Evolutionary history of *Leishmania killicki* (synonymous *Leishmania tropica*) and taxonomic implications

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Abstract

Background: *Leishmania* (*L.*) *killicki* is responsible for the chronic cutaneous leishmaniasis. The taxonomic status of this parasite is still not well defined. It was suggested on one hand to include this taxon within *L. tropica* complex but also on the other hand to consider it as a distinct phylogenetic complex. The present work represents the more detailed study on the evolutionary history of *L. killicki* relative to *L. tropica* and the taxonomic implications.

Methods: Thirty five *L. killicki* and 25 *L. tropica* strains isolated from humans and from several countries were characterized using the MultiLocus Enzyme Electrophoresis (MLEE) and the MultiLocus Sequence Typing (MLST) approaches.

Results: The genetic and phylogenetic analyses strongly support that *L. killicki* belongs to *L. tropica* complex. The study suggests the emergence of *L. killicki* by a funder effect followed by an independent evolution from *L. tropica*, but does not validate the species status of this taxon. In this context, we suggest to call this taxon *L. killicki* (synonymous *L. tropica*) until further epidemiological and phylogenetic studies justify the *L. killicki* denomination.

Conclusions: These findings provided taxonomic and phylogenetic informations on *L. killicki* and helped to better know the evolutionary history of this taxon.

Keywords

Leishmania killicki, *Leishmania tropica*, evolutionary history, phylogeny, isoenzymatic polymorphism

1. Background

Leishmaniases are tropical neglected diseases caused by *Leishmania* parasites and transmitted to mammals via infected bites of Phlebotomine sandflies of the genus *Phlebotomus* [1]. In humans, these diseases are responsible for cutaneous (CL), mucocutaneous (MCL) and visceral (VL) clinical manifestations.

Since the description of the genus *Leishmania* Ross, 1903, the classification methods have considerably evolved. Indeed, from 1916 to 1987, the classification was descriptive of Linnean type based on simple extrinsic characters, such as clinical forms, geographical distribution and epidemiological cycles. This method leads to the subdivision of the genus *Leishmania* into the two sub-genera *Leishmania* and *Viannia* [2, 3].

Since the eighties, the biochemical classification based on isoenzymes studies has been developed. This approach has itself evolved from the classical Adansonian type where isoenzymes were not considered as evolutionary characters to the numerical cladistic approach using isoenzymes as evolutionary markers [4-8]. These analyzes have participated to the description of several *Leishmania* complexes in the Old and the New Worlds. However, the complete numerical phenetic and phylogenetic study was performed by [9] who identified four main groups of the Old World *Leishmania* and [10, 11] who defined eight complexes and two groups of the New World *Leishmania* respectively.

Currently, the numerical taxonomic technique based on isoenzyme analysis is considered as the reference method for *Leishmania* genus classification and is continuously applied enabling the updating of some complexes classification and the analysis of the geographical distribution and the epidemiological features of several leishmaniasis foci [12, 13]. Nevertheless, the drawbacks of this approach such as the requirement of a mass culture of *Leishmania* and the relatively poor discriminatory power and the appearance of the DNA-based techniques conducted to the development of the genetic classification methods.

Since the nineties, several genetic approaches targeting nuclear and/or kinetoplastic markers have been used for *Leishmania* phenetic and phylogenetic studies including PCR- sequencing [14], nested-PCR [15], RAPD [16, 17], SSCP [18, 19], MLST [20], MLMT [21, 22], PCR-RFLP [23, 24], HRM [25], and AFLP [26]. These techniques have contributed to the epidemiological, control and therapeutic advances on leishmaniases. However, generated taxonomic data required the revision of the genus *Leishmania* classification [27, 28].

The Multi Locus sequence typing (MLST) is one of the most appropriate approaches for taxonomic studies because it provides data on genetic variations in housekeeping genes. Since 2006, this approach has been increasingly used for phylogenetic investigations helping to understand epidemiological and transmission features of many *Leishmania* complexes [20, 29-33]. Nevertheless, having regard to the complexity of leishmaniases and the rarity of realized studies using this technique, several taxa need to be deeply explored [34].

Leishmania killicki is a recently described taxon, compared to other *Leishmania* parasites, causing CL in Maghreb: Tunisia [35], Libya [36] and Algeria [37]. The taxonomic status and the evolutionary history of this parasite relative to *L. tropica* are based on very few studies and samples. The numerical taxonomic analysis using the Multilocus Enzyme Electophoresis (MLEE) approach has first included this parasite within *L. tropica* complex [9, 38]. Nevertheless, after the revision of the genus *Leishmania* classification, it was considered as a separate phylogenetic complex [39]. Recently, an updated study by [12] has confirmed the first classification and has included *L. killicki* within *L. tropica* complex. Phenetic and phylogenetic studies using the MLMT [40], PCR-sequencing [41] and MLST [31] techniques have classified *L. killicki* within *L. tropica* and have suggested a closer genetic link with *L. tropica* from Morocco. Nevertheless, these data were obtained by the analysis of a low number of *L. killicki* strains (seven in total): two strains were analyzed in [40], two in [41] studies and three in [31]. In this context, the present study had for objective to conduct a MLST study on a larger number of *L. killicki* and *L. tropica* strains. The main goal was to investigate the evolutionary history of *L. killicki* and to study the taxonomic

implications. The present data endorse undoubtedly that this taxon belongs to *L. tropica* complex and descends from *L. tropica* by a founder effect. This event would have led to a loss of genetic diversity and to a different population, both genotypically and phenotypically. This subpopulation, after its adaption in new ecosystems, now appears to evolve and spread independently from *L. tropica*. Nevertheless, currently no data allow validating the species status of *L. killicki*. These findings could help to have insights into the epidemiology, phylogeny and taxonomy of this parasite.

2. Methods

2.1. Origin of strains

In order to study the evolutionary history of *L. killicki* relative to *L. tropica* and the taxonomic implications, the strains were selected to belong to different geographic origins and to have various zymodemes. A total of 62 strains of *L. killicki* (n=35), *L. tropica* (n=25), *L. major* (n=1) and *L. infantum* (n=1) were analyzed. These strains were isolated from human cutaneous lesions except the strain of *L. infantum* which was isolated from a human visceral leishmaniasis case. Among these strains, 53 were selected from the Cryobank of the Centre National de Référence des leishmanioses (CNRL) (Montpellier, France) and nine *L. killicki* were collected by the team of the Laboratoire de Parasitologie - Mycologie Médicale et Moléculaire (Monastir, Tunisia) during epidemiological investigations.

Forty eight strains among which 34 of *L. killicki* (Algeria n=6, Libya n=1, Tunisia n=27) and 14 of *L. tropica* from Morocco were analyzed for the first time by the MLST technique.

The *L. killicki* strain (LEM163) MHOM/TN/80/LEM163 was previously analyzed by MLST and published in Genbank under the accession number KC158820 (see [31]). The eleven remaining *L. tropica* strains were chosen to be isolated from several countries (Egypt n = 1, Greece n = 1, Israël n = 2, Jordan n = 2, Kenya n = 3, Yemen n = 2). These strains were also previously typed by MLST and their sequences were published in Genbank under the following accession numbers: KC158621,

KC158637, KC158643, KC158677, KC158682, KC158683, KC158690, KC158696, KC158711, KC158722 and KC158761 (see [31]).

The two strains of *L. major* (LEM62) MHOM/YE/76/LEM62 and *L. infantum* (LEM75) MHOM/FR/78/LEM75 previously typed by MLST were used as out-groups [31].

2.2. Isoenzymatic identification

All studied strains were identified by MLEE using 15 enzymatic systems according to [9]. The 15 enzymes investigated were : malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), isocitrate dehydrogenase (ICD, EC 1.1.1.42), phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), glutamate dehydrogenase (GLUD, EC 1.4.1.3), diaphorase NADH (DIA, EC 1.6.2.2), nucleoside purine phosphorylases 1 and 2 (NP1, EC 2.4.2.1 and NP2, EC 2.4.2*), glutamate oxaloacetate transaminases 1 and 2 (GOT1 and GOT2, EC 2.6.1.1), phosphoglucomutase (PGM, EC 5.4.2.2), fumarate hydratase (FH, EC 4.2.1.2), mannose phosphate isomerase (MPI, EC 5.3.1.8) and glucose phosphate isomerase (GPI, EC 5.3.1.9).

2.3. DNA extraction

Genomic DNA of culture parasites was extracted using the QIAmp DNA Mini Kit (Qiagen, Germany) following the manufacturer's recommendations and eluted in 150 µl.

2.4. Analysis by Multi locus sequence typing (MLST)

The 48 strains of *L. killicki* (n=34) and *L. tropica* (n=14) were typed on the seven loci coding for single copy housekeeping genes developed and optimized by [31]. The step of amplification was performed by real-time PCR using the SYBR Green method (Light cycler 480 II, Roche). The amplified products were sequenced on both strands (Eurofins MWG, operon, Germany) and obtained sequences were aligned and checked in both directions using the CodonCode Aligner software v.4.0.1 (Codon Code Co., USA). Polymorphic sites (PS) and ambiguous positions corresponding to heterozygous sites (HS) were identified for each strain in each locus using the

same software. The DnaSP software v.5 [42] was used to calculate the number of haplotypes from concatenated sequences.

The Neighbor Joining (NJ) tree, where each strain represents a different haplotype, was constructed on duplicated nucleotide sites by the MEGA 5.10 software using the Maximum Composite Likelihood model and 500 bootstrap replications [43]. This software was also used for the identification of amino acid variations between *L. killicki* and *L. tropica*.

3. Results

3.1. Isoenzymatic identification

The isoenzymatic characterization of the 62 studied strains was conducted either during the present study or during previous investigations. Fifty three strains were previously analyzed by MLEE at the Centre National de Référence des leishmanioses and the nine strains collected by the team of the Laboratoire de Parasitologie - Mycologie Médicale et Moléculaire (Monastir, Tunisia) during the epidemiological investigations were identified for the first time by the same technique [12, 35-38, 44-46]. The first step of the study was to reanalyze the strains by MLEE at the Centre National de Référence des Leishmanioses (Montpellier, France).

In all, 17 zymodemes were identified with three for *L. killicki*, 12 for *L. tropica* and a single zymodeme for each strain of *L. major* and *L. infantum* (Table1). For *L. killicki*, a new zymodeme (MON-317) was identified for the first time from a single Tunisian strain (LEM6173) MHOM/TN/2010/MET300), and added to the known zymodemes MON-8 (n=28) and MON-301 (n=6). The enzymatic profile of the zymodeme MON-317 strain differs by FH from zymodeme MON-8 [35], MDH, ME, GOT1, GOT2 and FH from zymodeme MON-301 [37] and MDH, GOT1, GOT2 and FH from zymodeme MON-306 [47] (Table 2). For *L. tropica*, the zymodemes were previously identified and confirmed by [12, 44]: MON-54 (n=1), MON-71 (n=2), MON-102 (n=5), MON-109 (n=3), MON-112 (n=2), MON-113(n=3), MON-114 (n=1), MON-119 (n=3), MON-137 (n=2), MON-200 (n=1), MON-264 (n=1) and MON-265 (n=1) (Table 1).

3.2. Sequence analysis

The obtained sequences of *L. killicki* (n=34) and *L. tropica* (n=14) strains were analyzed in both directions for the seven loci and submitted to GenBank and accession numbers have been assigned: KM085998-KM086333.

The loci sizes were identical to those reported by [31], except for the locus 12.0010 for which the length of sequences was only 579 pb instead of 714 pb, giving a concatenated sequence of 4542 pb (Table 3). Chromatograms were clear at all investigated loci, polymorphic (PS) and heterozygous sites (HS) corresponding to ambiguous positions with two peaks were easily identified. No tri-allelic site was detected.

3.3. Genetic polymorphisms in *L. killicki* and in *L. tropica*

In all, 95 (2.09%) polymorphic sites (PS) of which 59 (1.3%) heterozygous positions (HS) were identified for the 60 *L. killicki* and *L. tropica* strains. The number of PS varied from six (1.23%) for the locus 31.2610 to 21 (2.6%) for the locus 31.0280 (Table 3).

For *L. killicki*, we obtained 11 (0.24%) PS corresponding all to heterozygous positions. The locus 31.2610 was the most polymorphic with three (0.61%) PS. However, for the locus 12.0010 no PS was identified. For *L. tropica*, 87 (1.91%) PS among which 48 (1.06%) HS were found. The number of PS varied from six (1.23%) for the locus 31.2610 to 19 (2.36%) for the locus 31.0280 (Table 4).

Mutations between these two taxa were also analyzed at the amino acid level and heterozygous mutations were excluded from this analysis. In all, 55 mutations were identified of which 29 silent substitutions and 26 resulted in altered amino acid residues (Table 5). All *L. killicki* mutations corresponded to a single amino acid. However, for *L. tropica* more than one amino acid was observed for the same mutation site.

3.4. Phylogenetic analysis of *L. killicki*

Totally, 32 haplotypes were obtained with 10 out of 35 strains for *L. killicki* and 22 out of 25 strains for *L. tropica*. Twenty seven unique haplotypes were identified among which eight for *L. killicki*

and 19 for *L. tropica*. No common haplotypes were obtained between these two taxa. The strain of *L. killicki* MON-317 had its own haplotype (Table 6). The Neighbor Joining tree using the 32 strains representing all identified haplotypes was constructed based on the concatenated sequences and duplicated nucleotide sites to avoid the loss of genetic information in ambiguous positions (Fig. 1). The phylogenetic tree showed that *L. killicki* belongs to *L. tropica* but creates a separate and monophyletic group sustained by 96% of bootstrap replicates. The *L. killicki* cluster groups together all the strains of this taxon from the different countries without clear structuring and low polymorphism (see Fig. 1). In contrast, *L. tropica* revealed a high polymorphism with strong structuring supported by high values of bootstrap accordingly to the country of origin for Kenya and Yemen. The larger group supported by a bootstrap of 98% was composed of all the strains from Morocco with other strains from various countries.

4- Discussion

Despite the small number of strains and the different molecular tools and analytic methods used previously for the study of *L. killicki* [9, 12, 31, 38, 40], this parasite was mostly included within *L. tropica* complex except in the study of [39] in which it was considered as a separate phylogenetic complex. In this context, the present study had for objective to improve the knowledge of the *L. killicki* phylogeny and its evolutionary history relative to *L. tropica* by using a larger sample of *L. killicki* from different countries.

The phylogenetic analyses performed in this study confirm the position of this taxon within *L. tropica* in agreement with the previous biochemical and genetic findings. The close phylogenetic relationships between these two taxa were also revealed by the low number of polymorphic sites compared to those found by [30, 31] between various *Leishmania* species. The phylogenetic tree shows that *L. killicki*, all countries included, creates an independent monophyletic group within *L. tropica* with high bootstrap value and no common haplotypes between them. Nevertheless, this taxon is included in *L. tropica* complex and from our data, nothing justifies the species status of *L. killicki*. Furthermore, regarding the diversity of *L. tropica* complex and the multiple monophyletic

branches in this complex, if we consider *L. killicki* as a species, *L. tropica* complex would be composed of many species. In this context, we suggest to call this taxon *L. killicki* (synonymous *L. tropica*) as it was done before for *L. chagasi* (synonymous *L. infantum*) [9, 11, 48, 49]. Further epidemiological and clinical studies in the different countries where this taxon was reported will say whether the *L. killicki* denomination should be maintained.

From an evolutionary point of view, these data suggest strongly that *L. killicki* would descend from *L. tropica* due to only one funder effect. This hypothesis is supported by the structure of the phylogenetic tree and biochemical and genetic data. Indeed, the isoenzymatic characterization showed a low number of *L. killicki* zymodemes compared to those of *L. tropica*. This low polymorphism in *L. killicki* was confirmed by the low numbers of PS, HS and haplotypes and amino acid variations. The analysis of the phylogenetic tree suggests that the origin of *L. killicki* could be *L. tropica* from the Middle East. This ancestor would have separated in *L. tropica* in Morocco and other countries on one hand and *L. killicki* in several countries on the other hand. Finally, the data suggest that *L. killicki* is now evolving independently from *L. tropica* by the lack of shared haplotypes and the identification of the new zymodeme MON-317 and its own haplotype. The independent evolution of these two taxa is probably due to different transmission cycles, zoonotic for *L. killicki* [50, 51] and both anthroponotic and zoonotic types for *L. tropica* [44, 52, 53].

Since all the *L. killicki* isolates from different countries are in the same cluster, presenting a low polymorphism and no structuring, we cannot define by these analyses the precise evolutionary history of this taxon and in particular the country in which it emerged for the first time. From the epidemiological data, higher genetic diversity and especially the relatively high number of cases described compared to the other countries [35, 54-56], it is likely that this taxon has emerged for the first time in Tunisia and then has spread in other Maghreb countries. Nevertheless, this should be studied further.

5- Conclusion

In conclusion, the present work allowed to better know the evolutionary history and the taxonomic implications of *L. killicki* in relation to *L. tropica*. However, more investigations need to be carried out on this model and particularly a detailed population genetics analysis would lead to a better understanding of the epidemiology and population dynamics in comparison to *L. tropica.*

Competing interests

The authors declare that they have no competing interests

Authors' contributions

This work was accomplished by the contribution of all authors cited: DC has done all the technical experiments and has drafted the manuscript; CR has contributed to the analysis, interpretation of data and have been involved in the revision of the manuscript; ALB has participated in the analysis, interpretation of data and has contributed to the draft and the revision of the manuscript; NH has been involved in the revision of the manuscript; PL and LT have participated in the technical experiments; FEB has contributed to data analysis; KJ and ZH have participated in samples collection; JPD has been involved the revision of the manuscript; HB has directed the study; FP has directed the study, revised and approved the manuscript. All authors have read and approved the final manuscript

Acknowledgements

This study was funded by the Institut Français de Coopération de Tunisie fellowship for PhD students

References

1. WHO: **Control of the leishmaniases: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases.** World Health Organ Tech Rep; 2010: Ser 949, Geneva.

2. Safjanova VM: **Le problème de taxonomie chez les *Leishmania***. In *Les Leishmanies*. Edited by Sér. Protozoologie, Académie Soviétique des Sciences. Science, Léningrad ; 1982 : 5-109

3. Lainson R, Shaw JJ: **Evolution, classification and geographical distribution.** *In The Leishmaniases Biology and medicine.* Edited by Peters W, Killicki- Kendrick R. London: Academie Press; 1987: 1- 120.

4. Lanotte G, Rioux JA, Maazoun R, Pasteur N, Pratlong F, Lepart J : **Application de la méthode numérique à la taxonomie du genre *Leishmania* Ross, 1903. A propos de 146 souches originaires de l'Ancien Monde. Utilisation des Allozymes. Corollaires épidémiologiques et phylétiques.** *Annls Parasitol Hum Comp* 1981, **56** : 575-592.

5. Lanotte G, Rioux JA, Lepart J, Maazoun R, Pasteur N, Pratlong F : **Contribution de la cladistique numérique à la phylétique de genre *Leishmania* Ross, 1903 (Kinetoplastida-Trypanosomatidae). Utilisation des caractères enzymatiques.** *C R Acad Sci*, Paris 1984, **299** : 769-772.

6. Lanotte G, Rioux JA, Serres E : **Approche cladistique du genre *Leishmania* Ross, 1903. A propos de 192 souches originaires de l'Ancien Monde. Analyse numérique de 50 zymodèmes identifiés par 15 enzymes et 96 isoenzymes**. *In Leishmania, Taxonomie et Phylogénèse*. Edited by Rioux JA. Montpellier : IMEE/CNRS/INSERM, Montpellier ; 1986 :269-288.

7. Le Blancq SM, Cibulkis R E, Peters W : ***Leishmania* in the Old World. 5. Numerical analysis of isoenzyme data.** *Trans R Soc Trop Med Hyg* 1986, **80**: 517-524.

8. Moreno G, Pratlong F, Velez I D, Restrepo M, Rioux JA: **Individualisation du complexe *Leishmania guyanensis*. A propos de l'analyse enzymatique de sept zymodèmes**. *In Leishmania, Taxonomie et phylogénèse*. Edited by Rioux JA. Montpellier: IMEEE/CNRS/INSERM; 1986:165-172.

9. Rioux JA, Lanotte G, Pratlong F, Bastien P, Perieres J: **Taxonomy of *Leishmania.* Use of isoenzymes. Suggestions for a new classification.** *Ann Parasitol Hum Comp* 1990, **65**: 111-115.

10. Thomaz- Soccol V, Lanotte G, Rioux JA, Pratlong F, Martini- Dumas A, Serres E: **Phylogenetic taxonomy of New Word *Leishmania*.** *Annls Parasitol Hum Comp* 1993, **68**: 104-106.

11. Cupolillo E, Grimaldi GJ, Momen H: **A general classification of New World *Leishmania* using numerical zymotaxonomy.** *Am J Trop Med Hyg* 1994, **50**: 296-311

12. Pratlong F, Dereure J, Ravel C, Lami P, Balard Y, Serres G, Lanotte G, Rioux JA, Dedet JP: **Geographical distribution and epidemiological features of Old World cutaneous leishmaniasis foci, based on the isoenzyme analysis of 1048 strains.** *Trop Med Inter Heal* 2009, **14:** 1071-1085

13. Pratlong F, Lami P, Ravel C, Balard Y, Dereure J, Serres G, El Baidouri F, Dedet JP : **Geographical distribution and epidemiological features of Old World *Leishmania infantum* and *Leishmania donovani* foci, based on the isoenzyme analysis of 2277 strains**. *Parasitology* 2013, **140**: 423-434.

14. Van Eys GJ, Schoone G J, Kroon NC, Ebeling SB: **Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites.** *Mol Bioch Prasitol* 1992, **51**: 133- 142

15. Noyes HA, Reyburn H, Bailey J W, Smith D: **A nested-PCR based schizodeme method for identifying *Leishmania* kinetoplast minicercle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan**. *J Clin Microbiol* 1998, **36**: 2877-2881

16. Bañuls AL, Jonquieres R, Guerrini F, Le Pont F, Barrera C, Espinel I, Guderian R, Echeverria R, Tibayrenc M : **Genetic analysis of *Leishmania* parasites in Ecuador: are *Leishmania* (*Viannia*) *panamensis* and *Leishmania* (*V.*) *guyanensis* distinct taxa?** *Am J Trop Med Hyg* 1999, **61** : 838-845.

17 **.** Hide M, Bañuls AL, Tibayrenc M: **Genetic heterogeneity and phylogenetic status of *Leishmania* (*Leishmania*) *infantum* zymodeme MON-1: epidemiological implications**. *Parasitology* 2001, **123**: 425-432.

18. Schönian G, Schnur L, El Fari M, Oskam L, Kolesnikov AA, Sokolowska- Köhler W, Presber W: **Genetic heterogeneity in the species *Leishmania tropica* revealed by different PCR- based methods.** *Trans R Soc Trop Med Hyg* 2001, **95**: 217-224.

19. Spanakos G, Piperaki ET, Menounos PG, Tegos N, Flemetakis A, Vakalis NC : **Detection and species identification of Old World *Leishmania* in clinical samples using a PCR-based method.** *Trans R Soc Trop Med Hyg* 2008, **102** :46-53.

20. Mauricio IL, Yeo M, Baghaei M, Doto D, Pratlong F, Zemanova E, Dedet JP, Lukes J, Miles MA: **Towards multilocus sequence typing of the *Leishmania donovani* complex: resolving genotypes and haplotypes for five polymorphic metabolic enzymes (ASAT, GPI, NH1, NH2, PGD).** *Int J Parasitol* 2006, **36** :575- 569.

21. Oddone R, Schweynoch C, Schönian G, de Sousa Cdos S, Cupolillo E, Espinosa D, Arevalo J, Noyes H, Mauricio I, Kuhls K: **Development of a multilocus microsatellite typing approach for discriminating strains of *Leishmania* (*Viannia*) species.** *Jour Clin Microbiol* 2009, **47**: 2818-2825.

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

23. Montalvo AM, Fraga J, Monzote L, Montano I, De Doncker S, Dujardin JC, Van der Auwera G: **Heat-shick protein 70 PCR-RFLP: a universal simple tool for *Leishmania* species discrimination in the New and Old World.** *Parasitology* 2010, **137**:1159-1168.

24. Haouas N, Garrab S, Gorcii M, Khorchani H, Chargui N, Ravel C, Mezhoud H, Babba H: **Development of a polymerase chain reaction-restriction fragment length polymorphism assay for *Leishmania major*/*Leishmania killicki*/*Leishmania infantum* discrimination from clinical samples, application in a Tunisian focus.** *Diagn Microbiol Infect Dis* 2010, **68**: 152-158.

25. Talmi-Frank D, Abedelmajeed N, Schnur LF, Schönian G, Toz S O, Jaffe CL, Baneth G: **Detection and Identification of Old World *Leishmania* by High Resolution Melt Analysis**. *PLoS Negl Trop Dis* 2010, **4**, e581.

26. Odiwuor S, Vuylsteke M, De Doncker S, Maes I, Mbuchi M, Dujardin JC, Van der Auwera G: ***Leishmania*** **AFLP: paving the way towards improved molecular assays and markers of diversity**. *Infect Genet Evol* 2011, **11**: 960-967.

27. Schönian G, Mauricio I, Cupolillo E: **Is it time to revise the nomenclature of *Leishmania*?** *Tren Parasitol* 2010, **26** : 466-469.

28. Van der Auwera G, Fraga J, Montalvo AM, Dujardin JC: ***Leishmania*** **taxonomy up for promotion.** *Tren Parasitol* 2011, **27** :49-50.

29. Zemanova E, Jirku M, Mauricio IL, Horak A, Miles MA, Lukes J: **The *Leishmania donovani* complex: genotypes of five metabolic enzymes (ICD, ME, MPI, G6PDH, and FH), new targets for multilocus sequence typing.** *Int J Parasitol* 2007, **37** : 149-160

30. Boité MC, Mauricio IL, Miles MA, Cupolillo E : **New insights on taxonomy, phylogeny and population genetics of *Leishmania* (*Viannia*) parasites based on multilocus sequence analysis**. *Plos Negl Trop Dis* 2012, **6**, e1888. Doi: 10.1371/journal.pntd.0001888.

31. El Baidouri F, Diancourt L, Berry V, Chevenet F, Pratlong F, Marty P, Ravel C: **Genetic structure and evolution of the *Leishmania* genus in Africa and Eurasia: What does MLST tell us?** *Plos Negl Trop Dis* 2013, **7**, e2255. Doi: 10.1371/journal.pntd.0002255.

32. Zhang CY, Lu XJ, Du XQ, Jian J, Shu L, Ma Y : **Phylogenetic and evolutionary analysis of Chinese *Leishmania* isolates based on multilocus sequence typing**. *Plos one* 2013, **8**, e63124. doi: 10.1371/journal.pone.0063124.

33. Marlow MA, Boité MC, Ferreira GEM, Steindel M, Cupolillo E: **Multilocus sequence analysis for *Leishmania braziliensis* outbreak investigation**. *Plos Negl Trop Dis* 2014, **8** (2):e2695. doi: 10.1371/journal.pntd.0002695.

34. Bañuls AL, Hide M, Prugnolle F: ***Leishmania* and the leishmaniases: A parasite genetic updates and advances in taxonomy, epidemiology and pathogeniecity in Humans**. *Adv Parasitol* 2007, **64** : 1-109.

35. Rioux JA, Lanotte G, Pratlong F : ***Leishmania killicki* n.*sp* (*Kinetoplastida*: *Trypanosomatidae*) *Leishmania*. Taxonomie et phylogenèse**. Application éco-épidémiologique (Coll. Int. CNRS/INSERM, 1984). IMEEE, Montpellier; 1986:139-142.

36. Aoun K, Bousslimi N, Haouas N, Babba H, El-Buni A, Bouratbine A: **First report of *Leishmania* (*L.*) *killicki* Rioux, Lanotte and Pratlong, 1986 in Libya**. *Parasite* 2006, **13**: 87-88.

37. Harrat Z, Boubidi SC, Pratlong F, Benikhlef R, Selt B, Dedet JP, Ravel C, Belkaid M: **Description of a dermotropic *Leishmania* close to *L. killicki* (Rioux, Lanotte & Pratlong 1986) in Algeria.** Trans *R Soc Trop Med Hyg* 2009, **103**:716-720.

38. Pratlong F, Lanotte G, Ashford RW, Rioux JA: **Le complexe *Leishmania tropica*. A propos de l'analyse numérique de 29 souches identifiées par la méthode enzymatique. *Leishmania*. Taxonomie et phylogenèse.** Applications éco-épidémiologiques (Coll. Int. CNRS/INSERM, 1984). Montpellier: IMEEE ; 1986 :129-137.

39. Rioux JA, Lanotte G: **Apport de la cladistique à l'analyse du genre *Leishmania* Ross, 1903 (*Kinetoplastida*: *Trypanosomatidae*)**. Corollaires épidémiologiques. *Biosystema* 1993, **8** : 79-80.

40. Schwenkenbecher JM, Wirth T, Schnur LF, Jaffe CL, Schallig H, Al-Jawabreh A, Hamarsheh O, Azmi K, Pratlong F, Schönian G: **Microsatellite analysis reveals genetic structure of *Leishmania tropica*.** *Int J Parasitol* 2006, **36**: 237-246.

41. Chaouch M, Fathallah-Mili A, Driss M, Lahmadi R, Ayari C, Guizani I, Ben Said M, Benabderrazak S: **Identification of Tunisian *Leishmania* spp. by PCR amplification of cysteine proteinase B (cpb) genes and phylogenetic analysis**. *Act Trop* 2013, **125**: 357-365.

42. Librado P, Rozas J: **DnaSP v5: a software for comprehensive analysis of DNA polymorphism data.** *Bioinformatics* 2009, **25**:1451- 1452.

43. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: **MEGA5 : molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.** *Mol Biol Evol* 2011, **28**: 2731- 2739.

44. Pratlong F, Rioux JA, Dereure J, Mahjour J, Gallego M, Guilvard E, Lanotte G, Perieres J, Martini A, Saddiki A : ***Leishmania tropica* in Morocco. IV-Intrafocal enzyme diversity**. *Ann Parasitol Hum Comp* 1991, **66**:100-104.

45. Haouas N, Chargui N, Chaker E, Ben Said M, Babba H, Belhadj S, Kallal K, Pratlong H, Dedet JP, Mezhoud H, Azaiez R: **Anthroponotic cutaneous leishmanisis in Tunisia: Presence of *Leishmania killicki* outside its original focus of Tataouine.** *Trans R Soc Trop Med Hyg* 2005, **99**: 499-501

46. Haouas N, Chaker E, Chargui N, Gorcii M, Belhadj S, Kallel K, Aoun K, Messaadi Akrout F, Ben Said M, Pratlong F, Dedet JP., Mezhoud H, Lami P, Zribi M, Azaiez R, Babba H: **Geographical distribution updating of Tunisian leishmaniasis foci: About the isoenzymatic analysis of 694 strains.** *Act Trop* 2012, **124**: 221-228

47. Mansouri R, Pratlong F, Bachi F, Hamrioui B, Dedet JP: **The first isoenzymatic characterizations of the *Leishmania* strains responsible for cutaneous leishmaniasis in the Area of Annaba (Eastern Algeria).** *The open Conf proceed J* 2012, **3** (Suppl 2-M2): 6-11.

48. Momen H, Gabriel Grimaldi JR, Leonidas MD : ***Leishmania infantum*, the aetiological agent of American visceral leismaniasis (AVL) ?** *Mem Inst Oswaldo Cruz* 1987, **82** :447-448.

49. Mauricio IL, Stothard JR, Miles MA: **The strange case of *Leishmania chagasi***. *Parasitology Today* 2000, **16**:188-189.

50. Jaouadi K, Haouas N, Chaara D, Gorcii M, Chargui N, Augot D, Pratlong F, Dedet JP, Ettlijani S, Mezhoud H, Babba H: **First detection of *Leishmania killicki* (*Kinetoplastida, Trypanosomatidae*) in *Ctenodactylus gundi (Rodentia, Ctenodactylidae*), a possible reservoir of human cutaneous leishmaniasis in Tunisia.** *Parasit Vect* 2011, **11**: 159

51. Bousslimi N, Ben-Ayed S, Ben-Abda I, Aoun K, Bouratbine A: **Natural infection of North African gundi (*Ctenodactylus gundi*) by *Leishmania tropica* in the focus of cutaneous leishmaniasis, Southeast Tunisia.** *Am J Trop Med Hyg* 2012, **86**: 962-965

52. Sang DK, Njeru WK, Ashford RW : **A zoonotic focus of cutaneous leishmaniasis due to *Leishmania tropica* at Utut, Rift Valley Province Kenya.** *Trans R Soc Trop Med Hyg* 1994, **88** : 35-37

53. Svobodova M, Votypka J, Peckova J, Dvorak V, Nasereddin A, Baneth G, Sztern J, KravchenkoV, Orr A, Meir D : **Distinct transmission cycles of *Leismania tropica* in 2 adacent foci, Northern Israel.** *Emerg Infect Dis* 2006, **12**: 1860-1868

54. Chaffai M, Ben Rachid MS, Ben Ismail R: **Formes clinico-épidémiologiques des Leishmanioses cutanées en Tunisie**. *Ann Dermatol Venereol* 1988, **115**: 1255-1260

55. Aoun K, Ben Abda I, Bousslimi N, Bettaieb J, Siala E, Ben Abdallah R, Benmously R, Bouratbine A: **Caractérisation comparative des trois formes de leishmaniose cutanée endémique en Tunisie.** *Ann Dermatol Venereol* 2012, **139**: 452-458

56. Jaouadi K, Depaquit J, Haouas N, Chaara D, Gorcii M, Chargui N, Dedet JP, Pratlong F, Boubabous R, Babba H: **Twenty-four new human cases of cutaneous leishmaniasis due to *Leishmania killicki* in Metlaoui, southwestern Tunisia: probable role of *Phlebotomus sergenti* in the transmission.** *Act Trop* 2012, **122** : 276-283.

Legends to Figures

Fig 1. Neighbor Joining tree constructed from the concatenated duplicated sequences of the seven loci for the 32 strains representing different identified haplotypes using the Maximum Composite Likelihood model and 500 bootstrap replications.

Legends to Tables

Table 1. Details of the 62 studied strains

Table 2. Enzyme profiles of the four zymodemes of *L. killicki*

Table 3. Genetic data on all studied *L. killicki* and *L. tropica* strains

Table 4. Comparison of genetic data between *L. killicki* and *L. tropica*

Table 5. Amino acid variations between *L. killicki* and *L. tropica*

Table 6. Identified haplotypes of *L. killicki* and *L. tropica*

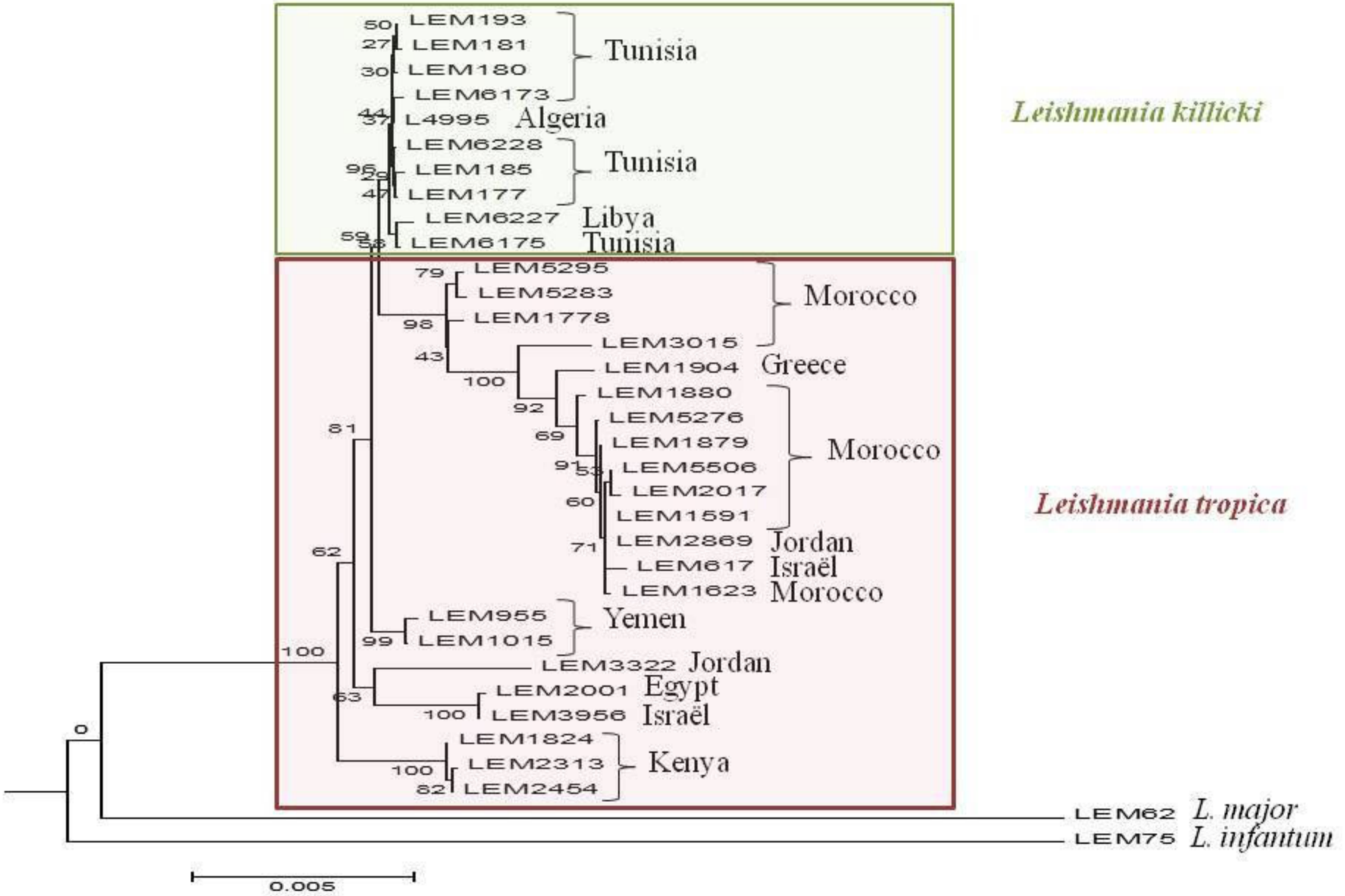

Figure1. Neighbor Joining tree constructed from the concatenated duplicated sequences of the seven loci for the 32 strains representing different identified haplotypes using the Maximum Composite Likelihood model and 500 bootstrap replications.

Figure 1

Additional files provided with this submission:

Additional file 1: Table1.xls, 35K
http://www.parasitesandvectors.com/imedia/2004650257142852/supp1.xls
Additional file 2: Table2.xls, 19K
http://www.parasitesandvectors.com/imedia/1142783989142852/supp2.xls
Additional file 3: Table3.xls, 18K
http://www.parasitesandvectors.com/imedia/1176325871142852/supp3.xls
Additional file 4: Table4.xls, 20K
http://www.parasitesandvectors.com/imedia/5747899971428521/supp4.xls
Additional file 5: Table5.xls, 24K
http://www.parasitesandvectors.com/imedia/1341860827142852/supp5.xls
Additional file 6: Table6.xls, 21K
http://www.parasitesandvectors.com/imedia/2227599461428521/supp6.xls