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To cite this version:
Myriam Harrabi, Wissem Ghawar, Mallorie Hide, Jihene Bettaieb, Rihab Yazidi, et al.. No evidence of interspecific genetic exchange by multi-locus microsatellite typing between Leishmania Killicki and Leishmania Major in a mixed focus of cutaneous Leishmaniasis in Southeast Tunisia. Journal of Infectious Diseases & Preventive Medicine, OMICS International, 2017, 5 (2), pp.1000163. 10.4172/2329-8731.1000163 . hal-02011014

HAL Id: hal-02011014
https://hal.umontpellier.fr/hal-02011014
Submitted on 7 Feb 2019

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No Evidence of Interspecific Genetic Exchange by Multi-Locus Microsatellite Typing Between *Leishmania Killicki* and *Leishmania Major* in a Mixed Focus of Cutaneous Leishmaniasis in Southeast Tunisia

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Received date: March 16, 2017; Accepted date: May 10, 2017; Published date: May 17, 2017

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**Abstract**

Sixty-four *Leishmania* samples were isolated from patients in several villages in the Tataouine governorate, southeast Tunisia. This region is known to be a mixed focus of human cutaneous leishmaniasis caused by *Leishmania* (L.) *killicki* (synonymous *L. tropica*) and *L. major*. To identify the *Leishmania* species in this governorate, a nested polymerase chain reaction based on the variable region of the kinetoplast minicircle was performed on each isolate. Multi-locus microsatellite typing using markers selected for their ability to amplify the two species was used to explore patterns of interspecific genetic exchange. Thirteen *L. major* and 51 *L. killicki* isolates were identified. The analysis of microsatellite data showed very low genetic diversity in each species with this set of microsatellites but a high differentiation between the two species. Nine *L. major* and five *L. killicki* strains revealed heterozygous genotypes with no shared allele between the two species. These heterozygotes probably resulted from genetic mutation events and not from interspecific genetic exchange. Specific and different epidemiological cycles at the sympatric level might explain the absence of genetic exchange between the two *Leishmania* species in the Tataouine governorate.

**Keywords:** *L. major*, *L. killicki*, Cutaneous leishmaniasis; Tunisia; Heterozygosity; Microsatellites

**Introduction**

Leishmaniasis is widespread in Tunisia and considered to be one of the major health problems for the human population [1]. In Tataouine, southeast Tunisia, *Leishmania* (L.) *major* and *L. killicki* (synonymous *L. tropica*) [2] are currently the causative agents of cutaneous leishmaniasis (CL) [3]. *Leishmania killicki* is responsible for the chronic CL observed in villages built on the flank of the Rocky Mountains [3-5]. *Leishmania major*, the causative agent of zoonotic CL, probably emerged as a human pathogen in this area as a result of recent establishment of a high density of susceptible human populations at the margins of villages [3].

In Tunisia, *Phlebotomus* (P.) *sergenti* and *P. papatasi* are the proven vectors of *L. killicki* (syn. *L. tropica*) and *L. major* species, respectively [6-8]. Recent studies on sandflies showed that *P. papatasi* is the most abundant in the burrows of rodents such as *Meriones*, which are known reservoir hosts of *L. major*. Indoors, *P. sergenti* and *P. papatasi*, known to be endophilic were co-dominant [8]. *Phlebotomus riouxi* (or *P. chabaudi*), which is suspected to be linked to *L. killicki* transmission [9], was the dominant species in the semi-natural rocky habitats associated with the presence of *Ctenodactylus* (C.) *gundl*, described as a potential reservoir [7-10]. Concerning the reproductive strategy, the current assumption, mainly based on population genetics studies, is that *Leishmania* alternates between three modes of reproduction: clonality, allogamy (interspecific recombination), and endogamy (intraspecific recombination), varying according to the species and the environment [11-18].

The occurrence of genetic exchange between different *Leishmania* species (corresponding to allogamy) is now largely accepted. Several cases of hybridization have been described in natural populations between both genetically close or distinct species (*L. braziliensis* and *L. panamensis/guyanensis* [19]; *L. panamensis* and *L. braziliensis* [20]; *L. braziliensis* and *L. guyanensis* [21]; *L. major* and *L. arabica* [22]; multiple hybrid genotypes of *Leishmania* (*Viannia*) species [23]; *L. infantum* and *L. major* [24]; *L. donovani* and *L. aethiopica* [25]; *L. donovani*, *L. major* and *L.intantum* [26]; *L.braziliensis* and *L.peruviana* [27]). All these hybrids were evidenced by the detection of heterozygous profiles reflecting genetic exchange between different species. Additionally, some reports also suggested that intraspecific hybridization events (corresponding to endogamy) could also occur [28-31]. Several in vivo studies demonstrated the capacity of *Leishmania* species to undergo intraspecific genetic exchange in sandflies [32-34]. Although genetic exchanges have been experimentally and empirically demonstrated, questions remain about its frequency in natural *Leishmania* populations and their impact on the evolution of these populations.

In this context, our objective was to explore the existence of hybridization patterns between *L. major* and *L. killicki* by multi-locus microsatellite typing (MLMT) in a CL focus, where the two species are...
spreading. A total of 64 Leishmania isolates from patients with CL in the southeast of Tunisia (Tataouine governorate) were analyzed.

Materials and Methods

Study area

The study was carried out in the governorate of Tataouine, located in far southeast Tunisia at 32°55′40.16″ North, 10°26′57.28″ East and an altitude of C. 300 m above sea level [35] (Figure 1). Tataouine is characterized by its vast area (23.7% of the country) and by its international frontiers (Libya and Algeria). This region covers an area of 38,889 km² with a population of 149,453 (2014 census) [36]. Tataouine is subdivided into three biogeographical zones: the desert, the mountain (Dhaher), and the plain (Djefara) (Republic of Tunisia Ministry of Development, Investment and International Cooperation, South Development Office). It has a Mediterranean-desert climate [35]; the average temperature is 22°C and annual rainfall varies between 88 and 157 mm [37]. The regional landscape is characterized by poor vegetation, dominated by steppe species, and rocky escarpments. The area is pastoral with olive groves and cereals cultivated around water points. Ctenodactylus gundi is the most prevalent wild rodent living in the stony mountains. However, gerbils (Meriones species) are found at the periphery of villages [38].

Leishmania identification

Some isolates were previously characterized by the multi-locus enzyme electrophoresis technique performed in the World Health Organization (WHO) reference center of leishmaniasis in Montpellier “Centre National de Référence des Leishmanioses”; they are indicated in Supplementary Table 2 with a WHO code (unpublished data). For our study, genomic DNA was extracted from the 64 parasite cultures (promastigotes) using the QIAamp® DNA Mini Kit (Qiagen, CA) according to the manufacturer’s protocol. DNA quality was checked by agarose gel electrophoresis and the concentration was measured with a Nano Drop spectrophotometer. For identification of the Leishmania species, a nested PCR, previously designed by Noyes et al. (1998) was performed with modifications [39,40].

The presence of a product of approximately 560-bp indicated L. major and a product of approximately 750-bp indicated L. tropica. Negative control were included in each reaction mix. Two reference strains, L. tropicaMHOM/SU/74/SAF-K27 and L. major MHOM/SU/73/ASKH, were included for comparison.

Multilocus Microsatellite Typing

Microsatellite markers

A panel of 11 microsatellite markers designed for the characterization of L. tropica species or L. major was selected for their potential ability to amplify the two species (Table 1). The selected microsatellite markers were previously described in Schwenkenbecher et al. [41]. As it was recently confirmed that L. killicki belongs to the L. tropica complex with close genetic relationships [2], we used the L. tropica microsatellite markers for genetic characterization of L. killicki. In addition to microsatellite markers designed for L. tropica with positive PCR products for L. major, we selected microsatellite loci originally designed for L. major and also able to be amplified in L. tropica.

All the selected loci were tested on three L. major strains (one reference strain and two isolates from our sample) and three L. tropica and L. killicki strains (one reference strain and two isolates from our sample). PCR conditions were optimized for the 11 markers to increase the probability of amplifying the loci for both species. Approximately 50 ng of genomic DNA was added to each PCR. A negative control was included in each experiment. Microsatellites were amplified using the following conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 30s, annealing temperature for 1 min (Table 1), and 72°C for 1 min (extension), followed by a final extension at 72°C for 10 min. Genotyping was performed by capillary electrophoresis using an ABI Prism 3130 XL automated DNA sequencer (Applied Biosystems, USA). Fragment size was determined automatically using GeneMapper 4.0 software (Applied Biosystems). Genescan 500 LIZ (Applied Biosystems, France) was used as internal size standard. The loci were then selected and evaluated according to the peak quality and sizing information.

Genetic and Phylogenetic Analyses

FSTAT Version 2.9.3.2 software [42] updated from [43] was used to analyze the data. Genetic polymorphism was measured based on the allelic richness (A) and the Nei’s unbiased estimate of genetic diversity within subsamples (Hs) [44]. FST measures the relative inbreeding in subpopulations attributable to the subdivision of the total population.
into subpopulations of limited size. FST also measures the genetic differentiation between subpopulations. The significant departure from zero of these parameters was tested by 10,000 randomization procedures with FSTAT. The significance of these estimates was confirmed by p-values ≤ 0.05. A neighbor-joining tree, based on Nei's minimum genetic distances was used to cluster the genotypes. Data were computed using POPULATION software to build the distance matrix (version 1.2.28; Centre National de la Recherche Scientifique, UPR9034, Langella, O) and the phylogenetic tree was generated using MEGA software, version 4.0.2 [45].

Results

Lesion type and clinical impression

The lesions were unique in 68.75% (n=44) of cases and showed a clinical polymorphism. The most frequent pattern of eruption was seen in 78.12% of cases (n=50) patients who had a nodular ulcerative crusting aspects localized predominantly in the limbs (65.62%), followed by nine patients who had plaque-like ulcerative eruption followed by 5 patients who had a dry plaque/nodular eruption. All dry lesions were found to be Leishmania killicki (syn. L. tropica) (see supplementary Tables 1,2 and Figure 2).

Table 1: Details of the 11 microsatellite loci selected for the characterization of L. major and L. killicki species [28].

| Primer tested | Repeat array | Flanking region size (bp) | Labeled Forward Primer (5’→3’) | Unlabeled reverse Primer (5’→3’) | AT (°C) | Fragment size (bpjc) | Applicability for both species
|---------------|--------------|--------------------------|---------------------------|---------------------------------|---------|---------------------|----------------------|
| GA1<sup>a</sup> | (GA)11       | 44                       | 6FAM-TGGAGTGACCTCGCAGGCGGC | GGTGGGCGAGGTAAGCGGCGGC          | 56      | 66                  | Applicable<sup>*</sup> |
| GA2<sup>a</sup> | (GA)8        | 46                       | VIC-GATGACAGCAGCGCTGAGTGAAG | CCTCTGACACCACATACCAGGC          | 56      | 62                  | Not applicable**     |
| GA3<sup>a</sup> | (GA)7        | 50                       | NED-GCGAGCGACACATACACAGCA   | CGCAGCTATTGTCGCGCGCCCGCGG       | 50      | 64                  | Applicable<sup>*</sup> |
| GA8<sup>a</sup> | (GA)8        | 45                       | PET-GTGTGAGCTAATCGGATTGGG   | CGCTCTTCTCGTCTGTCTGTCT          | 42      | 61                  | Not applicable**     |
| GA10<sup>a</sup> | (GA)19       | 50                       | 6FAM-CTCTACTCCCTGTTGAGTAT    | CGGCATATCTCATCCACT              | 42      | 89                  | Applicable<sup>*</sup> |
| Mix9<sup>a</sup> | (GC/GT)12    | 44                       | VIC-CGCGTGAAGAGGAGCGGCGCC    | GTGCTTGTGTCTGTGTGAGC           | 60      | 60                  | Applicable<sup>*</sup> |
| GTG3<sup>a</sup> | (GTG)5       | 50                       | NED-TAAGTGGACATCACAGGAGGAGG | GCGGACAGGTGCTGCTCGCCT            | 50      | 65                  | Applicable<sup>*</sup> |
| GT4<sup>a</sup> | (GT)12       | 50                       | PET-TCTGTGACATCCTCTGCCA      | TGAATCTCGGGGCGCGGGGCT           | 56      | 74                  | Applicable<sup>*</sup> |
| GACA4<sup>a</sup> | (GACA)3      | 50                       | 6FAM-ACCCACACAGGCACACAC     | CTCTCGGCGTCCGCTGTGTC            | 62      | 62                  | Not applicable**     |
| 4GTG<sup>b</sup> | (GTG)5       | 47                       | VIC-CGTTCTGGCGCCGTCGAGG      | CGTGGAGCGCGCGCGCGG             | 54.5    | 62                  | Applicable<sup>*</sup> |
| 27GTG<sup>b</sup> | (GTG)5       | 44                       | NED-GAGGGTGGGCTGTGGTGGG      | GCGCCTGACGCGTGCAGCCT            | 50      | 59                  | Applicable<sup>*</sup> |

<sup>a</sup>Originally designed for L. tropica; <sup>b</sup>Originally designed for L. major; <sup>c</sup>Fragment sizes as in the reference strain MHOM/PS/2001/ISL590; <sup>*</sup>Usable for both species, L. major and L. tropica. | Not usable for both species, L. major and L. tropica; AT: Annealing temperature.

Table 2: Genetic diversity indices estimated from microsatellite data (eight loci) for the 64 L.major and L. killicki isolates included in this study.

| Population (number of isolates) | Descriptive statistics |
|---------------------------------|------------------------|
|                               | A (Hs) | H o | H e |
| L. major population (n=13)      | 1.247  | 0.08 | 0.102 | 0.081 |
| L. killicki population (n=51)   | 1.118  | 0.012 | 0.012 | 0.012 |

A: Allelic richness per population based on the standardized minimal sample size; Hs: Gene diversity; Ho: Observed heterozygosity; He: expected heterozygosity.
Identification of *Leishmania* species by nested PCR

All isolates analyzed presented a single *Leishmania* kinetoplast minicircle amplicon of approximately 560-bp or 750-bp, corresponding to *L. major* and *L. tropica* species, respectively. These identifications corroborated the CNRL data for *L. killicki* and *L. major* strains of the 64 samples under study. 51 isolates showed an amplicon size corresponding to *L. tropica* and 13 to *L. major*. The identification of each sample is detailed in Supplementary Table 2 and the village distribution of the two species in Supplementary Table 1 and Figure 1. Both chronic and zoonotic CL co-exist in the Tataouine governorate with *L. killicki* (syn. *L.tropica*) as the predominant species. The majority of the villages under study contained the two species except Tlelet (seven *L. killicki* strains) and Oued Khil (one *L. killicki* strain) localities (see Figure 1).

Microsatellite genotyping

From the 11 microsatellite loci selected from Schwenkenbecher et al. [41], our choice was essentially based on the ability of primer pairs to amplify products from the two species. Eight markers were usable for our purpose including those originally designed for *L. major* (4GTG and 27GTG) [46] (see Table 1). Three markers (GA2, GA6, and GACA4) could not be used because of their inability to amplify both species (GA6), imperfect reproducibility (GACA4), or, in the case of GA2, under the same MLMT-PCR conditions; primer pairs amplified a 66-bp product for *L. killicki* and a 499-bp product for *L. major*, suggesting different genomic targets in the different species.

Forty of the 64 isolates yielded complete genotype data for the eight selected loci. In some cases, no PCR products were obtained in repeated PCR runs, and were treated as missing data for the statistical analyses (see supplementary Table 2). The eight microsatellite markers were polymorphic, based on number of microsatellites repeats, across the whole sample set, including the reference strains, and five of them revealed heterozygous patterns. Among the 51 *L. killicki* and 13 *L. major* isolates genotyped with the eight loci, a total of 20 different alleles were identified and allelic richness (A) was 1.12 and 1.25, respectively.

The number of alleles per locus ranged from two (GA1, GA3, GTG3, and 4GTG) to three (GA10, Mix9, GT4, and 27GTG). The level of unbiased gene diversity (Hs) across populations was very low for the two species (Hs=0.080 for *L. major*, Hs=0.012 for *L. killicki*) (Table 2). Overall, 20 different genotypes were detected among the 64 samples analyzed; 5 genotypes for *L. major* populations of 13 isolates and 15 genotypes for *L. killicki* populations of 51 isolates. Nine of the 13 *L. major* isolates from Medila, Ferech, and Guermessa displayed an identical heterozygous genotype (64/68) at locus Mix9 (see Supplementary Table 2 and samples with green dots on Figure 2). Four isolates from Gomrassen and Ksar Mourabtin differed from the first nine either by a distinct allele at locus 27GTG (GHO010) or were homoygous at the Mix9 locus (GHO022, KSM001, and KSM006). Among the *L. killicki* isolates, we obtained two clusters of 28 and 9 isolates showing identical genotypes in each cluster, of which 11 samples had missing data at one or more markers. Seventeen strains had unique genotypes, of which five were heterozygous at loci GA10 (79/85) or GT4 (72/78) (see Supplementary Table 2 and Figure 2). The *L. tropica* reference strains (SAF-K27) revealed four different heterozygous patterns at loci GA10, GT3, GT4, and 27GTG.

Homoygous genotypes prevailed in our samples. As described above, heterozygous profiles were recorded for 14 isolates (without including *L. tropica* reference strain, MHOM/SU/74/SAF-K27) and on only one locus for each (MIx9 or GT4 or GA10). Ranged from 0 to 0.818 and he ranged from 0 to 0.506 per locus in the *L. major* population. For *L. killicki*, observed and expected values ranged from 0 to 0.663 and from 0 to 0.062, respectively (data not shown). The mean observed heterozygosity was very low for the *L. killicki* population and slightly higher for the *L. major* population (Table 2). Mean expected heterozygosity within the *L. killicki* population was similar to the mean observed heterozygosity and different to that of the *L. major* population (see Table 2).

Furthermore, it is worth noting that all the heterozygotes displayed one allele observed in the homoygous state in the same species and a second allele absent in the homoygous state. For example, the heterozygotes observed for *L. major* isolates at the locus Mix9 displayed allele (68) found in the homoygous state in the *L. major* population (68/68) and allele (64), which is absent in the homoygous state in the whole population (L. major+ L. killicki) (see Supplementary Table 2). The same pattern is observed in the *L. killicki* population for the GT4 and GA10 markers. The mean FST value estimated was 0.966 (p-value ≤ 0.05), showing, as expected, a significant level of differentiation between the two species [47]. The data from the MLMT analysis on the 64 *L. major* and *L. killicki* isolates and the two reference strains were used to create matrix distances and construct a neighbor-joining tree representing the relationships among all the strains used in this study. As expected, the MLMT data classified isolates according to the species taxonomy (see supplementary Table 2). Strains with heterozygous genotypes (tagged with green dots) clustered either in *L. killicki* cluster or in *L. major* one in agreement with their species identification. The phenetic tree did not reveal any cluster with an intermediate position between the two species (see Figure 3).
The of L. major in Tataouine were only recorded in 1991 [49,50], but now that L. major and L. killicki (syn. L. tropica) are the only species difference chronic and zoonotic CL co-exist in this governorate [3-5]. In transmission in humans of this taxon can explain the low level of genetic diversity. Nevertheless, conversely to the data of Chaara et al. geographical spread of Leishmania [3,7,9]. Contrary to previous agreement with previous epidemiological reports, our analysis found of our sampling. This probably owing to genetic mutations for L. killicki or to L. major, revealed heterozygous genotypes, with no shared allele between the two species. The accommodation of mutations, consisting of gain/loss of repeat units in microsatellites [61,62] may explain these heterozygous profiles. Both species (L. major and L. killicki) were collected in Ghomrassen, Gueremessa, Farech, and Mdhila villages. The absence of interspecific genetic exchange might be explained by different epidemiological cycles at the sympatric level. These two species seem to have overlapped geographical foci but different vector species and different mammalian reservoir hosts [7,9,10,63], limiting the interactions between them. As reported by Tabbabi et al. the incriminated vectors for L. major and L. killicki in the Tataouine region would be species specific [9]. Thus, the most likely opportunity for these two species to interact is during mixed infections in humans. According to published data, cross-species genetic exchange between Leishmania strains would mainly occur in the invertebrate stage [32-34,64]. These epidemiological and biological aspects could explain the absence of interspecific genetic exchange between L. killicki and L. major in the Tataouine governorate. Nevertheless, from our data we cannot infer any hypothesis on intraspecific genetic exchange.

Conclusion
In our study, we detected two sympatric species responsible for CL in southeast Tunisia, Tataouine governorate: L. major and L. killicki. Each species revealed very low intraspecific diversity but high genetic differentiation. Nine L. major strains and five L. killicki strains displayed heterozygous profiles probably owing to genetic mutations and not to genetic exchange. Specific and different epidemiological cycles at the sympatric level might explain the absence of cross-species genetic exchange between the two species in the Tataouine governorate.

Acknowledgment
The authors thank the Regional Health Directorate of the Tataouine governorate (Tunisia), the "Institut Pasteur Tunis" and the "Institut de Recherche pour le Développement" (Montpellier, France), and the
“Centre National de la Recherche Scientifique” (France) for this cooperative and collaborative project. We acknowledge the field staff of the Department of Medical Biology, Institut Pasteur Tunis, for strain collection and cryobank management; Adel Gharbi and Mongi Dellagi, for ensuring strain and patient data collection in accordance with current Good Clinical Practice guidelines. We gratefully acknowledge the “Centre National de Référence des Leishmanioses” for the multilocus enzyme electrophoresis typing of some strains.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Citation: Harrabi M, Ghawar W, Hide M, Bettaieb J, Yazidi Y, et al. (2017) No Evidence of Interspecific Genetic Exchange by Multi-Locus Microsatellite Typing Between Leishmania Killicki and Leishmania Major in a Mixed Focus of Cutaneous Leishmaniasis in Southeast Tunisia. J Infect Dis Preve Med 5: 163. doi:10.4172/2329-8731.1000163

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