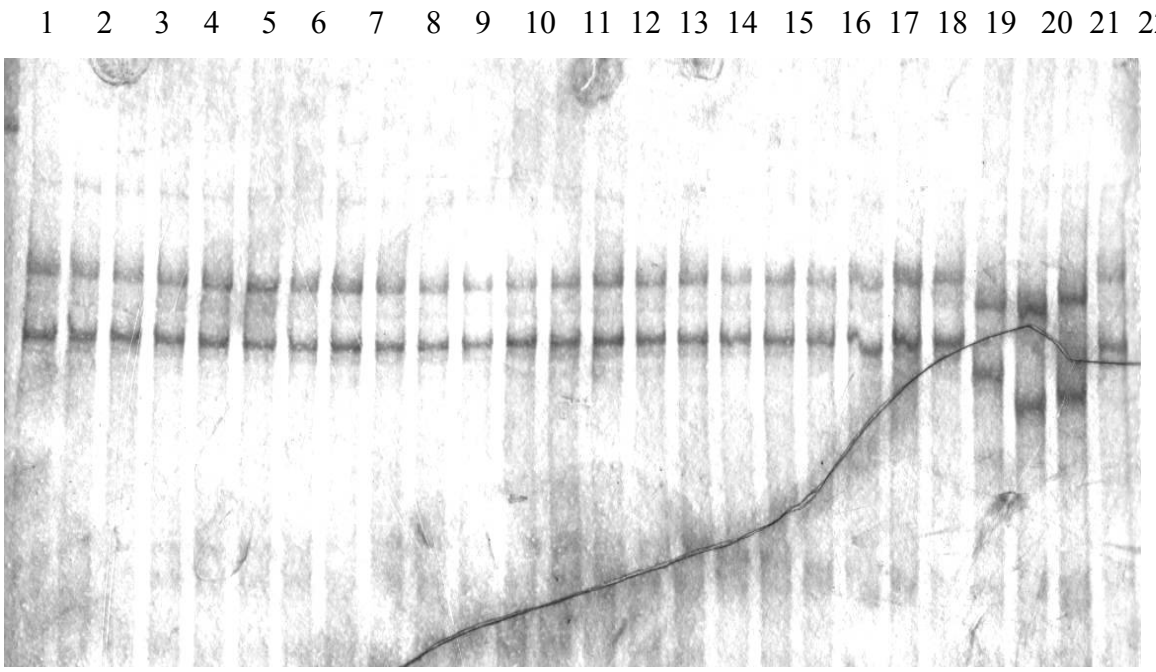


Figure 12: SSCP pattern of the PCR product of the primer pair L1112. Lanes 1-20, S(d)2-S(d)21; Lane21, MHOM/TN/80/IPT1; Lane 22, MHOM/ES/87/Lombardi; Lane 23, MHOM/CN/78/D2; Lane 24, MHOM/FR/80/189; Lane 25, MHOM/IL/89/LRC-L571. All lanes contain amplicons of canine isolates that were identified to belong in the *L.infantum* by PCR fingerprinting with arbitrary primers.

5. DISCUSSION

The main aim of this work has been the evaluation of PCR methods for detection, species identification and determination of genetic variation in *L. infantum*. In that frame a greater scale of *Leishmania* isolates from patients, humans and dogs were processed through for detection of the parasite, detection of the species and further to search genetic variation in *L. infantum*. The combination of ITS-RFLP, fingerprinting with single arbitrary primers and PCR-SSCP with codominant markers, produced results that contributed to all directions. In particular the experiments aimed to characterise isolates from Greece and the greater Mediterranean area.

Concerning identification of leishmanial parasites in Greece very little is known although the disease is endemic there. There is only one recent paper by Aransay *et al*. (2000), applying a semi-nested PCR, based on the minicircle sequence of *L. donovani* described by Smyth *et al*. 1992. By comparison of the sequence of the PCR products with *Leishmania* sequences of the Genomic data bank in the internet, they concluded that the amplification products most probably belonged to *L. infantum* and *L. donovani*. Earlier works using isoenzyme analysis revealed the existance of *L. infantum* (MON-1) in both human and canine patients, and *L. tropica* (MON-57 and MON-114) in humans (Frank *et al*., 1993). *L. infantum* was isolated from *Phlebotomus neglectus* on the island of Corfu (Léger *et al*., 1988).

The PCR technique applied in this study to detect the parasites was originally developed by Meredith *et al*., (1993). Osman used it in greater scale epidemiological studies, for diagnosis (Osman *et al*., 1997; 1997b; 1998) and management (Osman *et al*., 1997; 1997b; 1998; 1998b; 1998c) of the disease. They amplified a 560 bp variable fragment of the ssu rRNA gene.

The diagnostic PCR (§3.1) in our hands proved to be reliable test. It gave a positive result to all the isolates that had been previously identified as *L. infantum* and all the *L. infantum* characterised strains from Table 2 (§ 3.1). Also all the 24 samples from dogs (BS1-BS24) diagnosed with leishmaniasis in Germany after having travelled in South Europe (21 bone marrow, 1 lymph node and 2 blood) (§ 3.1), were positive when processed through this PCR. In the latter cases it corresponds to 100 % with previous diagnosis by axenic culture inoculation (Steuber, personal communication).

The assay used in the present study seems a very promishing *Leishmania* detection method and it should maybe considered becoming part of the officially by the WHO recommented diagnostic routine methods for the *L. donovani* complex.

Kinetoplastid specific sequences were previously used in hybridization assays but the results were not as good as expected because of the intrataxon variations in restriction patterns. Hybridization efficiency and sequence of the minicircle made kDNA an unlikely candidate for a simple classification of *Leishmania* (Rodgers *et al*., 1986; van Eys *et al.*, 1989). The ssu rRNA gene was originaly used by van Eys *et al*., 1992. The DNA as well as the RNA of the ssu rRNA could be used a target for a PCR assay. The nuclear DNA contains about 160 copies of the ssu rRNA gene and the cytoplasm contains more than 104 ssu rRNA molecules (Leon *et al.*, 1978).

In the dendrogrammes produced from the results of the fingerprinting with single arbitrary primers isolates of the same species do cluster together. The patients' isolates (Results § 4.3, Table 6) can be correlated to an isolate of the reference strains when the ITS product is digested with either restriction enzymes HaeIII or CfoI (Materials and Methods § 3.1, Table 2). Based on these results the same isolates were further prosessed to the fingerprinting with the single arbitrary primers.

The ITS region seems to be very concerved in all representatives of *L. infantum*. Nineteen (19) well characterised *L. infantum* strains (INF-01 to INF-19), the isolate CHA-01, 21 isolates from dogs (S1-S21) and 2 human isolates that were processed through the ITS-RFLP. They were identical not only with the *L. infantum* but also with the *L. donovani* and *L. chagasi* reference strains. Of those 42 were were further characterised via fingerprinting with single arbitrary primers as *L. infantum* and one as *L. chagasi*. Corresponding results were obtained for *L. donovani* isolates with ITS-RFLP by Schönian (personal communication), they observed some variation which we could not find in our isolates. The heterogeneity reported by El Tai *et al*. (2000), was due to point mutations that did not affect the digestion sites of the sequence. So overall it seems that there were almost no intra-complex, or intra-species variations, in the *L. donovani* complex (including *L. infantum*, *L. donovani* and *L. chagasi*), when refering to the ITS region. This is not the case for the New World *Leishmania* species (Cupollilo *et al*., 1995), or the *L. aethiopica* and *L. tropica* (Schönian *et al*., 2000; Schönian *et al*., personal communication).

When the PCR-fingerprinting with the single arbitrary primers was applied, the polymorphisms of different species and strains of *Leishmania* and merely *Leishmania infantum* were assesed. It has been suggested that polymorphic DNA markers amplified with single non specific primers may be very accurate indicators of genetic distances because this PCR randomly samples sequence polymorphisms distributed in the genome (Welsh *et al*., 1992). In most cases this

method detects DNA polymorphisms that are generated from primer sites in one sample beeing exactly complementary to the primer, whereas the priming site of another sample is not an exact complement to the primer.

Polymorphisms may be due to single base changes in genomic DNA, deletions and insertions that change the size of the DNA fragment, deletions of a priming site, or insertions that render the priming sites too distant to support amplification. Since amplification perameters influence strongly the resulting patterns, it is necessary that the protocols used are well optimised, especially concerning the primer/template ratios, annealing temperature and Mg^{2+} ions concentration.

Our data indicate that relationships of *Leishmania* species, as compared the PCR profiles, were consistent with previous taxonomic studies (Beverley *et al*., 1987; Makedo *et al*., 1992; Schönian *et al*., 1996). Dinstinctive and reproducible sets of amplified DNA fragments were obtained for all *Leishmania* isolates tested. The PCR profiles within the main *Leishmania* complexes (*L. donovani, L. mexicana*, and *L. brasiliensis*) are clustering together but they are still distinct with one another. The unrooted distance tree based on the combined data matrices (Dendrogramme 5) that was obtained with each primer reveal that all *Leishmania* species tested clustered into two major group of *Leishmania* species: the subgenera *Viannia* (*L.braziliensis* complex) and *Leishmania*. This branching is well supported by bootstrapping. Within the human isolates S(h)22-S(h)30, isolates S22-S25 belonged to *L. aethiopica* and *L. tropica* when using the M_{13} primer, to *L. aethiopica* in case of the T_3B primer, while when employing the $(GACA)_4$ primer, S(h)22, S(h)24 and S(h)25 belong to *L. tropica* and S(h)23 to *L. aethiopica.* In the ITS-RFLP S(h)22, S(h)24 and S(h)25 seemed to belong to *L. tropica* and S(h)23 to *L. aethiopica* whereas in the Dendrogramme 5 of the combined data they cluster together in a greater cluster that is shaped by the clusters of *L. tropica* and *L. aethiopica*. However it is possible that those isolates derive from a mixed infection from both species. They are to a great part co-endemic. That is also possible in the case of S(h)26 and S(h)28, which primed with M_{13} and T_3B, cluster with *L.brasiliensis* and with $(GACA)_4$ with *L.guyanansis.*

The *L. infantum* isolates, INF-01 to INF-19 and S(d)1-S(d)21, a total stock of 40 isolates, 9 human and 31 canine ones, was easily identified as *L. infantum* with each of the 4 single primers. As obvious from dendrogrammes 1, 2, 3 and 4 grouping and subdivisions of the isolates were correlated merely to the geographical origin. Combined data for the *L. donovani* complex only (*L. infantum, L. donovani, and L. chagasi*), were obtained when the isolates were primed with M_{13}, T_3B and $(GACA)_4$ (Dendrogramme 6). Groupings are supported very well

from bootstrap values and again the subgroupings of each isolate correlates to the geographical distribution

An interesting isolate is S(h)29. It derives from a patient who within a year travelled to Spain, Mexico and Brasil, countries where both American and Old World VL are endemic. This geographic origin is not of any help in this case. When it was processed through the ITS-RFLP it gave patterns of the *L. donovani* complex. When it was afterwards processed through the fingerprinting, it clustered with the CHA-01 when primed with M_{13}, T_3B and $(GACA)_4$ with very high bootstrap values in all three cases. Nevertheless it stands very close to the other two species within the *L. donovani* complex. However, the codominant marker L1112 was a great help because it led to a different set of pattern for the isolates CHA-01 and the S(h)29. This result supports the opinion that *L. chagasi*, the causative agent for American VL, has been imported by humans from the Old to the New World (Momen *et al*., 1993).

The RFLP analysis of the ITS region after digestion with the restriction enzymes HaeIII and CfoI enabled to distinguish the parasites at complexes level. It is a method that in the future can be also used in clinical samples (biological material as blood, bone marrow etc. infected with the parasite) since the primers are specific for the *Leishmania* genus.

The fingerprinting with the arbitrary primers distinguished and identified the isolates at species level even within the complex. Concerning species identification, the results of each primer are confirmed by the results of the others. In the fingerprinting with single arbitrary primers pure cultured parasites are needed because the primers are not species specific .

Concerning the primer pair L1119, it produces results that agree with these of the four fingerprinting systems with the single arbitrary primers eventhough the bootstrap values are low. The different species are clustering separate while species of the same complex are shaping greater clusters. Concerning *L. infantum,* predominantly the Greek canine isolates group together. In conclusion there seems to be very little variation within the *L. infantum*, group.

The codominant markers are able to detect both alleles in a diploid. Those used in this study were designed for epidemiological and population genetic studies in *L. donovani* (*L. donovani, L. infantum* and *L. chagasi*) complex (Schönian, personal communication). Seven anonymous markers were applied to the INF-01 to INF-19, CHA-01, S(d)1-S(d)21 and S(h)29. In those samples only the marker L1112 revealed polymorphisms. Two French human isolates (INF-05

and INF-06) give a common pattern, a Chinese human (INF-04) a second, a Spanish canine (INF-19) a third and interestingly the CHA-01 and the S(h)29 another fourth one. The latter two are interesting because up to now differences between *L. infantum* and *L. chagasi* were rarely found and the one is mostly considered to be a geographical deportation of the other (Burns *et al*., 1993; Carreira *et al*., 1995). The ability to detect both alleles in a diploid organism such as *Leishmania* is desirable for population studies. Information about the heredity mode of genes, whether dominant tendency of expression or not in case of heterogygous populations and heterogygoty itself in case of recombinal mode of propagation of the parasite are of major importance. Furthermore, those markers can be possibly amplified directly from clinical samples without cultivation of the parasites, while PCR fingerprinting methods need pure parasite material.

Is is interesting to note that in the case of combined data used for Dendrogramme 5, four isolates INF-05, INF-04, INF-01 and INF-03, coming from France, China, Tunisia and Spain, respectively are clustering together in the same subgroup with a bootstrap value of 100 %. This is a good indication that the variation within *L. infantum*, in general, is not very high, no matter the geographical region most probably even the host of origin of the isolate and fingerprinting allows differenciation due to the geographical origin. At the fingerprinting with the single arbitrary primer T3B (Dendrogramme 2) all the Greek canine isolates have identical patterns. The same is true for the isolates INF-15 and INF-17 both canine from Israel. Identical patterns gave also INF-09 INF-10 INF-13, INF-14, and INF-11 (4 canine and 1 human respectively), one isolate from Portugal (INF-09), two from Turkey (INF-09, INF-10) and two from Israel (INF-13, INF-14), i.e. parasites from distant areas and different hosts.At the fingerprinting with the primer $(GTG)_5$ the 20 of the 21 Greek canine *L. infantum* isolates gave once again identical patterns. The fingerprintings of the *L. infantum* isolates with the M13 and $(GACA)_4$ primers gave few sporadic identical patterns between isolates. Differences due to the geographical origin are supported even better in the combined data Dendrogramme 6 for the *L. donovani* complex. The denrogramme gives distinct clades to each strain, showing that each strain is formated by a unique group of molecular characteristics. The bootstrap values are also very high in all clades of the tree.

Independent assortment or recombination by crossing over during meiosis can scramble the parental alleles to give non parental combinations. The farther apart two genes are on a chromosome, the more likely recombination by crossing over between them will be. That's how theoritically recombination might simplisticaly take place. In 1998 Britto *et al*., showed

that the haploid genome of Old World *Leishmania* contains 36 chromosomes, while the number is different in the *Viannia* and the New World *Leishmania* subgenera. Along with that there are studies that suggest that *Leishmania* chromosomes are largely diploid with some aneuploid chromosomes (Andrews *et al.,* 1988). On the other hand if reproduction was strictly clonal the genotype would be replicated as a unit and independently propagating clonal lineages could evolve highly divergent karyotypes. Since the absolute ploidity and sexuality of *Leishmania* are uncertain, the importance of chromosomal recombination in the evolution of *Leishmania* also remains an area of speculation. Of course there is always the possibility that the different developmental stages are accompanied by chromosomale rearrangements eventhough there are no such reports yet.

The very limited degree of genetic variation among the strains of *L. infantum* from Greece in this study is consistent within the results of all the methods that were used (ITS-RFLP, fingerprinting with single arbitrary primers, PCR-SSCP). In a recent work from Spain (Jimenez *et al*., 1995) certain zymodemes of *L. infantum* were found exclusively in immunnocompromised patients and were absent from the typical cases of canine VL and CL. It was suggested that there is either an anthroponotic pattern of leishmaniasis where e.g. drug users act as the reservoirs for the new zymodemes, or that the cellular immune system could select virulent from non-virulent zymodems in immunocompetent VL patients. In a follow up study (Jimenez *et al*., 1997) the parasite population isolated from AIDS patients was of the same dominant "clonal" genotype (MON1). Thus once again strong linkage disequilibrium, over-representation of genotypes and overall lack of genotype diversity was shown.

Despite the broad enzymatic heterogeneity (WHO, 1990) that has been demonstrated from other workers among strains of *L.infantum* a clonal mode of propagation has been proposed for this species (Tibayrenc *et al*., 1990; Jimenez *et al*., 1997; Banuls *et al*., 1999). This hypothesis was based on (a) the existence of over-represented, geographically widely distributed genotype (zymodeme MON-1), and (b) the strong linkage disequilibrium in this species. However, occasional genetic exchanges are not excluded in predominantly clonal populations.

In the present study the hypothesis of clonality within *L. infantum* is supported from the combination of the ITS-RFLP results with the results of the fingerprinting with the single arbitrary primers and the PCR-SSCP. Particularly the ITS-RFLP fails to show variation within the *L. donovani* complex, while there is some heterogeneity demonstrated with the other two

methods. In principle this implies a tendency of conservation within the *L. donovani* complex that which suggests a more clonal development within *L. infantum.* It might be important in the future, to compare and combine species phylogenies obtained from single-primed PCR profiles with those derived from DNA sequencing data.

Clonality may be of importance not only for diagnosis of the disease but also for therapy, control methods, monitoring and management of the applied measures. Since we suggest that the *L. infantum* is in the majority clonal, it is easier to plan, apply and monitor strategic control methods, especially in an area like the Medditeranean. The problems of arising resistance development due to multiple recombination and mutation, after long time of drug therapy, are at least potentially reduced. All those factors together, are assembling a good potential when epidemiology some time will be used for systematic control of the infantile leishmaniasis.

When we applied the ITS-RFLP and the fingerprinting with single arbitrary primers we had two main goals: first to discrinimate amongst often closely related species and second to establish the quantitative degree of evolutionary relationship. The use of ITS-RFLP is an excellent tool to aquire an original direction and to discriminate the complex an isolate belongs to. The further exact identification of the species is resulting by the use of the PCR assay with the single arbitrary primers. The results can be confirmed by the parallel use of more than one single arbitrary primers. The combination of the two methods is a great weapon in the diagnostic quiver of leishmaniasis. Both are not complicated and after establishing them in the laboratory routine one can easily process a great number of isolates through. It would be worth while to apply them in an even greater scale using more isolates and comparing patterns of different species in a greater scale. It would be definitely a fascinating exploration of the variation also in other *Leishmania* species.

6. SUMMARY

The aim of this study was the evaluation of PCR methods for detection, species identification and determination of genetic variation in *L.infantum*.

For this reason a total of 36 well-characterised *Leishmania* strains, 21 canine isolates from Northern Greece, 9 human *Leishmania* isolates from various locations and 24 clinical samples from German dogs that had travelled to South Europe, suffering from clinical leishmaniasis, were used.

First the internally transcribed spacer (ITS) was amplified and the amplification products were digested with the restriction enzymes HaeIII and CfoI. The 36 well-characterised *Leishmania* strains, the 21 canine and 9 human isolates were tested. This process allowed to characterise the isolates to the complex level and some to the species level (*L. aethiopica*, *L. tropica*). The patterns acquired for the *L. donovani* complex (*L. infantum, L. donovani, L. chagasi*) were identical.

The same isolates were subsequently tested with fingerprinting with single arbitrary primers. The primers used derived from the core sequence of the phage M13, intergenic tRNA genes' spacers (T3B) and the repeat sequences $(GTG)_5$ and $(GACA)_4$. The 21 canine isolates were all identified as *L. infantum* three (3) of the human isolates corresponding with *L. tropica*, one (1) with *L. aethiopica*, one (1) with *L. donovani*, one (1) with *L. chagasi*, one (1) with *L. brasiliensis* and two (2) with *L. guayanensis*. All produced polymorphic patterns which were grouping depending on the species they belonged to, next to the relevant well-characterised strains of the same species. Within the *L. infantum* group the subgroupings formed were mainly related to the geographical origin of the strains.

A polymerase chain reaction-single strand conformation analysis (PCR-SSCP) using a set of 8 codominant primer pair markers was employed to test a total of 42 *L. donovani* complex strains (38 *L. infantum*, 2 *L. chagasi* and 2 *L. donovani*). Only one primer pair marker gave some variation patterns at the SSCP for the *L. chagasi* reference strain and a human isolate of debatable origin. Both had the same SSCP-pattern as well as the same fingerprinting pattern with single arbitrary primers.

A simple PCR detection system for *L. donovani* which amplifies a 560 bp variable fragment of the ssu rRNA gene was tested for general applicability in diagnostics. The 42 *L. donovani* complex strains and the 24 canine samples from clinical cases were processed and the sensitivity of this PCR was 100 %.

Digestion of the ITS in combination with the fingerprinting with single arbitrary primers was able to distinguish the parasites to the species level and revealed strain variations within the species. The variations detected with PCR fingerprinting with arbitrary primers were mainly depending to the geographical origin of the isolates.

Data analyses, which combined the results from the ITS-RFLP and fingerprinting with single arbitrary primers and PCR-SSCP, suggest that among the strains of *L. infantum* there is most probably a clonal mode of propagation. Nevertheless, occasional genetic exchanges can not be totally excluded in predominantly clonal populations.

7. ZUSAMMENFASSUNG

Ziel dieser Studie war die Überprüfung der PCR als Methode zür Erfassung und Identifizierung von Arten der *Leishmania* (incl. *Viannia*) sowie Bestimmung von genetischen Varianten in *L.infantum.*

Züdiesem Zweck wurden insgesamt 36 gut bekannte Leishmanien-Stämme, 21 Isolate aus nordgriechischen Hunden, 9 Isolate aus Menschen unterschiedlicher geographisches Herkunft und 24 Proben aus erkrankten Hunde naus Deutschland, die sich zuvor in Südde Europa aufgehalten hatten, untersucht.

In einen ersten Serie wurde in den Probender ITS (Internally Transcribed Spacer) amplifiziert. Die restriktionsenzymatische verdaüng der Amplifikate mit HaeIII und CfoI. Gestattete es die Isolate bis auf die Ebene derArten-Komplexe und zum Teil auch auf die Ebene der Spezies zu charakterisieren/identifizieren (*L. aethiopica*, *L. tropica*). Die Muster, die für *L. donovani*-Isolate gefunden wurden, waren fast identisch.

Die Isolate wurden anschließend mit Hilfe genetischer „Fingerprints“ mittels willkürliche er (arbitrary)Primer getestet. Die benutzten Primer stammten aus der Kernsequenz des Phagen M13, des intergenetischen „tRNA genes’ spacers“ (T3B) und der Wiederholungssequenzen (GTG)5 und (GACA)4. Die 21 Isolate aus den Hunden wurden alle als *L. infantum* identifiziert. Aus den 9 menschlichen Proben wurden drei als *L. tropica*, jeweils eine als *L. aethiopica*, *L. donovani*, eine (1) als *L. chagasi*, eine (1) als *L. brasiliensis* und zwei (2) als *L. guayanensis* identifiziert. Sie alle zeigten polymorphe Muster, die mit den Referenzstämmen gepaart werden konnten. Innerhalb der Species *L. infantum* wurden Untergruppen unterschieden, die hauptsächlich mit ünterschiedlichen geografischen Herkunft der Isolate züsammenliegen.

Eine PCR-SSCP (Polymerase Chain Reaction - Single Strand Conformation Analysis) benutzte einen Satz aus 8 codominanten „Primer Pair“-Makern und würde zür untersuchung von 42 Isolaten/Stämmen aus dem *L. donovani*-Komplex (38 *L. infantum*, 2 *L. chagasi* und 2 *L. donovani*) herausgezogen. Nur ein Primer Pair Maker zeigte einige unterschiedliche Muster in der SSCP an, die ein Isolat als *L. chagasi* Stamm und vier (4) als *L. infantum* Stamm identifizierte.

Im Sinne eines praktischen diagnostischen Verfarens züm Nachwers von *L. donovani* läß sich für ein einfaches PCR-süchsystem, beidem ein variables 560 bp fragment des ssü rRNA-Gens amplifiziert wird, eine 100 % ige sensitivität zeigen, d.h. alle als *L. donovani* bekannten Identifizierten Stämme/Isolate würden erfaßt. Eine Kombination der restriktionenzymatischen analyse des ITS-Amplifikate mit dem genetischen „Fingerprint“ ermöglichte eine ünterscheidung der arten und zeigte varianten auf. Die beobachteten varianten läßen sich der geographischen Herkunft der Stämme/Isolate züordnen.

Die Analyse der Daten ünter Einbeziehüng der Ergebnisse aus dem ITS-RFLP, dem genetischen „Fingerprinting“ und der PCR-SSCP läßt vermuten, daß es innerhalb der Art *L. infantum* einen klonalen Verbreitungsmodus gibt. Dennoch kann gelegentlicher genetischer Austausch in sonst hauptsächlich klonalen Populationen nicht ausgeschlossen werden.

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Acknowledgements

This work has been the combilation of efforts of more people than myself. Without them it would definitely not be the same!

I would like to thank my “Doktor Vater” Prof. Dr. vet. med. H. Zahner from the Institute of Parasitology of the Justus Liebig University, Giessen, Germany. His litteraly fatherly embracement and instruction to this work and to myself has been a firm steering wheel at very critical times. His intervention was always timely to turn the ship to the right direction. Thank you very much for receiving me so warmheartedly!

I would like to thank Prof. Dr. med. H.M. Seitz from the Institute for Medical Parasitology, University Clinics of the Rheinische Friedrich-Wilhelm University, Bonn, Germany. His trust and support during the whole time has been important especially at times of dead ends where he would find a way out as long as I would do the work as he would tell me. Thank you very much for having me in Bonn. It has been a previledge to know somebody like you so sharp in his job as a parasitologist!

I would like to thank Dr. rer. nat. G. Schönian from the Institute for Microbiology and Hygiene, Charité Hospital, Humboldt University, Berlin, Germany. Gabriele has mothered this work and supervised it very effectively even from a distance. Her positive attitude that it will work out when it seemed that it wouldn’t, has been something to learn from. Thank you Gabi for been an example for me not only as a scientist but also as a leader and a woman!

It has been a previledge in times like the ones we live in, to have the chance to be in Germany and to follow up all the latest developments, be so close to places where discussions are taking place and decisions are taken. In that context I would like to thank all those friends like Andrea and Elsa Tuchen and Gerhard Pürschel that lovingly welcomed me, supported me in different ways in this country and believed that in the end there would be a completed work. To those people that made this country a home for me all those years I will be endeabted always! Thank you for being here!

Last but not least I would like to thank my family, my parents and my brother in Greece. Their love, support and faith in me has always been a light to the dark nights of my soul and I know that it will always continue to be. They have been instrumental for all my life, they have been instrumental for this work. Thank you for being my family!

I wish that the experience I gained from this work will profit myself and others to the good in the future!

Köln 07.01.2003

Maria Kokozidou