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Multilocus Microsatellite Typing reveals intra-focal genetic diversity among strains of *Leishmania tropica* in Chichaoua Province, Morocco

Lena Krayter [a,*], Mohammad Zahangir Alam [a], Mohamed Rhajaoui [b], Lionel F. Schnur [c], Gabriele Schönian [a]

[a] Institute of Microbiology and Hygiene, Charité-University Medicine Berlin, Hindenburgdamm 30, 12203 Berlin, Germany
[b] Département de Parasitologie, Institut National d'Hygiène, POB 769, Avenue Ibn Batouta, 11400, Rabat – Agdal, Rabat, Morocco
[c] Department of Parasitology, The Kuvin Center for the Study of Infectious and Tropical Diseases, Hebrew University-Hadassah Medical School, Jerusalem, Israel

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ABSTRACT

In Morocco, cutaneous leishmaniasis (CL) caused by *Leishmania* (*L.*) *tropica* is a major public health threat. Strains of this species have been shown to display considerable serological, biochemical, molecular biological and genetic heterogeneity; and Multilocus Enzyme Electrophoresis (MLEE), has shown that in many countries including Morocco heterogenic variants of *L. tropica* can co-exist in single geographical foci. Here, the microsatellite profiles discerned by MLMT of nine Moroccan strains of *L. tropica* isolated in 2000 from human cases of CL from Chichaoua Province were compared to those of nine Moroccan strains of *L. tropica* isolated between 1988 and 1990 from human cases of CL from Marrakech Province, and also to those of 147 strains of *L. tropica* isolated at different times from different worldwide geographical locations within the range of distribution of the species. Several programs, each employing a different algorithm, were used for population genetic analysis. The strains from each of the two Moroccan foci separated into two phylogenetic clusters independent of their geographical origin. Genetic diversity and heterogeneity existed in both foci, which are geographically close to each other. This intra-focal distribution of genetic variants of *L. tropica* is not considered owing to *in situ* mutation. Rather, it is proposed to be explained by the importation of pre-existing variants of *L. tropica* into Morocco.

1. Introduction

Three species of *Leishmania* cause human cutaneous leishmaniasis (CL) in Morocco: *Leishmania* (*Leishmania*) *major*, *L.* (*L.*) *tropica*, and, rarely, in the north of the country, *L.* (*L.*) *infantum*, which is usually the cause of human and canine visceral leishmaniasis (VL) in all the countries surrounding the Mediterranean Sea (Amro et al., 2013; Rhajaoui et al., 2007). Of these, *L. tropica* is geographically the most widely spread (Rhajaoui et al., 2007). Since 1997, CL caused by *L. tropica* has been considered a major public health hazard in Morocco as the number of human cases of CL caused by it has constantly increased during the previous decade and more (Alvar et al., 2012). *Phlebotomus* (*Paraphlebotomus*) *sergenti* and *Phlebotomus* (*Paraphlebotomus*) *chabaudi*, which are anthropophilic vectors, transmit *L. tropica* in Morocco (Alvar et al., 2012; Guernaoui et al., 2005; Guilvard et al., 1991). In Chichaoua Province, western Morocco, *L. tropica* causes human CL, *P. sergenti* is the vector, and the disease occurs predominantly in the area south and south- east of the Atlas Mountains (Fig. 1) (Guernaoui et al., 2005; Rhajaoui, 2011; Rhajaoui et al., 2012). Anomalous cases of leishmaniasis have also been reported from Morocco: two cases of canine VL caused by *L. tropica* (Guessous-Idrissi et al., 1997; Lemrani et al., 2002) and seven cases of canine CL caused by *L. tropica* (Dereure et al., 1991); and several of human (Rhajaoui et al., 2007; Rioux et al., 1996) and at least one of canine CL caused by strains of *L. infantum* (Dereure et al., 1991).

Strains of *L. tropica* are very heterogeneous, displaying considerable serological, biochemical, and molecular biological diversity, and also showed extensive genetic variation divulged by Multilocus Microsatellite Typing (MLMT) done prior to this study (Le Blancq and Peters, 1986; Schnur et al., 2004; Schonian et al., 2001; Schwenkenbecher et al., 2006). Multilocus Enzyme Electrophoresis (MLEE) revealed the existence of ten different zymodemes of *L. tropica* in Morocco among 178 strains, which indicated a particularly high degree of polymorphism (Pratlong et al., 2009). By comparison and underscoring the high degree of heterogeneity of the species *L. tropica*, only one zymodeme of *L. major* (MON-25) and two of *L. infantum* (MON-1 and MON-24) have been found in

* Corresponding author. Tel.: +49 30 450524058.
E-mail addresses: lena.krayter@charite.de (L. Krayter), mzalam12@yahoo.com (M.Z. Alam), rhajaouimed@yahoo.fr (M. Rhajaoui), schnurl@cc.huji.ac.il (L.F. Schnur), gabriele.schoenian@charite.de (G. Schönian).

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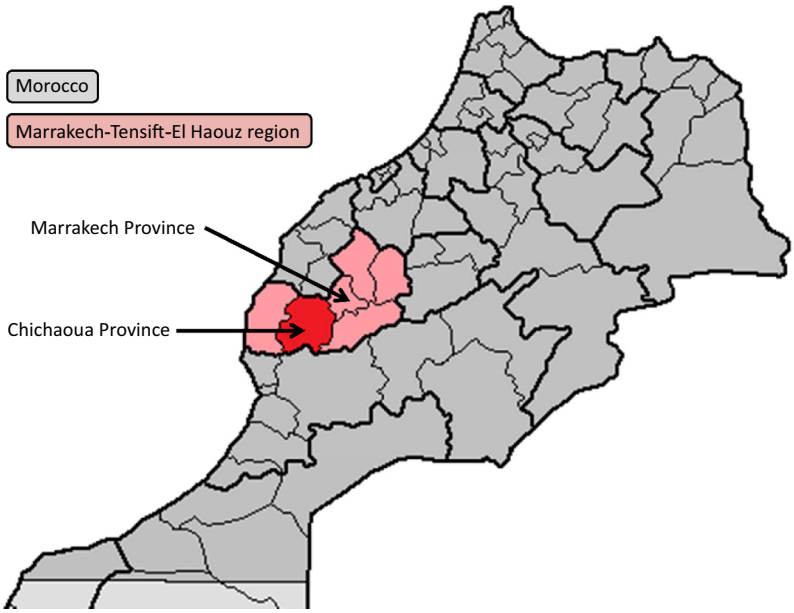

Fig. 1. The study area. Map of Morocco showing the Chichaoua and Marrakech Provinces, which are parts of the Marrakech-Tensift-Al Haouz region and from where the strains came. Modified from *Die Provinz Chichaoua in der Region Marrakech-Tensift-Al Haouz* by Vincent van Zeijst/Wikimedia Commons/CC BY-SA 3.0.

Morocco (Pratlong et al., 2009; Rioux et al., 1996; Ait-Oudhia et al., 2011).

On applying MLMT to Moroccan strains of *L. tropica* from the Marrakech Province, Schwenkenbecher et al. found that they separated into two genetically distinct clusters (Schwenkenbecher et al., 2006). Pratlong et al., combining the numerous results of MLEE, exposed isoenzyme diversity within geographical foci (Pratlong et al., 1991). The aim of this study was to analyse the intra-focal diversity of Moroccan strains of *L. tropica*, and to draw conclusions about the population dynamics of *L. tropica* in these Moroccan foci. Nine Moroccan strains of *L. tropica* from the Chichaoua Province (Fig. 1) were analysed by MLMT to see if they formed a single genetic entity congruent with their geographical origin or, like the strains from Marrakech Province, separated into distinct intra-focal genetic clusters. For this, 12 unlinked microsatellite markers, previously shown capable of revealing population structure at the intra-species level, were used (Krayter et al., 2014). The resulting fragment sizes were compared to those of previously typed strains of *L. tropica* of diverse geographical origins within the range of the species (Krayter et al., 2014; Schwenkenbecher et al., 2006).

2. Materials and methods

2.1. Ethical clearance

This study was approved by the Ethics Committee of the National Reference Laboratory for Leishmaniasis (LNRL) of the National Institute of Hygiene, Rabat, Morocco.

2.2. Leishmanial strains

The nine strains of *L. tropica* were isolated from skin biopsies of patients diagnosed with CL at the local health care centres in the Chichaoua Province, Morocco, in 2000 (Fig. 1). After 4 weeks of cultivation they were cryopreserved until the isolation of their gDNA. Their WHO codes are: MHOM/MA/2000/INH-W02, MHOM/MA/2000/INH-W04, MHOM/MA/2000/INH-W05, MHOM/MA/2000/INH-W09, MHOM/MA/2000/INH-W10, MHOM/MA/2000/INH-W13, MHOM/MA/2000/INH-W14, MHOM/MA/2000/INH-W16, and MHOM/MA/2000/INH-W17. The strains from Marrakech were collected in the frame of a field study of Pratlong et al. (1991). Both, skin biopsies of the human cases and the intestinal content of the sand flies were cultured before cryopreservation.

For population genetic analyses, 156 microsatellite profiles of other strains of *L. tropica* from many geographical regions where CL is endemic were used for comparison (Table A.1). The strain of *L. tropica* MHOM/PS/2001/ISL590, sequenced previously (Schwenkenbecher et al., 2004), served as a reference in all experiments.

2.3. Microsatellite typing

The repeat numbers of 12 unlinked microsatellite markers were determined as described in (Krayter et al., 2014). Briefly, microsatellite regions were amplified by PCR and, subsequently, their sizes were determined by fragment analysis, using an ABI sequencer and peak evaluation, using GeneMapper software version 3.7 (Applied Biosystems, Foster City, USA). Both, homo- and heterozygous loci were included in the following analyses. DNA from the reference strain of *L. tropica.*

MHOM/PS/2001/ISL590 was included in each run as a standard for fragment size in comparing DNA samples from different PCR amplifications and fragment analyses. Microsatellite calculations were carried out with virtual fragment sizes. These were created by first calculating the repeat numbers in comparison to the reference DNA, then multiplying them by the size of the microsatellite repeat (di- or tri-nucleotide repeat) and, finally, adding the size of the flanking regions.

2.4. Population genetic analyses

Several programs for population genetic analysis were used, each working with a different algorithm.

Bayesian clustering implemented in STRUCTURE software version 2.3.4 (Pritchard et al., 2000) was used to infer the population structure. Analysing allele frequencies identifies genetically

distinct groups and estimates the membership of each strain to the groups. The length of the Burnin period and the number of Markov Chain Monte Carlo Repeats after Burnin were set to 20,000 and 200,000, respectively. The most likely number of distinct populations was determined by calculating deltaK (Evanno et al., 2005), using 10 replicate runs for each K.

Factorial correspondence analysis (FCA) is a multidimensional statistical method implemented in Genetix 4.05 (Belkhir et al., 1996–2004) and presents an additional tool to evaluate the number of genetic groups.

Genetic distances were calculated with the Chord distance (Cavalli-Sforza and Edwards, 1967) settings in POPULATIONS software version 1.2.34 (http://bioinformatics.org/~tryphon/populations/). MEGA 5.1 was used to visualise the resulting Neighbour Joining (NJ) tree (Tamura et al., 2011).

A phylogenetic network was created, using SplitsTree 4.12.8 (Huson and Bryant, 2006), which accounts for reticulation events such as hybridization, horizontal gene transfer and recombination.

F_{ST} values (genetic distances between populations), mean number of alleles (A), observed (H_o) and expected (H_e), and the inbreeding coefficient (F_{IS}) were calculated, using MSA 4.05 and GDA 1.1 (Lewis and Zaykin, 2001).

CONVERT 1.31 (Glaubitz, 2004) and MSA 4.05 (Dieringer and Schlötterer, 2003) were used to convert the input file into the required formats.

3. Results

The analysed Moroccan strains encompassed 12 different microsatellite profiles: four from Chichaoua Province; eight from the Marrakech Province. The 165 strains encompassed by the study comprised 112 different microsatellite profiles. The fragment size for one marker (4GTG) was consistent for all the Moroccan strains. All the other markers displayed variation in size in at least one of the Moroccan strains (Table A.1).

Several algorithms were applied to the raw data generated to reveal the population structure of the Moroccan strains of *L. tropica* and discern their placement in the global perspective of the species.

The Bayesian statistics as implemented in STRUCTURE and the calculation of the most probable number of populations, deltaK (Fig. A.1) suggested the existence of two main populations, A and B, each of which divided into two sub-populations, A1, A2 and B1, B2, some of which, in turn, divided into sub-sub-populations, A1a, A1b and A2a, A2b and B2a, B2b (Fig. 2). In general, the clustering of the strains correlated with their geographical origins. Population A comprised all the Moroccan strains and strains of other North African and African, origins, i.e. Tunisia, Kenya and Namibia. A group of strains of different geographical origins, most of which were collected before 1995 and are called 'old strains' as well as 34 strains from Turkey and six from the Palestinian Authority, also fell into this population. Population B consisted almost exclusively of strains from Israeli and Palestinian foci. In addition, one strain from the Sinai Peninsula, Egypt, was assigned to this population. The Kenyan strain MHOM/KE/1984/NLB297 displayed a hybrid genotype with membership coefficients of 0.524 and 0.476 for populations A and B, respectively. For further sub-structuring it was included in the population A as the membership coefficient was slightly higher for that population.

The results of population genetic analyses are summarised graphically in Figs. 2–5. When population A was analysed separately by STRUCTURE, it split into two sub-populations, A1 and A2. Sub-population A1 comprised all the Turkish strains, the old strains and six Palestinian strains isolated in 2002. Sub-population A2 comprised all the African strains, including the Moroccan ones, two Turkish strains and an Israeli one. When these sub-populations were analysed again with STRUCTURE, the Moroccan strains separated into two different sub-sub-populations, A2a and A2b. Seven strains from the Chichaoua Province and six from the Marrakech Province now collectively referred to as the 'Morocco A' cluster, formed together with the two Turkish and one Israeli strain mentioned just above the sub-sub-population A2a. The sub-sub-population A2b comprised two strains from the Chichaoua Province and three from the Marrakech Province, now collectively referred to as the 'Morocco B' cluster, and 11 African strains, five Kenyan, four Namibian, and two Tunisian. The 26 Turkish strains isolated during a localised outbreak of human CL near Sanliurfa formed the separate sub-sub-population A1a, and the sub-sub-population A1b comprised the old strains, six Palestinian and six Turkish strains. On sub-structuring the population B, strains from a focus at the northern side of the Sea of Galilee formed the sub-population B1. The remaining strains from Israel and the Palestinian Authority were assigned to sub-population B2, which was sub-divided further into the sub-sub-populations B2a and B2b, albeit, with what appeared to be no correlation to geographical origins. However, the sub-sub-populations B2a and B2b were not supported by the downstream analyses, and were considered invalid.

The factorial correspondence analysis (FCA) was performed with the presumption of six genetic entities being present in the strain set based on the separation of strains by STRUCTURE. The

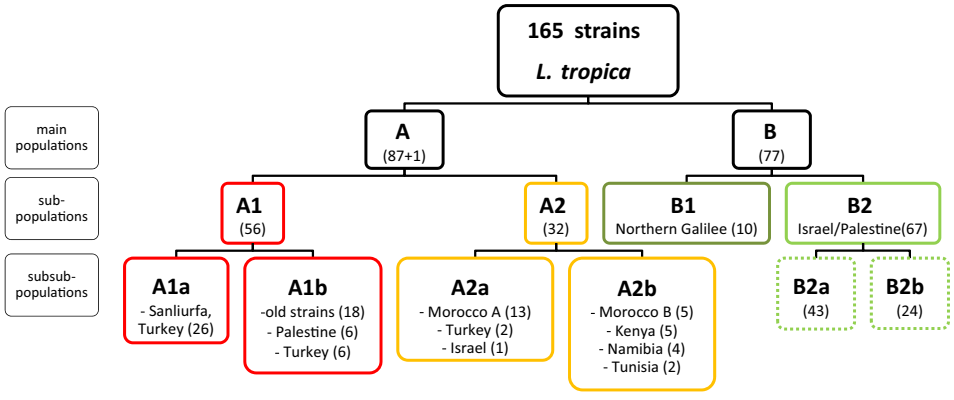

Fig. 2. Bayesian clustering. This shows the hierarchical structure of the strains of *L. tropica* down to the level of sub-sub-populations as inferred by the Bayesian statistical analysis. The numbers in parentheses give the number of strains in the respective population or sub-population. The clusters circled with the dashed lines were not supported by the other algorithms and are not considered distinct groups in the other analyses.

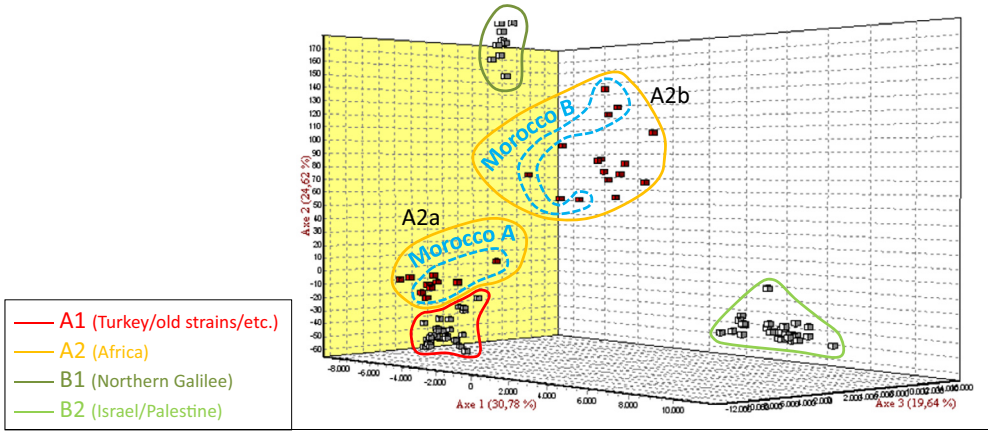

Fig. 3. Factorial correspondence analysis. The two groups of Moroccan strains are circumscribed by dotted lines. The sub-populations inferred by Bayesian results are circumscribed by different colours. The strains, one representative for each genotype, were assigned to the populations proposed by Bayesian clustering before applying Factorial correspondence analysis (FCA).

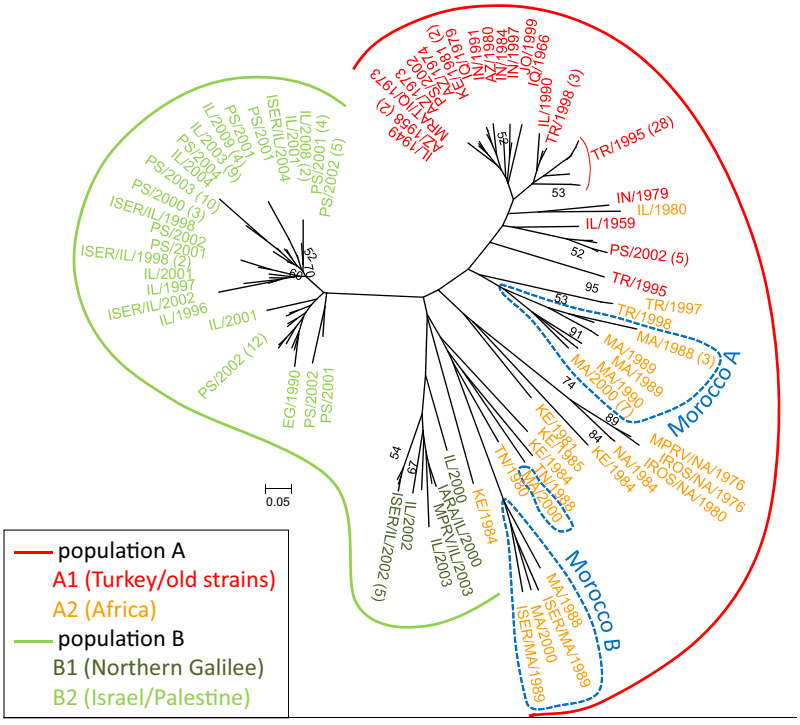

Fig. 4. Neighbour joining tree. Phylogenetic relationship between strains of *L. tropica* based on Chord distance estimates. Bootstrap values >50 are indicated at the nodes. Strain designations specify, respectively, the host, the country of origin, and the year of isolation. The numbers in parentheses after strain designations indicate the number of strains sharing the same designation. If not indicated otherwise, samples were isolated from human patients. The full WHO codes of all the strains are given in Table A.1. The two Moroccan clusters are circumscribed by broken lines. Bayesian results are indicated by colours: main populations are indicated by the green and red lines, sub-structures are differentiated by the colour of the designations.

strains in sub-population A2, which were mainly African, were dispersed in the three dimensional box as a 'cloud', the spatial arrangement of which corresponded to the two groupings, sub-sub-populations A2a and A2b, proposed by the Bayesian approach (Fig. 3). The sub-sub-population A2a encompassed the 'Morocco A' cluster plus two Turkish strains and one Israeli strain, and sub-sub-population A2b encompassed the 'Morocco B' cluster and the other African strains, which came from Tunisia, Kenya and Namibia. This approach revealed a closer relationship between the 'Morocco A' cluster and the sub-population A1 that consisted of all the other Turkish strains and the old strains. This might have been influenced by the proximity of the two separate Turkish strains to the 'Morocco A' cluster. The sub-populations B1 and B2, which contained most of the Israeli and Palestinian strains, and the sub-population A1, which contained Turkish, Palestinian and the old strains, were genetically close entities. No further sub-structures were distinguished by the FCA approach. The strains in sub-population B1 that came from the Galilean focus appeared to be very distantly related to the Israeli and Palestinian strains in sub-population B2, and showed a closer genetic relationship to the African strains in sub-sub-population A2b.

In the distance-based Neighbour Joining (NJ) tree (Fig. 4), strains in sub-population A1 and, especially, those in sub-population A2, again, presented as different paraphyletic groups. Strains

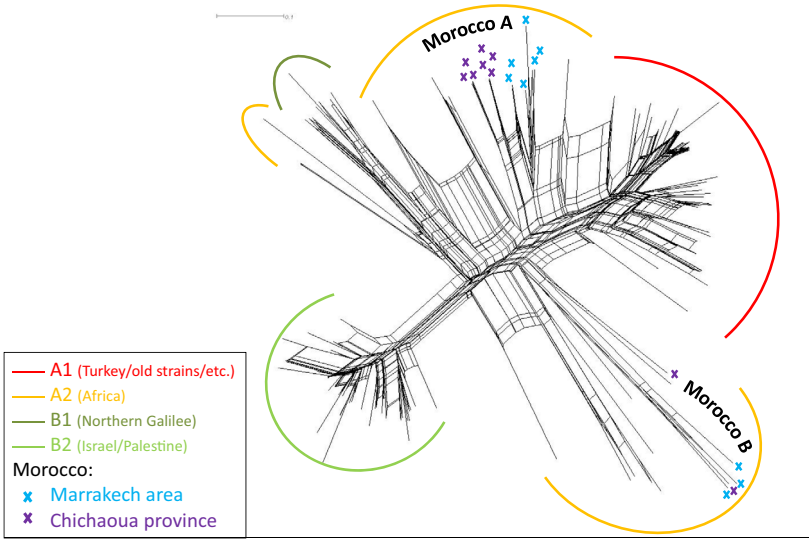

Fig. 5. Neighbour network. Neighbour network presenting the genetic relationship among strains of *L. tropica* calculated by SplitsTree 4. Cross connections indicate probable reticulation events like hybridisation, recombination, or horizontal gene transfer between the strains. The strains from Chichaoua Province are indicated by purple crosses; those from Marrakech Province by blue crosses. Sub-populations resulting from Bayesian clustering are indicated by coloured lines.

in the 'Morocco A' cluster together with the Turkish strains MHOM/TR/1997/YO001 and MHOM/TR/1997/YO002 formed an independent monophyletic branch that corresponded to the sub-population A2a discerned by STRUCTURE. The strains of sub-sub-population A2b were separated into two monophyletic groups: one that contained four Namibian and two Kenyan strains; the other that contained strains of the 'Morocco B' cluster and three from Kenya and two from Tunisia. Interestingly, strains from the Galilean focus that fell into the sub-population B1 when applying the Bayesian approach now fell to a separate branch of the second monophyletic group discerned by NJ. While this conflicted with the Bayesian approach, it did accord with the FCA.

The Neighbour network (Fig. 5) unravelled high levels of genetic variation and interaction. Overall, the network had no tree-like structure except for the Israeli-Palestinian entity labelled B2 in Fig. 5, which clearly demarcated a distinct monophyletic group. The two entities encompassing the Moroccan strains were clearly distinct from each other. This analysis confirmed the close relationship mentioned above of the 'Morocco B' cluster and the strains from Kenya, Namibia and Tunisia. Nevertheless, both the 'Morocco A' cluster and the 'Morocco A' cluster were not seen demarcated as separate monophyletic groups.

Calculation of the mean fixation index (F_{ST}) revealed considerable genetic differentiation among the various genetic entities (Table A.2). *F*-statistics revealed substantial genetic differentiation, $F_{ST} = 0.339$, between the sub-sub-populations A2a and A2b, both of which contained Moroccan strains. Strains of the sub-sub-populations A2a and A2b showed maximal genetic differentiation, $F_{ST} = 0.713$ and $F_{ST} = 0.583$, respectively, compared to sub-population B2, which contained Israeli and Palestinian strains.

The number of alleles per locus (A) ranged from 3 (GA6, GA9n, 4GTG) to 17 (LIST7039) with an average of 7.33 (Table 1). The observed heterozygosity (H_o), which ranged from 0.000 to 0.358, was lower than the expected heterozygosity (H_e), which ranged from 0.071 to 0.815, for all loci. For the sub-sub-population A1a, H_o was higher than H_e, displaying a negative inbreeding coefficient F_{IS}, −0.849. For the sub-sub-population A1b, H_o and H_e were nearly identical, giving a F_{IS} value of 0.036, which is close to 0. The inbreeding coefficient (F_{IS}) by locus varied from 0.278 (GA9n) to 1.000 (GA6 and 4GTG) and by population from −0.849 for the sub-sub-population A1a to 0.895 for the sub-sub-population B1.

Table 1
Descriptive statistics (A) per locus and (B) per population. A, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, inbreeding coefficient (−1 = outcrossing, 0 = random mating, +1 = inbreeding).

Locus	*A*	H_o	H_e	F_{IS}
(A)				
GA1	4	0.012	0.071	0.830
GA2	9	0.079	0.676	0.884
GA6	3	0.000	0.171	1.000
GA9n	3	0.348	0.481	0.278
LIST7010	10	0.181	0.814	0.778
LIST7011	8	0.006	0.753	0.992
LIST7027	10	0.358	0.815	0.562
LIST7033	7	0.006	0.613	0.990
LIST7039	17	0.158	0.806	0.805
LIST7040	9	0.313	0.661	0.527
4GTG	3	0.000	0.191	1.000
27GTGn	5	0.224	0.667	0.665
All	7.33	0.140	0.560	0.750
(B)				
Population				
A1a	1.42	0.318	0.176	−0.849
A1b	2.83	0.340	0.353	0.036
A2a	2.50	0.116	0.328	0.654
A2b	4.67	0.096	0.678	0.862
B1	1.83	0.025	0.226	0.895
B2	2.42	0.030	0.140	0.788
Mean	2.61	0.154	0.317	0.517

4. Discussion

Microsatellite typing separated the Moroccan strains of *L. tropica* from the neighbouring Provinces of Marrakech and Chichaoua into two separate populations that did not correlate with the two geographical areas. The microsatellite profiles of the nine strains from human cases of CL from the Chichaoua Province were compared to those described previously for nine strains from

Marrakech Province, which were shown to belong to two genetically distinct groups (Pratlong et al., 1991; Schwenkenbecher et al., 2006). All the algorithms employed grouped the strains from Chichaoua together with those from Marrakech but as two genetically distinct clusters: the 'Morocco A' cluster, consisting of six strains from Marrakech and seven from Chichaoua, and, also, two from Adana, Turkey, and one from Israel although the Israeli one was not confirmed in the NJ tree; and the 'Morocco B' cluster consisting of three strains from Marrakech and two from Chichaoua, which were genetically closer to strains from other African foci of human CL, i.e. Tunisia, Kenya, and Namibia. The inbreeding coefficient indicated a relatively high rate of inbreeding for the sub-sub-populations A2a and A2b, both of which contained Moroccan strains.

The same various methods applied to analyse the microsatellite profiles of all the other strains of *L. tropica* not mentioned just above placed them, more or less, in the same geographically defined populations. However, a few discrepancies were seen between the FCA and the distance-based methods on the one hand and the Bayesian statistical approach on the other. The former placed the Palestinian and Israeli strains, excluding those from the Galilean focus, into one population; the latter assigned most of the Palestinian and Israeli strains, including those from the northern Galilean focus, into one population, population B, that was divided by further sub-clustering into the sub-population B1 and sub-population B2. However, the splitting of sub-population B2 into two further groups by Bayesian and F_{ST} statistics was not confirmed by the distance-based and FCA methods.

Those results that were supported by all or most of the four approaches applied to the data can be considered as trustworthy, while those results that were only obtained with one of the methods are questionable and need further investigation.

Most of the strains analysed clustered according to their geographic origins with few exceptions. Six Palestinian strains were assigned to sub-population A1, while two Turkish strains and, depending on the algorithm used, one Israeli strain were assigned to sub-population A2. These discrepancies might be owed to people getting infected while in other countries but being diagnosed as local cases back home, thus importing foreign strains of *L. tropica*. Worthy of note is the negative value for the inbreeding coefficient, −0.849, obtained for sub-sub-population A1a, which consisted exclusively of strains isolated in 1995 during an outbreak of CL in Sanliurfa, Turkey, and confirmed the clonal expansion of a strain causing an epidemic in this focus.

Three possibilities are proposed to explain the genetic diversity existing in single foci: (i) the introduction into an originally uninfected area of distinct and separate genetic entities from other geographical regions; (ii) mutation occurring within a single original entity, possibly associated with genetic exchange; and (iii) recent hybridisation followed by recombination to give genetically distinct separate progenies as has been reported for *L. infantum* (Rogers et al., 2014). It is unlikely that mutations alone account for the complete separation of the strains of *L. tropica* from the Chichaoua and Marrakech foci. The factors opposing a high rate of genetic exchange are the low heterozygosity and relatively high F_{IS} values of 0.654 and 0.862, indicating high inbreeding rates. For *L. infantum*, Rogers et al. showed that meiosis is a rare event (7.5×10^4 mitotic cell divisions per meiosis) (Rogers et al., 2014). Provided the rates of sexual reproduction are about the same low level as in *L. tropica*, the hypothesis of consecutive introduction from different origins is favoured.

With so much time available from when the strains from Marrakech Province were isolated, 1988/89, to when those from Chichaoua Province were, 2000, one might wonder why the two populations have not 'interbred' as a result of their closer geographical proximity. Genetic exchange, if and when it occurs, is said to do so inside the female sand fly vector (Akopyants et al., 2009). For interbreeding to have occurred in the context described here, the interbreeding variants would have to be imbibed by the same female sand fly at about the same time, a possible but rather unlikely event. Also, there is the possibility that the transmission cycles in the two Moroccan foci rely on different species or even sub-populations of the same species of sand fly vector. *P. sergenti* is the species of sand fly most widely involved in the transmission of *L. tropica* but other sand fly species have been incriminated in some instances. For example, in the Galilean focus mentioned above and from where some of the strains included here came from, *P. arabicus* is a vector of the species *L. tropica* as well as is *P. sergenti*, with each species of sand fly transmitting a different sub-population of the species *L. tropica* as separated by Multilocus Enzyme Electrophoresis (MLEE) and antigenic analysis by applying, separately, species-specific monoclonal antibodies and excreted factor (EF) serotyping (Svobodova et al., 2006). The strains from the Galilean focus used in the genetic analysis described here also constituted a separate sub-population. In Morocco, the sand fly species *P. sergenti* and *P. chabaudi* were both found to be transmitters of *L. tropica* (Alvar et al., 2012). Two strains from Marrakech assigned to sub-sub-population A2b were isolated from female sand flies of the species *P. sergenti*. All the other strains were isolated from human cases of CL. Possibly, the vector species involved in the transmission of strains in the sub-sub-populations A2a and A2b are different for each of the two, preventing possible genetic exchange between their individuals.

To conclude, MLMT as applied here revealed, through microsatellite profiles, two genetically distinct populations among Moroccan strains of *L. tropica*, each mainly associated with a particular Moroccan geographical focus, but with some strains from each population falling in the other population's focus.

These findings confirm that the clustering in genetic entities is not exclusively based on geographical or temporal factors. There has to be another variable which accounts for this intra-focal diversity, which we propose is the subsequent introduction into a new focus. Future studies can figure out from where the Moroccan strains of *L. tropica* were introduced into the country by analysing more strains from various possible regions of origin.

Author contributions

LK performed the microsatellite typing and subsequent data analysis, and wrote the manuscript. RM collected the Moroccan strains of *Leishmania*. MA prepared the DNA samples and determined the strains' species. GS designed the study and participated in writing the manuscript. LS participated in writing the manuscript. All the authors have read and approved the final manuscript.

Competing interests

We confirm that there are no conflicts of interest associated with this publication and there has been no significant financial support for this study that could have influenced its outcome. The manuscript has been read and approved by all authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.09.037.

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