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DISSERTATION

Population genetic study for evaluating the differentiation and gene flow among Eastern Mediterranean strains of the *Leishmania donovani* complex

zur Erlangung des akademischen Grades
Doctor rerum medicarum (Dr.rer.medic)

vorgelegt der Medizinischen Fakultät Charité - Universitätsmedizin Berlin

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Datum der Promotion: 30 May 2015

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Acronyms and abbreviations

AL	Albania
BG	Bulgaria
CanL	canine visceral leishmaniasis
CL	cutaneous leishmaniasis
CY	Cyprus
DNA	deoxyribonucleic acid
ES	Spain
FCA	factorial correspondence analysis
F_{IS}	inbreeding coefficient
FR	France
F_{ST}	F-statistics, fixation index
GDA	genetic data analysis
GR	Greece
HCL	human cutaneous leishmaniasis
*H*e	expected heterozygosity
HIV	human immunodeficiency virus
*H*o	observed heterozygosity
HR	Croatia
HVL	human visceral leishmaniasis
L.	*Leishmania*
MEGA	molecular evolutionary genetic analysis
MLEE	multilocus enzyme electrophoresis
MLMT	multilocus microsatellite typing
MON	Montpellier system
NJ	neighbor-joining tree
P	proportion of polymorphic loci
P.	*Phlebotomus*
PCR	polymerase chain reaction
PT	Portugal
TR	Turkey
VL	visceral leishmaniasis

1. Abstract

The present study aimed to assess the population genetic structure of *L. donovani* complex strains from eastern Mediterranean focusing on southeastern (SE) Europe, where leishmaniasis represents a major public health problem. *L. infantum* zymodeme MON-1 is the main causative agent of human (HVL) and canine visceral leishmaniasis (CanL), and occasionally of human cutaneous leishmaniasis (HCL) in this area. The evident re-emergence of cases in classical endemic foci and reports of imported or autochthonous cases in formerly non-endemic regions calls for new epidemiological studies using highly discriminating markers.

Leishmania strains were isolated from HVL, CanL and HCL cases in six countries ranging from Croatia to Turkey, and from *Phlebotomus tobbi* sand flies in Turkey. These strains were initially typed by the *K26*-PCR assay, which amplifies the HASPB region, and multilocus enzyme electrophoresis (MLEE). Both methods corroborated the predominance of *L. infantum* MON-1 in SE Europe. MLEE exposed also a considerable number of zymodeme MON-98 strains in Turkey and Greece. All human isolates from Cyprus were identified as *L. donovani sensu stricto MON-37*. The Turkish strains from Cukurova and one HVL isolate from Kusadasi represented two novel zymodemes, MON-308 and MON-309.

Multilocus microsatellite typing (MLMT) was performed to exploit genetic diversity and population structure of the SE *L. donovani* complex strains. It assigned the SE and southwestern (SW) European strains to clearly distinct genetic populations. Croatian strains were divided in-between reflecting the geographic position of this country. All Cypriot canine isolates constituted a monophyletic group suggesting the circulation of a homogenous MON-1 population among dogs. MLMT did not support the differentiation between MON-1 and MON-98 zymodemes. Strains of these zymodemes from Turkey, Bulgaria, Greece and Albania grouped together in different highly heterogeneous populations. Our data point to recombination events and substantial gene flow among them. MLMT showed that strains of zymodeme MON-37 are paraphyletic. The Cypriot MON-37 strains formed a unique genetic group and were clearly distinct from MON-37 strains from the Indian subcontinent, Middle East and East Africa. Thus, the Cypriot MON-37 strains could possibly be autochthonous. When the Turkish MON-308 and MON-309 strains and the Cypriot MON-37 were compared to other *L. donovani* complex strains, the Turkish and Cypriot strains formed a new monophyletic group, clearly distinct from all other previously identified groups. Our observations suggest the existence of hybrids, aneuploidies or mixed infections for these Cypriot and Turkish strains thus raising concerns about their spreading to neighboring Mediterranean countries.

Zusammenfassung

Das Ziel der vorliegenden Studie war eine populationsgenetische Untersuchung des *L. donovani*-Komplexes mit dem Fokus auf Südost-Europa (SO), wo Leishmaniosen ein wichtiges Gesundheitsproblem darstellen. *L. infantum*-Stämme des Zymodems MON-1 sind die häufigsten Erreger humaner (HVL) und caniner (CanL) viszeraler Leishmaniosen, verursachen aber auch gelegentlich humane kutane Leishmaniosen (HCL) in diesem Gebiet. Das Wiederaufleben der Erkrankungen in klassischen Endemiegebieten und Berichte über importierte oder autochthone Erkrankungen in früher nicht-endemischen Gebieten erfordern neue epidemiologische Studien und die Anwendung hoch-diskriminierender Marker.

In dieser Studie wurden *Leishmania*-Stämme untersucht, die von HVL-, CanL- und HCL-Fällen in 6 Ländern, von Kroatien bis zur Türkei, und von *Phlebotomus tobbi* Sandmücken in der Türkei isoliert wurden. Die Stämme wurden zunächst mit der *K26*-PCR, die den HASPB-Lokus amplifiziert, und der Multilokus-Enzym-Eelektrophorese (MLEE) typisiert. Beide Methoden haben die Prädominanz von *L. infantum* MON-1 in SO bestätigt. Die MLEE hat auch eine große Anzahl von Zymodem MON-98 Stämmen in Griechenland und der Türkei nachgewiesen. Alle zypriotischen humanen Isolate wurden als *L. donovani sensu stricto* MON-37 identifiziert. Die türkischen Stämme aus Cukurova und ein HVL-Isolat aus Kusadasi repräsentieren zwei neue Zymodeme, MON-308 und -309.

Die genetische Diversität und die Populationsstruktur der SO-Stämme des *L. donovani*-Komplexes wurde mittels Multilokus-Mikrosatelliten-Typisierung (MLMT) untersucht, welche die Stämme aus SO und Südwest-Europa (SW) genetisch unterschiedlichen Populationen zuordnete. Kroatische Stämme waren in beiden Populationen zu finden, in Übereinstimmung mit der geografischen Lage dieses Landes. Alle Hundeisolate aus Zypern bildeten eine monophyletische Gruppe, was auf die Zirkulation einer homogenen MON-1-Population unter diesen Hunden schließen lässt. Die MLMT konnte keine Unterschiede zwischen MON-1- und MON-98-Stämmen feststellen. Stämme beider Zymodeme aus der Türkei, Bulgarien, Griechenland und Albanien wurden verschiedenen sehr heterogenen Populationen zugeordnet, mit deutlichen Hinweisen auf Rekombinationen und Genfluss zwischen ihnen. Mittels MLMT wurde gezeigt, dass die MON-37-Stämme paraphyletisch sind. Die zypriotischen Stämme bildeten eine eigenständige genetische Gruppe, unterschieden sich klar von den MON-37-Stämmen vom indischen Subkontinent, vom Mittleren Osten und Ostafrika und könnten autochthonen Ursprungs sein. Der Vergleich der türkischen MON-308- und MON-309-Stämme sowie der zypriotischen MON-37-Stämme mit Stämmen anderer Herkunft ergab, dass diese türkischen und

zypriotischen Stämme zu einer neuen monophyletischen Gruppe im *L. donovani*-Komplex gehören, die sich klar von allen bisher nachgewiesenen Gruppen unterscheidet. Die Existenz von Hybriden, Aneuploidie und Mischinfektionen in dieser Gruppe ist wahrscheinlich und ihre weitere Ausbreitung in benachbarten Ländern zu befürchten.

2. Introduction

Leishmaniasis constitutes a complex of vector-borne diseases caused by obligatory intra-macrophage protozoan parasites of the genus *Leishmania* and transmitted by the bite of female phlebotomine sand flies. Leishmaniasis remains a major public health problem, grossly underestimated for years and currently classified as one of the world's most neglected tropical diseases, whilst affecting mostly the poorest of the poor in developing countries. An estimate of 310 million people are at risk of infection, 1.3 million new cases and over 20,000 deaths are reported annually in 101 countries and territories widespread on all continents except Antarctica [1].

For centuries, leishmaniasis has been endemic across the Mediterranean basin and most often exhibits one of two clinical entities in humans, HVL and sporadic HCL. *Leishmania infantum*, one of the two taxa of the *L. donovani* complex, is the main causative agent of human and canine visceral leishmaniasis (HVL and CanL respectively) and also accountable for sporadic human cases of cutaneous leishmaniasis (HCL) in the Mediterranean area. *L. donovani*, the other species of the *L. donovani* complex, is known to exist in East Africa and the Indian subcontinent causing most of leishmaniasis burden in terms of mortality and morbidity. The concurrent dermotropic species *L. tropica* is present in Greece, Turkey, the Middle East and North Africa, and *L. major* is highly prevalent in the east and south Mediterranean region being responsible for zoonotic CL. In contrast to the anthroponotic species *L. donovani*, the transmission of *L. infantum* depends on a zoonotic cycle, in which dogs are the principal reservoir hosts [1].

The natural spread of VL and CL due *L. infantum* species is one of the current risks in European Mediterranean countries [2]. Being previously confined to coastal Mediterranean biotopes, autochthonous leishmaniasis is not limited to these habitats anymore but expands towards new biotopes at northern latitudes and higher altitudes, representing an emerging threat for central and northern European countries [3]. The re-emergence of leishmaniasis in the Mediterranean region of Europe is another challenging current risk that is boosted by the mounting number of HIV/*Leishmania* co-infections in some countries [4]. A sharp re-emergence is being witnessed in traditionally endemic southwestern (SW) European countries, for

example in Spain [5]. Looking at southeastern (SE) European countries, both VL and CanL have re-emerged across the mainland and the island of Crete in Greece and an HVL outbreak with an unexpectedly high morbidity rate (50.7%) was recorded in south Bulgaria (for review see [6]). HVL is now widespread across north and central Albania with high morbidity and lethality affecting mostly children and both HVL and CanL are present in central and southern Dalmatia (for review see [6]). Despite this dynamic recurrence of VL in southern Europe, the epidemiology of VL there has been inadequately or never examined so far.

Another alarming issue rises from the potential introduction of exotic *Leishmania* species into Europe. In Cyprus, five HVL and HCL cases were reported in 2006. The isolates obtained were identified as *L. donovani* zymodeme MON-37 rather than *L. infantum* MON-1 found in canine infections. This suggested the existence of two separate concurrent transmission cycles; zoonotic transmission of *L. infantum* involving dogs as reservoir and anthroponotic transmission of *L. donovani*. The first report of *L. donovani* in Europe [7] raised the question whether the strains were autochthonous or imported into Cyprus. In addition, a canine isolate obtained from the same district in Cyprus presented a mixed MON-1/MON-37 genotype. Whether this dog was being co-infected with both parasite strains or by a hybrid strain requires validation. Since natural *L. major/L. infantum* [8] and *L. donovani*/*L. infantum* [9] hybrids have been reported, the scenario of possible genetic recombination and generation of hybrids within the *L. donovani* complex and their spread among human and dog populations in Cyprus is alarming and needs to be elucidated. In neighboring Turkey, strains isolated from *Phlebotomus tobbi* sand flies and one isolate from HCL were identified as *L. infantum,* but clearly different from MON-1 strains and rather similar to *L. infantum* strains of zymodeme MON-188 [10]. Taken together, the epidemiology of leishmaniasis in SE Europe is complex and merits investigation.

All the above prompted us to perform a comprehensive study of the genetic diversity of the *L. donovani* complex in the Eastern Mediterranean at species, zymodeme and strain level. To achieve this, we applied different approaches. Multilocus enzyme electrophoresis (MLEE) is the old ‘‘gold standard’’ for *Leishmania* species identification and zymodeme characterization. It is however, time-consuming, expensive and requires large amounts of protein. It has also proven insufficient to highlight genetic diversity for micro-epidemiological studies in the Mediterranean and South America [11]. In contrast, the *K26*-PCR assay is a rapid, sensitive and cost-effective molecular typing method using the repeat region of the *L. donovani* complex HASPB protein (also known as K26) as target sequence [12]. The *K26* typing is

specific for the *L. donovani* complex and discriminates between various zymodemes of the *L. donovani* complex, including *L. infantum* MON-1 and, at least, nine non-MON-1 zymodemes [12].

Multilocus microsatellite typing (MLMT) is considered the most highly discriminatory approach for assessing the genetic diversity, exploring the population genetic structure and accommodating the taxonomic revision of the *L. donovani* complex [11]. This approach was utilized to carry out three individual and interrelated population genetic studies based on microsatellite profiles of different strains of the *L. donovani* complex from the eastern Mediterranean.

3. Objectives

1. To establish a *Leishmania* strain collection from the eastern Mediterranean focusing on SE European countries and to characterize these *Leishmania* parasites at species and zymodeme level using the *K26*-PCR assay complementary to MLEE analysis.
2. To perform microsatellite typing and MLMT data analysis for this *L. donovani* complex strain collection and to elucidate population structuring by combining Bayesian model- and distance-based approaches. Particular objectives were:

2.1. To (re)-evaluate the epidemiology of VL due to *L. infantum* in the eastern Mediterranean by exploring the genetic diversity and inferring the population structure of *L. infantum* strains isolated in our study area and comparing them to those from SW Europe. To identify correlations between the observed structuring and zymodeme type, host specificity, place of isolation and other geo-epidemiological characteristics.

2.2. To address the genetic composition and assess the genetic polymorphism of *L. donovani* complex MON-37 strains isolated from Cypriot HVL and HCL cases. To search for the existence of geographically- and tropism-defined populations within zymodeme MON-37 and determine the origin of Cypriot MON-37 isolates by comparing them to other *L. donovani* strains, including those of MON-37 from India, Sri Lanka, Kenya and Sudan.

2.3. To assess the genetic composition, elucidate the population structure and phylogenetic placement of Turkish *L. donovani* complex strains isolated from HVL and HCL cases and *P. tobbi* in Cukurova region, and identified as non-MON-1 or MON-98 zymodemes, by comparing them to other *L. donovani* complex strains of different zymodemes and geographical origins. To evaluate the scenario of potential introduction of MON-37 to Cyprus from

Turkey and appraise the possible establishment in and the spread of *L. donovani* complex MON-37, MON-308 and MON-309 zymodemes to Europe.

4. Materials and Methods

4.1 Study area and *Leishmania* strains

***Leishmania* strains from Turkey** (TR)**:** Of the 23 strains from Turkey, 14 were obtained from CanL cases, one from a HCL case and three from HVL cases and five strains were isolated from *P. tobbi* sand fly vectors. Most CanL and HVL isolates (EP strains) originated from the Aegean region in south-western Turkey whereas the single HCL and all sand fly isolates (CUK strains) were collected from the Cukurova region in south-eastern Turkey.

***Leishmania* strains from Cyprus** (CY)**:** Of the 28 strains from Cyprus, 23 were canine isolates (CD strains) collected from distinct and relatively distant bio-geographical regions of all governmental prefectures. Five human isolates (CH strains) were obtained from two HVL and three HCL cases. All but one CH strains originated from Paphos prefecture.

***Leishmania* strains from Bulgaria, Greece, Albania and Croatia:** A single canine isolate originated from Blagoevgrad prefecture in Bulgaria (BG). Out of the 61 Greek strains (GR), 25 and 36 strains were obtained from HVL and CanL cases, respectively, in different regions of the Greek mainland and islands. Five strains were isolated from Albania (AL); three from HVL cases and two from CanVL cases. Ten canine isolates were obtained from various regions in Croatia (HR).

4.2 Parasite cultures, cloning and DNA extraction

Leishmania promastigotes were cultured and *Leishmania* DNA was extracted from mass cultures or clinical samples based on established protocols (cited in [6]). Parasite clones were obtained for a canine isolate (strain CD44) from Cyprus and of an HVL isolate (strain EP59) from Turkey.

4.3 *K26*-PCR assay and Multilocus enzyme electrophoresis (MLEE)

The *K26*-PCR assay was performed to discriminate *L. infantum* MON-1 from other *L. donovani* complex zymodemes, as cited in [6]. This approach was also applied to clones of strain CD44 (CD44cl.1-cl.5) and strain EP59 (EP59cl.1-cl.4). The MLEE analysis was carried out at the University of Montpellier (France).

4.4 Multilocus microsatellite typing (MLMT)

Fourteen microsatellite markers (Li22-35, Li23-41, Li41-56, Li45-24, Li46-67, Li71-5, Li71-7, Li71-33, Lm2TG, Lm4TA, TubCA, CS20, LIST7031 and LIST7039) distributed

randomly throughout the genome and previously found highly discriminative within the *L. donovani* complex were amplified using established PCR protocols (as cited in [6]). Microsatellite-containing fragments were either resolved on high-resolution MetaPhor® gels and screened for length polymorphisms using the AlphaImager® imaging system, or analyzed by capillary electrophoresis using the CEQ 8000 automated genetic analysis system. Control strains were included in both PCR protocols and reading methods to confirm the reproducibility of the results.

4.5 MLMT data and population genetic analyses

The MLMT datasets were processed by models based on Bayesian statistics and genetic distances. Model-based analysis was performed by STRUCTURE v2.3.1 [13] to infer the population structure and identify genetically distinct *Leishmania* clusters. Based on multilocus genotypes and independent of a mutation model, this algorithm determines patterns of allele frequencies per analyzed locus and estimates the most probable degree of differentiation. Individuals are assigned in discrete genetic populations and expose their membership to each estimated population by genotypic fractions. The allele frequencies among populations were correlated by admixture modeling for a series of runs using a 'burn-in' period of $2x10^5$ iterations and probability estimates based on 2×10^6 of MCMC repeats. For each possible number of populations between K=1 and K=10-12, ten independent simulations were conducted to estimate the delta (Δ)K values. The likelihood of population number was calculated based on the second-order rate of change in the log probability of data with respect to successive K values. The major population structure was captured at the plateau (maximum) of the derived Gaussian graph. Microsatellite-based genetic distances were calculated by MICROSAT software using the proportion of shared alleles distance measure (DAS). The resulting distance matrix input was processed by PHYLIP v3.6 to construct a Neighbour-joining (NJ) tree with confidence intervals by bootstrapping (100-1000 replications). A consensus tree with the corresponding bootstrap values and branch lengths was re-constructed using GENEIOUS v5.4 and the derived mid-point rooted NJ tree was edited by FIGTREE (http://tree.bio.ed.ac.uk/software/figtree). For studying *L. infantum* strains from SE Europe, genetic relatedness and recombination were evaluated through NeighborNet networks constructed by SplitsTree4 software and through the population membership coefficients estimated by STRUCTURE. The MLMT data were analyzed by GDA (http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php) with respect to the proportion of polymorphic loci (P), allelic variation (A), inbreeding coefficient (F_{IS}), expected (H_e) and observed (H_o) heterozygosity. The FST values (pairwise Wright's fixation index) with their corresponding p-values (confidence test) were

calculated using the microsatellite analyser (MSA) tool. The spatial distribution of the observed genetic structure at population and individual level was assessed based on allelic similarity by factorial correspondence analysis (FCA) using GENETIX v4.03.

5. Results

5.1 MLEE and *K26*-PCR typing of *L. donovani* complex strains from SE Europe

5.1.1 *L. donovani* complex strains from Cyprus and Turkey

Five human isolates from HVL and HCL cases in Cyprus (CH strains) were MLEE-typed as *L. donovani* MON-37 and presented a 700bp *K26* amplicon. Six Turkish strains isolated from one HCL case and five *P. tobbi* sand flies in Cukurova region (CUK strains) demonstrated a new *K26* amplicon size of 480bp. Strain EP59 isolated from a HVL case in Kusadasi (Aegean region of Turkey) presented a 385 bp *K26* amplicon, which was previously observed for an *L. infantum* MON-78 strain (MHOM/MT/85/BUCK) isolated from an HVL patient in Malta [12]. The same 385bp *K26* amplicon was observed after cloning the parental strain EP59 (EP59cl.1-cl.4). MLEE analysis revealed two novel *L. donovani* zymodemes. Strain EP59 was typed as MON-308 and presented enzymatic patterns identical to *L. infantum* MON-1 except for the two glutamate-oxaloacetate transaminases (GOT_1 and GOT_2). The latter were heterozygous between the *L. infantum* GOT_{100} and the *L. donovani* GOT_{113}. Strains CUK1, CUK2 and CUK10 presented an identical enzymatic pattern and were typed as *L. donovani* MON-309 showing the same isoenzyme electrophoretic mobilities as for *L. donovani* MON-3 zymodeme apart from malate dehydrogenase (MDH). In fact, CUK strains presented a relative mobility of 145, which is distinct from all other known MDH variants among the *L. donovani* complex. Interestingly, the novel MON-309 zymodeme differed from zymodeme MON-37 only in the electrophoretic mobilities of MDH and glucose phosphate isomerase (GPI) isoenzymes.

5.1.2 *L. infantum* strains from SE Europe

Overall, 122 of the 128 *L. infantum* strains isolated in different SE countries were characterized at species and zymodeme level by MLEE. Of these, 101 strains were typed as MON-1 and 21 strains from Turkey and Greece as MON-98; the zymodeme type of 6 Turkish *L. infantum* strains was not defined. Notably, we observed the so far highest percentage of MON-98 strains (30%) in Turkey (6 CanL isolates from Kusadasi) and Greece (15 HVL and CanL isolates from mainland regions and the island of Crete).

The *K26*-PCR showed that the 626bp *K26* amplicon characteristic for *L. infantum* MON-1 zymodeme was presented by all Cypriot, Croatian and a few Turkish and

Greek strains. The 940bp *K26* amplicon, and, occasionally, a second 870bp non-specific PCR product, previously found only in Greek strains and corresponding to either *L. infantum* MON-1 or MON-98 zymodemes was observed in all strains from Bulgaria, Albania, and all but two Turkish strains.

5.1.3 *K26*-PCR typing of the parental CD44 strain and the isolated CD44 clones

The observation of two *K26*-PCR products one corresponding to MON-1 and the other to MON-37 and the unusual profile at nine microsatellite markers for the canine isolate CD44 from Cyprus suggested that the dog was co-infected with both parasites and prompted us to isolate and re-analyze CD44 parasite clones. Of the five CD44 clones isolated, three clones (CD44cl.1-cl.3) presented the single 700bp *K26* amplicon found for all MON-37 strains isolated from human cases in Cyprus and two clones (CD44cl.4-cl.5) presented the single 626bp *K26* MON-1 amplicon.

5.2 Genetic diversity and population structure of *L. infantum* from SE Europe as revealed by MLMT

Assessment of the inter- and intra-zymodeme genetic diversity among 128 *L. infantum* MON-1 and MON-98 strains from SE demonstrated different degrees of polymorphism, with the highest allelic richness, expected and observed heterozygosities observed at markers Lm4TA, Lm2TG and Li22-35. Alleles characteristic for only the Cypriot strains were identified at markers Lm2TG and Lm4TA, for the Croatian strains at marker Li71-7, and for the Greek strains at markers Li22-35 and Li23-41. When MON-1 and MON-98 strains from Greece and Turkey were analyzed separately to investigate intra-zymodeme diversity, the measures of genetic variability demonstrated extensive inbreeding in both zymodeme populations and just trivial differences between the zymodemes MON-1 and MON-98.

Unique microsatellite profiles were observed for 50 strains, whereas 78 strains shared the same MLMType with at least another strain. One or more matching pairs were detected within each endemic country and between strains originating from different countries, except for those from Croatia and Cyprus. Notably, the same MLMT profile was identified for 17 out of 23 canine isolates from different prefectures of Cyprus. The MON-1 CD44 clones (CD44cl.4 and CD44cl.5) revealed common allelic sizes to those of other Cypriot MON-1 strains, except for one allelic variant at marker Li22-35. Moreover, the same MLMT profile was identified among MON-98 canine isolates from Kuşadasi district (Turkey). Of the overall collection of 61 Greek strains, 54 GR strains were used for studying *L. infantum* from SE Europe. Of these, only 20 strains presented individual MLMTypes and same profiles were shared

among strains from Greek mainland regions and islands, among human and dogs as well as among both zymodemes.

The Bayesian statistic approach implemented in the STRUCTURE software indicated the existence of 4 distinct genetic populations (POP) in our data set consisting of 128 *L. infantum* strains isolated in different SE countries. POP1 comprised all MON-1 strains from Cyprus (CY) including the MON-1 CD44cl.5. POP2 was formed by the majority of strains from Greece (GR) and Albania (AL), the single isolate from Bulgaria (BG) and half of the strains from Turkey (TR); vast majority were MON-1 strains. POP3 enclosed all strains from SW Europe, more than half of the strains from Croatia (HR) and the only TR strain from the Black Sea region; all were MON-1 strains. POP4 enclosed the remaining TR/GR/AL/HR strains and the majority of the MON-98 strains.

Different genetic distance analyses corroborated the existence of the 4 populations determined by the model-based analysis, except for the assignment of HR strains to POP3 and POP4 that was inconsistent with their placement at the mid-point rooted NJ tree and distribution in the phylogenetic network (NeighborNet). The three-dimensional FCA plot reflected the genetic isolation of CY strains, the diversification of TR/HR strains from SW European strains and the clear differentiation of the latter from all other populations. Further subdivision was supported by the different genetic distance methods only for POP3 and POP4. Re-analysis of POP3 revealed three distinct subpopulations based on geographical origin. Almost all strains from the islands of ES (sub3A) were separated from the strains from FR, PT and the mainland of ES (sub3B1), and also from the HR strains in sub3B2. The distinct homogeneous subpopulation of HR strains was supported by all analysis methods. Three single strains from TR, HR and Majorca that grouped with strains from the mainland ES (sub3B1) were placed in intermediate positions in the Splitstree and FCA plots indicating considerable gene flow among them. Re-analysis of POP4 revealed four stable subpopulations based on both zymodeme type and geographical origin. Sub4A solely consisted of GR/TR MON-98 strains and sub4B enclosed only HR strains and one AL strain. Other GR/TR strains whether of zymodeme MON-1 or MON-98 were assigned to sub4C1 whereas sub4C2 enclosed only GR MON-1 strains. All different distance-based analyses demonstrated extensive gene flow and recombination among the TR and GR strains of both zymodemes.

The highest degree of polymorphism and allelic richness in all loci was observed within POP3 and POP4, whereas CY MON-1 strains in POP1 presented the lowest polymorphism and allelic variation. All main populations were characterized by much lower observed heterozygosities than the expected ones (mean H_e=0.17 and mean

H_o=0.02) as well as high inbreeding coefficients (mean F_{IS}=0.86). *F*-statistics indicated strong differentiation between the main populations and POP1 presented the greatest genetic isolation from all other populations (F_{ST}=0.546-0.655). Among all subpopulations, the lowest levels of polymorphism, heterozygosity and inbreeding were found in sub3B2 (HR strains) and sub4B (AL/HR strains).

These results have been published in the following paper: **Gouzelou, E.**, Haralambous, C., Antoniou, M., Christodoulou, V., Martinković, F., Živičnjak, T., Smirlis, D., Pratlong, F., Dedet, J.P., Özbel, Y., Toz, S.Ö., Presber, W., Schönian, G. and Soteriadou, K. (2013). Genetic diversity and structure in *Leishmania infantum* populations from southeastern Europe revealed by microsatellite analysis. *Parasites & Vectors*, 5(6): 342.

5.3 MLMT and population structure of *L. donovani* complex MON-37 strains

The five MON-37 isolates from Cyprus isolated from HVL and HCL cases were compared to 42 *L. donovani* strains of various other zymodemes, including ten strains of MON-37 from the Indian subcontinent, the Middle East, China and Africa. Of the 15 MON-37 strains, two strains from Cyprus (strains CH33 and CH35), two from Sri Lanka (strains L60b and L60c) and two from India (strains LEM4537 and LEM4527) shared identical microsatellite profiles, whereas nine MON-37 strains presented unique MLMTypes. Considerable heterozygosity was presented by two HCL isolates from Cyprus (strains CH32 and CH34), the single HVL isolate from Israel (strain LRC-L740) and one *P. martini* isolate from Kenya (strain LRC-L57), however the observed heterozygous loci were randomly distributed. The mid-point rooted NJ tree demonstrated four main clusters; India-1/Bangladesh/Nepal, Sudan/Ethiopia, Kenya/India-2 and Iraq/China. Interestingly, the MON-37 strains did not form a distinct genetic group but were rather related to other *L. donovani* zymodemes based on their geographic origin, except for the Cypriot MON-37 strains that were clearly separated from all other strains investigated. The FCA plot confirmed these observations and further illustrated the formation of a unique genetic group enclosing all Cypriot MON-37 strains, apparently distinct from all other MON-37 strains. The Bayesian model-based approach implemented in STRUCTURE assigned the *L. donovani* strains to eight distinct populations. Consistent with genetic distance-based and FCA analyses however, the MON-37 strains were found in five of these distantly-related geographically-defined genetic groups and the differentiation of Cypriot MON-37 strains was highlighted.

These results have been published in the following paper: Alam, M.Z., Haralambous, C., Kuhls, K., **Gouzelou, E.**, Sgouras, D., Soteriadou, K., Schnur, F., Pratlong, F. and Schönian, G. (2009). The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. *Microbes and Infection*, 11(6-7): 707-715.

5.4 Genetic diversity and population structure of Turkish *L. donovani* complex MON-308 and MON-309 strains: Comparison with the Cypriot MON-37 strains

Unique microsatellite profiles were observed for all Turkish CUK strains of zymodeme MON-309 pointing to a significant genetic diversity of these strains. The MON-308 strain from Kusadasi presented extensive heterozygosity at 9 out of 14 markers. One allele was always characteristic for *L. infantum* MON-1 and the second corresponded to those observed for the MON-309 strains. This finding had prompted us to isolate and re-analyze clones of the EP59 strain. Identical MLMT profiles were observed for all four clones (EP59cl.1-cl.4) strongly suggesting that it may be a true MON-1/MON-308 hybrid. MLMT analysis of the three MON-37 CD44 clones (CD44cl.1-CD44cl.3) also revealed heterozygosity in six out of the 14 loci. The fact that allelic sizes observed were either as in other Cypriot MON-37 or in MON-1 strains confirms the previous suggestion that the dog was co-infected with both MON-1 and MON-37 strains [7] and the generation of a putative MON-1/MON-37 hybrid.

The MLMTypes of the one TR MON-308 and of the six TR MON-309 strains was compared to those of six CY MON-37 strains, and of 63 other *L. donovani* complex strains from different geographic regions (Indian subcontinent, Africa and South Europe) reflecting the zymodeme diversity of both *L. infantum* and *L. donovani* species. The population structure was assessed by various methods revealing different assumptions. Based on Bayesian statistics analysis, the uppermost hierarchical level of population structure indicated the existence of two main populations, separating the *L. infantum* MON-1 strains from all other *L. donovani* complex non MON-1 strains. At K=3, the *L. donovani* strains formed a distinct population, and at K=6 the TR MON-309 and CY MON-37 strains along with one CY MON-37 CD44 clone (CD44 cl.1) diversified into a separate population, onwards named TR/CY non MON-1. Interestingly, the TR MON-308 strain could not be assigned to one population and presented shared membership to both the GR/TR MON-1 and TR/CY non MON-1 populations. All populations were well-defined at K=8 and generally corresponded to the geographical origin of the strains (Indian subcontinent, Africa, TR/CY non MON-1, SW European *L. infantum* non MON-1, SW

European *L. infantum* MON-1 and SE European *L. infantum* MON-1).The mid-point rooted NJ tree illustrated the same main clusters including that of TR/CY non MON-1, which was formed by two geographically-defined subclusters (TR non MON-1 and CY non MON-1). In the FCA plot the TR/CY non MON-1 strains were placed in between the MON-1 and the *L. infantum* non MON-1 populations, while the TR MON-308 strain was found between the TR non MON-1 and *L. infantum* MON-1 populations, which illustrates the hybrid nature of this strain. Thus, a new monophyletic TR/CY population being genetically distinct from other *L. donovani* complex populations was identified by MLMT. Expected heterozygosity was higher in the TR/CY non MON-1 population (H_e=0.518) than in the SW and SE European MON-1 (H_e=0.276 and 0.246, respectively) and comparable to that of the *L. infantum* non MON-1 population (H_e=0.710). This high genetic variability was also evident by the greater genetic distances in the mid-point rooted NJ tree. For all populations, the observed heterozygosity values were significantly lower than the expected ones. High inbreeding coefficients were observed for all main populations (F_{IS}=0.662-0.925) implying population subdivision (Wahlund effect) or a high rate of gene conversion.

These results have been published in the following paper: **Gouzelou, E.**, Haralambous, C., Amro, A., Mentis, A., Pratlong, F., Dedet, J.P., Votypka, J., Volf, P., Toz, S.O., Kuhls, K., Schönian, G. and Soteriadou, K. (2012). Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic *L. donovani sensu lato* group. *PLoS Neglected Tropical Diseases*, 6(2): e1507.

6. Discussion

6.1 Population structure of *L. infantum* strains from SE Europe

This is the first comprehensive study that investigates the genetic diversity of *L. infantum* in SE Europe. We have identified distinct genetic *L. infantum* populations and demonstrated clear differentiation between SE and SW European strains.

Notably, in contrast to the clear diversification between Spanish strains from the mainland and the Balearic islands, the strains from the Greek mainland and islands always grouped together in the same genetic groups. The Cypriot canine isolates were, however genetically isolated and formed a monophyletic group, mirroring the natural isolation of the island of Cyprus and suggesting the circulation of a rather homogenous MON-1 population there. The epidemiology of CanL in Cyprus has

been doubtless affected by eradication campaigns held against malaria and echinococcosis resulting in the massive reduction of vector and dog populations, respectively. Most probably, this caused severe bottleneck events in *Leishmania* populations leading to a limited parasite genetic pool which is evident in our study by the significant inbreeding and genetic similarity.

Croatian strains were assigned to both SE and SW populations reflecting the geographic position of Croatia. Croatian strains could have been introduced from any neighboring country. The microclimate, the presence of vectors and reservoirs favor the establishment and circulation of parasites in the Dalmatian coastal regions. The exportation from Croatia to neighboring countries is equally possible, as adult VL cases acquired at the Dalmatian coast have been reported in Austria and Switzerland [14].

The identification of highly heterogeneous populations enclosing all MON-1 and MON-98 strains from Turkey, Bulgaria, Greece and Albania and their successive sub-structuring upon finer analysis indicated substantial differentiation and gene flow among them probably driven by various evolutionary effects. Our findings did not correlate to host specificity or year of strain isolation, but seem to reflect intensive host migration over long periods of time and common eco-epidemiological conditions, including a similar sand fly fauna. Despite analyzing the largest number of MON-98 strains so far, Bayesian statistic-based sub-clustering, phylogenetic and Splitstree analyses demonstrated extensive gene flow and putative recombination events, and did not support the differentiation between MON-1 and MON-98 zymodemes. The existence of groups of genotypically identical MON-1 as well as MON-98 strains could indicate selection of specific microsatellite genotypes, as previously observed for MON-1 strains from Madrid or Majorca and Ibiza [15]. Altogether our results corroborate and extend earlier studies [16] by including a larger strain set from Turkey and Greece and strains from endemic Balkan countries. The observed structuring of *L. infantum* strains seems to be mainly associated with the composition of the sand fly fauna, geo-ecological characteristics, historical background and important events that have influenced the epidemiology of VL in the endemic countries. Re-evaluation and comparison of population structure of *L. infantum* in SE and SW Europe has provided valuable insight into the dynamics of VL in the re-emerging endemic foci in south European countries.

6.2 The paraphyletic composition of zymodeme MON-37 and the novel *L. donovani sensu lato* group from Turkey and Cyprus

The MLMT approach was employed to assess the genetic diversity of strains belonging to the most widespread zymodeme of *L. donovani* MON-37 and to

compare the newly identified MON-37 strains from Cyprus to MON-37 strains from the Indian subcontinent, the Middle East, China and East Africa as well as to strains of other related *L. donovani* zymodemes. It was expected that the Cypriot MON-37 were genetically related to those of other origins and had been introduced by infected immigrants from any of these countries. To our surprise, MON-37 strains from India, Sri Lanka, Kenya, Israel and Cyprus were assigned by all algorithms used for the analyses of the MLMT profiles to different distantly-related genetic subgroups that corresponded to their geographical origin. In fact, the Kenyan, Sri Lankan and Indian MON-37 strains were genetically closer to strains of other zymodemes from the same regions than to MON-37 strains from other areas. Moreover, the Cypriot and Israeli MON-37 strains were found heterozygous at 5-7 microsatellite loci and clearly different among themselves and also to all the other MON-37 strains, indicating that they could be autochthonous although their origin remained enigmatic. Thus, this study demonstrated the paraphyletic composition of zymodeme MON-37, which neither represents a single genetic entity nor reflects any genetic relationship between strains of different geographical origin.

We then decided to compare the Cypriot MON-37 strains with the Turkish strains belonging to the newly identified zymodemes MON-308 and MON-309, along with a set of *L. donovani* complex strains of different zymodemes and geographical origins. The six MON-309 strains from Çukurova, Turkey, were quite similar to *L. infantum* zymodeme MON-188 and identical between them [10]. The only previous isolation of *L. infantum* from *P. tobbi* has been reported in Cyprus [17]. Our study demonstrated that the Cypriot MON-37 strains were closely related to the novel MON-308 and MON-309 strains from southeast Turkey. Thus, the MON-37 strains might have been introduced to Cyprus recently, probably from mainland Turkey where human leishmaniasis is widespread, by Turkish immigrants and/or the army following the war in 1974. Moreover, these Cypriot strains were not clonal but shared distinct microsatellite profiles, including multiple heterogeneous microsatellite loci, suggesting the existence of hybrids, aneuploidies or mixed infections. In addition to this, our findings have demonstrated a putative MON-1/MON-308 hybrid isolated from a HVL patient in Turkey that was found to be related to both the new population and that of *L. infantum* MON-1 from SE strains.

The identification of a ‘new’ main cluster within the *L. donovani* complex consisting of both Turkish and Cypriot strains, which is differentiated from the *L. donovani, L. infantum* MON-1 and non-MON-1 populations observed previously [16, 18, 19] is the most striking finding of the present study. It demonstrates the genetic isolation of the Turkish CUK/Cypriot CH population from the *L. infantum* MON-1, *L. infantum* non-

MON-1 and *L. donovani* populations, and designates the Turkish and Cypriot strains as a new monophyletic group of the *L. donovani* complex. Very recently, the first genome-wide assessment of a vector-isolated population of *Leishmania* parasites was reported [20] on a sample-set that included the HCL isolate (CUK1) and the *P. tobbi* isolates (CUK2, CUK3, CUK4, CUK7 and CUK10) that were also analyzed in the context of our study. Interestingly, the CUK strains presented high genome-wide heterozygosity and SNP density as well as variation in chromosome copy numbers both between them and across the reference strains of *L. infantum*, *L. major* and *L. donovani* to which they were compared. This is in agreement with the much higher microsatellite variability of CUK strains which differed in up to 8 of the 14 microsatellite loci and the higher expected heterozygosity observed herein, in contrast to the low genome-wide heterozygosity found for other Old World *Leishmania* populations. Whole-genome sequencing confirmed that the Turkish CUK strains display considerable genetic divergence from the *L. infantum* and *L. donovani* reference genomes and form a unique population genetically distinct from the other *L. donovani* complex isolates fully sequenced so far [20]. Whole-genome SNP data suggest that the CUK strains originate from a single out-crossing event between one parasite strain related to the reference genome of *L. infantum* MON-1 zymodeme and 'another unknown strain'.

Our findings based on MLMT indicate that the uppermost hierarchical level of population structure is shaped by two major homogeneous gene pools. The first ancestral source population enclosed all *L. infantum* MON-1 strains and the second all *L. donovani* and *L. infantum* non MON-1 strains including those from Turkey and Cyprus. Next, the *L. donovani* population split to become the third ancestral population. Taken together, these results suggest that the 'other unknown strain' could have derived from an *L. infantum* non MON-1 rather than an *L. donovani* strain. Our findings reflect the complexity of the epidemiology of leishmaniasis in Turkey and Cyprus whilst rising concern about the spreading of these new, anthroponotic *L. donovani sensu lato* non-MON-1 strains to neighboring countries. In areas where sand fly vectors are well-established, introductions such as that of *L. donovani* in Cyprus and Turkey could enhance the potential of genetic exchange between different species/strains. Consequently, new hybrids may be generated with different epidemiology, pathogenicity or drug resistance. Our results do not correspond to the current nomenclature of the *L. donovani* complex according to MLEE typing, which may lead to discrepant results, and support the revision of *Leishmania* taxonomy.

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Affidavit

"I, Stavroula Gouzelou certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "Population genetic study for evaluating the differentiation and gene flow among Eastern Mediterranean strains of the *Leishmania donovani* complex". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

30 May 2015

Agreement

This doctoral thesis (publication thesis) is based on the publications published in the journals listed below.

1. Alam M.Z., Haralambous C., Kuhls K., Gouzelou E., Sgouras D., Soteriadou K., Schnur L., Pratlong F. and Schönian G. (2009) The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. Microbes Infect 11(6-7): 707-715.

2. Gouzelou E., Haralambous C., Amro A., Mentis A., Pratlong F., Dedet J-P., Votypka J., Volf P., Toz SO., Kuhls K., Schönian G. and Soteriadou K. (2012) Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic *L. donovani sensu lato* group. PLoS Negl Trop Dis 6(2): e1507.

3. Gouzelou E., Haralambous C., Antoniou M., Christodoulou V., Martinković F., Živičnjak T., Smirlis D., Pratlong F., Dedet J-P., Özbel Y., Toz SÖ., Presber W., Schönian G. and Soteriadou K. (2013) Genetic diversity and structure in *Leishmania infantum* populations from southeastern Europe revealed by microsatellite analysis. Parasit Vectors 5(6): 342.

Erklärung über den Eigenanteil an den Publikationen

Stavroula Gouzelou (EG, Evi is the short name for Stavroula) had the following share in the following publications:

Publication 1: Alam, M.Z., Haralambous, C., Kuhls, K., Gouzelou, E., Sgouras, D., Soteriadou, K., Schnur, F., Pratlong, F. and Schönian, G., The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing, Microbes and Infection, 2009.

Contribution in detail (33%): EG was responsible for parasite culturing, cell banking, extracting DNA and performing MLMT for the Cypriot MON-37 strains. For microsatellite analysis she carried out the PCR assays using fluorescence conjugated primers or non-labelled primers. She prepared the samples for fragment analysis by capillary electrophoresis or resolved the amplicons by Metaphor® gel electrophoresis and determined the repeat numbers. She participated in manuscript drafting. The MLMT results were compared to those obtained by Alam M.Z.

EG also applied the *K26*-PCR assay to these Cypriot MON-37 strains. Preliminary results by MLMT analysis of these strains were included in Antoniou, M., Haralambous, C., Mazeris, A., Pratlong, F., Dedet, J.P., Soteriadou, K., *Leishmania donovani* leishmaniasis in Cyprus, Lancet Infectious Diseases, 2008.

Publication 2: Gouzelou, E., Haralambous, C., Amro, A., Mentis, A., Pratlong, F., Dedet, J.P., Votypka, J., Volf, P., Toz, S.O., Kuhls, K., Schönian, G. and Soteriadou, K., Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic *L. donovani sensu lato* group, PLoS Neglected Tropical Diseases, 2012.

Contribution in detail (50%): EG contributed in designing the study, was responsible for parasite culturing, obtaining parasite clones, cell banking and DNA extraction. She performed *K26* and microsatellite typing of the Turkish MON-309 strains, the parent and clones of the Turkish MON-308 strain, the MON-1 and MON-37 clones of a CanL case being co-infected with *L. infantum* MON-1/*L. donovani* MON-37 strains. She performed the PCR assays for microsatellite typing using fluorescence conjugated primers or non-labelled primers and carried out the fragment analysis by capillary or Metaphor® gel electrophoresis, respectively. She determined the repeat numbers and carried out part of the microsatellite data analysis, manuscript writing and subsequent manuscript revision.

Publication 3: Gouzelou, E., Haralambous, C., Antoniou, M., Christodoulou, V., Martinković, F., Živičnjak, T., Smirlis, D., Pratlong, F., Dedet, J.P., Özbel, Y., Toz, S.Ö., Presber, W., Schönian, G. and Soteriadou, K., Genetic diversity and structure in *Leishmania infantum* populations from southeastern Europe revealed by microsatellite analysis, Parasites & Vectors, 2013.

Contribution in detail (70%): EG designed the study and was responsible for parasite culturing, cell banking at LN_2 and DNA extraction from *L. infantum* strains from Turkey, Cyprus, Bulgaria, Greece, Albania and Croatia. For this sample set she performed *K26* typing and carried out the PCR assays for microsatellite typing (MLMT) using (a) fluorescence conjugated primers or (b) non-labelled primers, prepared the samples for (a) fragment analysis by capillary electrophoresis or (b) amplicon resolution by Metaphor® gel electrophoresis and determined the repeat numbers. She carried out the microsatellite data and phylogenetic analysis and compiled the results. She was responsible for the manuscript writing and review processing.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

Erläuterung des Impact Faktor in den publizierten Journalen

Impact factor	Publikation	Nr.
2.92	Alam M.Z., Haralambous C., Kuhls K., **Gouzelou E.**, Sgouras D., Soteriadou K., Schnur L., Pratlong F. and Schönian G. (2009) The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. Microbes Infect 11(6-7): 707-715.	1
4.57	**Gouzelou E.**, Haralambous C., Amro A., Mentis A., Pratlong F., Dedet J-P., Votypka J., Volf P., Toz SO., Kuhls K., Schönian G. and Soteriadou K. (2012) Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic *L. donovani sensu lato* group. PLoS Negl Trop Dis 6(2): e1507.	2
3.25	**Gouzelou E.**, Haralambous C., Antoniou M., Christodoulou V., Martinković F., Živičnjak T., Smirlis D., Pratlong F., Dedet J-P., Özbel Y., Toz SÖ., Presber W., Schönian G. and Soteriadou K. (2013) Genetic diversity and structure in *Leishmania infantum* populations from southeastern Europe revealed by microsatellite analysis. Parasit Vectors 5(6): 342.	3

The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing.
Alam M.Z., Haralambous C., Kuhls K., **Gouzelou E.**, Sgouras D., Soteriadou K., Schnur L., Pratlong F. and Schönian G. (2009) Microbes Infect 11(6-7): 707-715.

http://dx.doi.org/10.1016/j.micinf.2009.04.009

Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic *L. donovani sensu lato* group.
Gouzelou E., Haralambous C., Amro A., Mentis A., Pratlong F., Dedet J-P., Votypka J., Volf P., Toz SO., Kuhls K., Schönian G. and Soteriadou K. (2012) PLoS Negl Trop Dis 6(2): e1507.

http://dx.doi.org/10.1371%2Fjournal.pntd.0001507

Genetic diversity and structure in *Leishmania infantum* populations from southeastern Europe revealed by microsatellite analysis.
Gouzelou E., Haralambous C., Antoniou M., Christodoulou V., Martinković F., Živičnjak T., Smirlis D., Pratlong F., Dedet J-P., Özbel Y., Toz SÖ., Presber W., Schönian G. and Soteriadou K. (2013) Parasit Vectors 5(6): 342.

http://dx.doi.org/10.1186%2F1756-3305-6-342

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Acknowledgements

Foremost, I wish to express my sincere gratitude to Prof. Wolfgang Presber for accepting to supervise my thesis and for providing significant reviewing and support.

It is difficult to overstate my appreciation to Dr. Gabriele Schönian, an admirable person and scientist who I was lucky to meet from the beginning of this research work. She has given me all good reasons to think of her as a role model of a successful researcher. Dear Gabi, thank you for the tremendous assistance and thoughtful supervision throughout this study.

I wish to express my deep gratitude to Dr. Ketty Soteriadou, for giving me the opportunity to carry out this research study at Laboratory of Molecular Parasitology at Hellenic Pasteur Institute and for taking me on board to a great journey of life lessons. I thank her for sharing her knowledge and experience in this research field, for the efficient cooperation and overall supervision that have been undoubtedly valuable.

I am grateful to the Hellenic Pasteur Institute for providing a PhD studentship and the essentials to perform and accomplish this work. I also acknowledge the General Secretariat for Research and Technology (GSTR) of the Hellenic Republic Ministry of Education and Religious Affairs and the Scientific and Technological Research Council of Turkey (TÜBİTAK) for the research grants that have majorly supported this work.

I am thankful to all past and current members of the Laboratory of Molecular Parasitology and especially to Dr. Christos Haralambous for his scholastic guidance, the insightful comments and hard questions as well as for his trust and motivation from the very beginning of this journey.

I am tempted to individually thank my colleagues for our enlightening scientific and non-scientific discussions as well as my good friends for always being there to share excitements and concerns. However, this list would be quite long so I simply wish to Thank You All.

My life partner Avraam however, is a bit luckier. He has put so much effort to look excited every time I talk about *Leishmania* that I can't miss to thank him in particular. My dearest, thank you for giving me reasons to keep happy and fulfilled, for encouraging me to do my best in all life matters.

Lastly and most importantly, as a small token of gratitude for always believing in me and supporting my decisions whether right or wrong, for their unconditional love and every sacrifice they made for me, this work is truly dedicated to my beloved parents Spyro and Eleftheria.