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

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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

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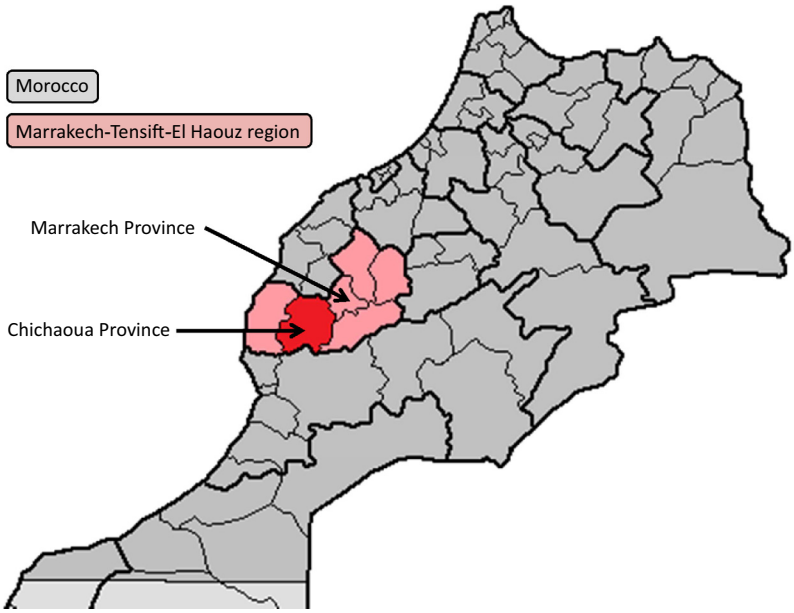

Fig. 1. The study area. Map of Morocco showing the Chichaoua and Marrakech Provinces, which are parts of the Marrakech-Tensift-Al Haouz region and from where the strains came. Modified from *Die Provinz Chichaoua in der Region Marrakech-Tensift-Al Haouz* by Vincent van Zeijst/Wikimedia Commons/CC BY-SA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