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Genetic diversity of *Leishmania tropica*: Unexpectedly complex distribution pattern

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ABSTRACT

In this study, we characterized a collection of clinical samples obtained from Syrian and Turkish patients with cutaneous leishmaniasis using internal transcribed spacer 1 (ITS1) sequences. All obtained sequences belonged to *Leishmania tropica*. Combining them with those available from GenBank allowed us performing a broad-scale analysis of genetic diversity for this species. We demonstrated that *L. tropica* has a complex phylogeographic pattern with some haplotypes being widespread across endemic countries and others restricted to particular regions. We hypothesize that at least some of them may be associated with alternative vectors or animal reservoirs.

1. Introduction

Leishmaniasis is one of the poverty-related neglected tropical diseases with 1.3 million people infected every year (WHO, 2015). It is caused by over 20 *Leishmania* spp. transmitted by phlebotomine sandflies in about 100 countries in tropical and subtropical regions (Maroli et al., 2013). There are three main clinical forms of leishmaniasis: visceral (VL), cutaneous (CL), and mucocutaneous (MCL). VL is the most severe, often fatal, disease affecting the liver, spleen, and bone marrow. It is mainly caused by *L. donovani* and *L. infantum* and occasionally by *L. martiniquensis, L. orientalis* and *L. tropica* (Alborzi et al., 2008; Jariyapan et al., 2018; Liautaud et al., 2015; Ready, 2014). MCL affects the skin and mucosa, leading to the destruction of the tissues of the nose, mouth, and throat. It is reported mostly in Latin America and caused mainly by *L. braziliensis*, followed by *L. guyanensis, L. peruviana, L. panamensis,* and *L. amazonensis* (Akhoundi et al., 2016; McGwire and Satoskar, 2014). Over 18 *Leishmania* spp. are implicated as causative agents of CL, the most common form of infection, usually associated with skin-resident macrophages (Akhoundi et al., 2016; Ameen, 2010). The major factors determining the epidemiology of leishmaniases are socioeconomic conditions, impaired immunity, human mobility, urbanization, deforestation, ineffective vector control, and climate change (Georgiadou et al., 2015; Oryan and Akbari, 2016).

Members of the genus *Leishmania* show significant genetic diversity and infections with different genotypes of the same species may have distinct clinical manifestations (Krayter et al., 2014; Mahnaz et al., 2011; Schönian et al., 2011; Schriefer et al., 2004). The genetic exchange between *Leishmania* populations, producing intra- and interspecific hybrids may change population structure and, thus, complicate molecular epidemiological studies (Akopyants et al., 2009; Chargui et al., 2009; Volf and Sádlová, 2009). Various molecular markers, including nuclear and kinetoplast genes, microsatellites, and internal transcribed spacers (ITS) have been used to assess the genetic diversity of *Leishmania* spp. (Botilde et al., 2006; Fotouhi-Ardakani et al., 2016;

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Ghatee et al., 2020; Schönian et al., 2012). However, most such studies were confined to a particular geographic region and/or included a limited set of isolates, not allowing investigating a species diversity over its whole habitat.

Here we analyzed a vast collection of samples from Syrian and Turkish patients with cutaneous leishmaniasis. All ITS1 sequences obtained from these samples belonged to *L. tropica* and, after combining them with those available from GenBank, we performed a broad-scale analysis of genetic diversity for this species across many endemic countries.

2. Materials and methods

2.1. Material collection

Eighty-seven specimens were collected from 68 Syrian refugees and 2 local Turkish CL-suspicious patients administered at the Parasitology Department of the Medical Faculty of Erciyes University, Kayseri, Turkey (Table S1), and the following data were recorded for each patient: age, gender and lesion location. After cleaning the skin with 70 % ethanol, the biopsy specimens were collected with disposable scalpel blades from the margins of suspected lesions into vials with phosphate-buffered saline. Part of this material was used to prepare Giemsa-stained smears, later examined under a light microscope to confirm the presence of leishmanial amastigotes. The rest was frozen for subsequent DNA extraction.

2.2. DNA isolation, amplification and sequencing

DNA was isolated from the clinical samples using the GeneAll Exgene Cell SV Mini Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's protocol. The ITS1-containing fragment was amplified using semi-nested PCR with the primers LITSR, LITSV, and L5.8S as described previously (Mahdy et al., 2010). The amplicons were cloned using the CloneJET PCR Cloning kit and the plasmid DNA was isolated using the GeneJET plasmid miniprep kit (both from ThermoFisher Scientific, Waltham, US). All plasmid inserts were sequenced at Macrogen Europe (Amsterdam, Netherlands). The sequences were submitted to GenBank under accession numbers MG515728-MG515730, MN872253-MN872262, and MT353714-MT353755.

2.3. Sequence analysis

The ITS1 sequences obtained in this work and those retrieved from the GenBank (Table S2), were aligned in MAFFT using the E-INS-i algorithm (Katoh and Standley, 2013). The resulting alignment containing 239 nucleotide positions was purged of the sequences with incomplete ITS1 and suspicious ones, containing multiple substitutions or indels in highly conserved flanking regions. The final dataset included 294 sequences of *L. tropica*. DnaSP v.6 was used to identify haplotypes and estimate nucleotide and haplotype diversities with their standard deviations (Rozas et al., 2017). Construction of the haplotype network was performed using the median joining method in PopART v. 1.7 (Leigh et al., 2015).

3. Results and discussion

Amastigotes were microscopically detected in samples from only 17 patients, whereas PCR detected leishmanial DNA in 55 samples. In agreement with the previously published data on the incidence of CL in other endemic countries (El Hamouchi et al., 2019; Fakhar et al., 2016; Ghatee et al., 2014), the majority of infected Syrians (78%) were under 16 years of age (Table S1). Typically (in 80% of individuals), there was a single lesion, but in some cases, there could be up to four. Most of these lesions were situated on exposed parts of the body, such as the face, arms, and hands. ITS1 sequences could be obtained for 55 samples from 53 patients. BLAST analysis showed that all these sequences belong to *L. tropica*, which is consistent with the results of earlier studies, showing that *L. tropica* is the main species responsible for CL in Syria and Turkey (Muhjazi et al., 2019; Özbilgin et al., 2019). In both cases, when the sequences were obtained from two different lesions of the same patient, they were not identical. In total, there were 11 sequence variants (haplotypes), of which two were represented by more than one sequence, eight proved to be unique and novel, and one (H_11) has been previously recorded in other countries (Tables S1, S2).

The analysis of *L. tropica* ITS1 sequences, obtained in this work and those retrieved from the GenBank, showed that they have a relatively low level of divergence. There were 54 polymorphic sites producing 50 haplotypes (Table 1), only 12 of which were non-unique (i.e. with >1 instance in the dataset). The median joining haplotype network constructed from these sequences showed rather short distances. The non-unique haplotypes typically separated from each other by no more than 2 substitutions, which complicated the separation of haplogroups (Fig. 1). In order to have a clearer picture, we created a map showing the distribution of non-unique haplotypes (Fig. 2). The most abundant and widespread are the closely related haplotypes H_8 and H_ 9, which can be found throughout the whole area from Africa in the West to China and India in the East. Most other haplotypes represented by more than one sequence, are directly connected to H_9 and likely represent its local derivatives (Fig. 1). Of these, only H_25 found from Morocco to China has a distribution comparable in width to its parent haplotype, while others are restricted to particular areas, such as Tunisia and Lybia (H_7), Middle East (H_11), Egypt (H_12), Israel (H_16), Iran and Afghanistan (H_18), or Turkey (H_19). The presence of the latter is the only difference between the sets of non-unique haplotypes observed in Syria and Turkey.

Of special interest is the well-separated group of haplotypes H_2 – H_6, which have been found only in Morocco (Fig. 2). Such geographic restriction of an isolated haplogroup suggests that they may be associated with a separate vector, being endemic in this area. Indeed, while *Phlebotomus sergenti*, the main confirmed vector of *L. tropica*, has a wide distribution coinciding with that of the parasite, *P. chabaudi* was shown to transmit the latter in Morocco and Tunisia (Maroli et al., 2013; WHO, 2010). However, many of the sequences belonging to this group originated from *P. sergenti*, while *P. chabaudi* has never been tested (Table S2). Several other sand fly species, such as *P. aculeatus, P. arabicus, P. guggisbergi, P. saevus, P. similis,* and *P. rossi*, were implicated in the transmission of *L. tropica* in other African countries (Depaquit et al., 2002; Maroli et al., 2013). Yet, the information on the epidemiology of these vectors for *Leishmania* spp. is patchy (Cunze et al., 2019) and the data on the genetic diversity of African populations of *L. tropica* are too scarce to make any conclusions now.

The analysis of the genetic polymorphism in the populations with more than 10 available sequences showed that both haplotype and nucleotide diversities in Iran are significantly lower than in the rest of the *L. tropica* geographic range (Table 1). This is apparently due to the essential predominance of the haplotype H_9, which may be indicative of its recent expansion. The reason for this phenomenon is obscure, but it may be explained by the fact that in Iran, *L. tropica* competes with another, more prevalent, etiological agent of CL, *L. major*. In the Moroccan population, the same polymorphism parameters are slightly higher (although not reaching the threshold of statistical significance). These differences are better noticeable if one compares the numbers of observed segregating sites and haplotypes, which are expected to be proportional to the number of analyzed sequences (Suppl. Fig. 1). These numbers in Morocco are noticeably higher than expected, and that is likely associated with the presence of the endemic haplogroup H_2 – H_6 in this population. Pakistan is another country with divergent *L. tropica*

Table 1
Genetic polymorphism in the whole dataset and countries with n>10.

country	number of samples	number of variable sites	number of haplotypes	haplotype diversity	nucleotide diversity
Iran	94	11	13	0,41±0,06	0,0026± 0,0005
Morocco	41	13	13	0,76±0,06	0,0095± 0,0010
Syria	58	10	11	0,65±0,04	0,0040± 0,0005
Turkey	13	5	5	0,71±0,12	0,0050± 0,0014
China	27	13	12	0,80±0,07	0,0066± 0,0012
All countries	294	54	50	0,67±0,03	0,0067± 0,0006

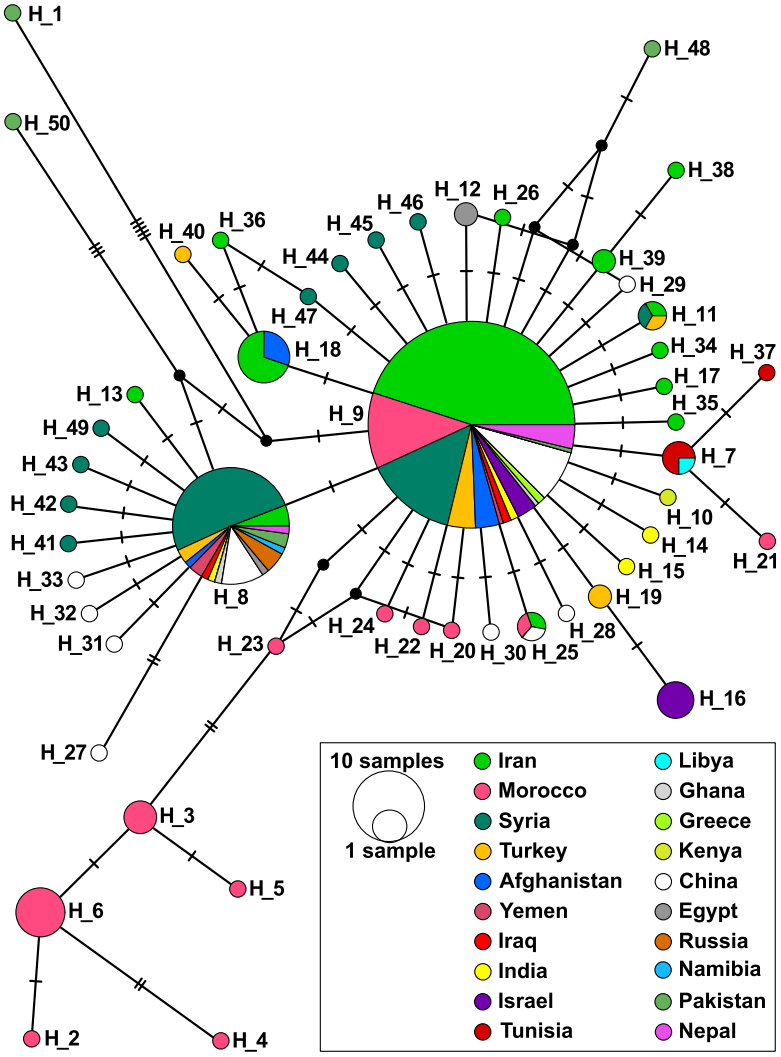

Fig. 1. A Median-joining haplotype network of ITS1 sequences of *L. tropica* isolates, sequenced in this study and retrieved from the GenBank. Circles represent individual haplotypes; their color indicates the country of origin, while their size is proportional to the haplotype frequency. Branch lengths are proportional to the number of substitutions, which are additionally indicated by crossing marks.

haplotypes, which, however, are unique and do not form a separate haplogroup.

The CL caused by *L. tropica* is considered mainly anthroponotic, but, in some territories, animals can be used as reservoirs (WHO, 2010). The low proportion of the ITS1 sequences of *L. tropica*, isolated from non-human hosts, has limited our ability to analyze whether the transmission of parasites with particular haplotypes was preferentially anthroponotic or zoonotic. Such isolates were obtained from rodents, canids, and lizards. Most of them (23/41) belonged to the major haplotypes H_8 and H_9 (Table S2). Two other haplotypes found both in humans and non-human vertebrates were H_25 detected in a Chinese lizard and H_7 documented in Tunisian sand rats along with a related unique haplotype H_37 (Table S2). This suggests that, at least in the latter case, specific haplotypes may have zoonotic transmission (Ben Othman et al., 2018). Of special interest is the case of *L. tropica* in Israel, where it is considered to be a zoonotic pathogen (Gandacu et al., 2014). The two distinct haplotypes H_9 and H_16, of which the former has been detected in humans and dogs, while the latter – in wild canids (foxes and jackals) may indicate the existence of two independently circulating parasite lineages.

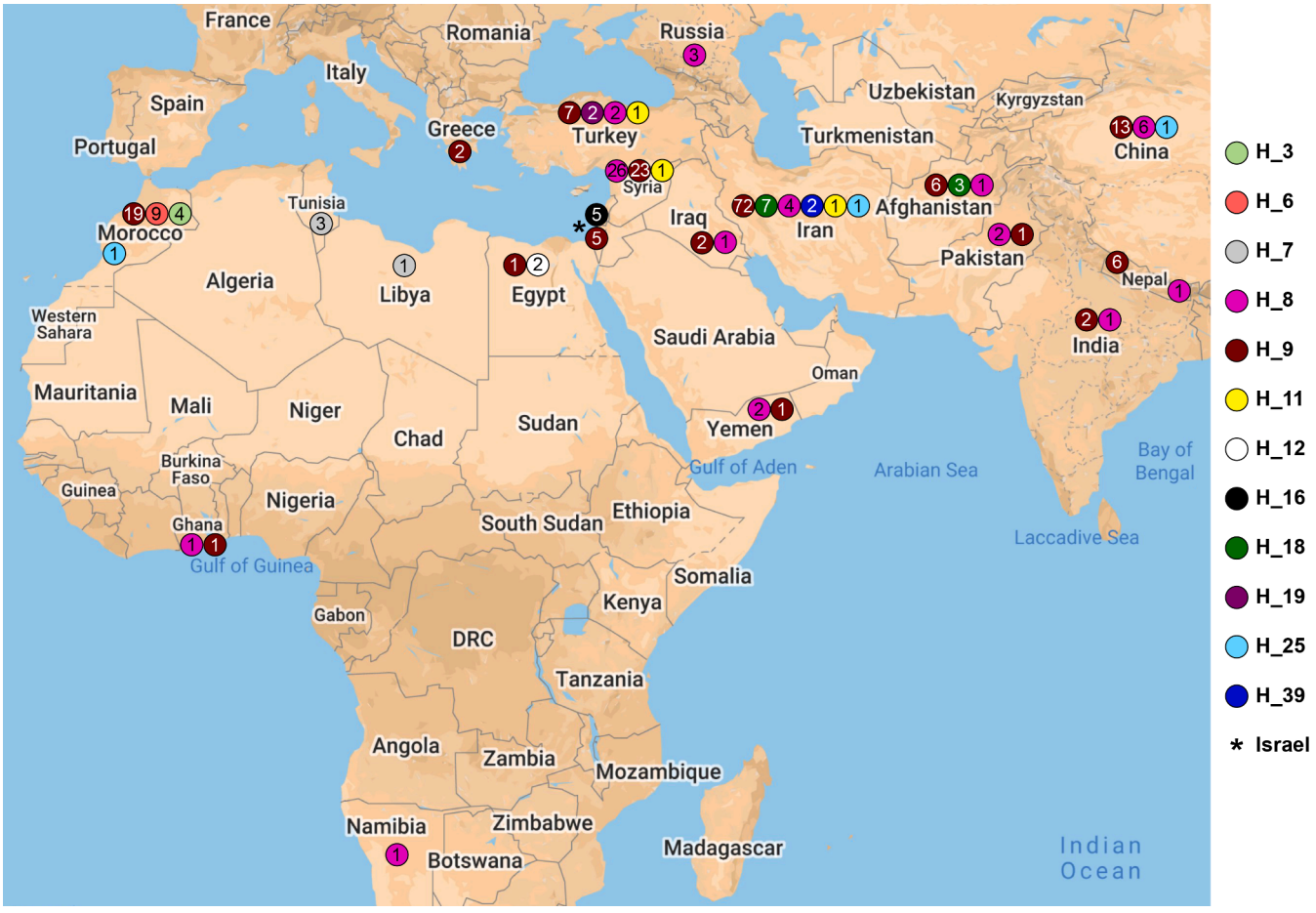

Fig. 2. A geographical distribution of *L. tropica* haplotypes present in at least two isolates. Each color represents a distinct haplotype. Numbers of local isolates belonging to particular haplotypes are shown in circles. Position of circles within a country does not indicate a certain locality.

4. Conclusion

While the studied collection of samples from Turkey and Syria did not demonstrate any specific features, the inclusion of material from the central part of the parasite's geographic range allowed a better understanding of the complex phylogeographic structure of *L. tropica*. Our analyses revealed several haplotypes characterized by distinct distributions, and we propose that some of them can be associated with additional vectors and/or animal reservoirs. More data from undersampled countries on the periphery of *L. tropica* geographic range, as well as from the wild animals and phlebotomine vectors, should shed more light on these issues.

Ethical considerations

This study has been approved by the Ethics Committee of the Faculty of Medicine, Erciyes University, Turkey (# 2016/167).

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding authors.

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CRediT authorship contribution statement

Arzuv Charyyeva: Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Ülfet Çetinkaya:** Conceptualization, Investigation, Resources, Funding acquisition, Project administration. **Bora Özkan:** Investigation, Data curation. **Serkan Şahin:** Investigation, Data curation. **Nermin Yaprak:** Investigation, Data curation. **Izzet Şahin:** Investigation, Data curation. **Vyacheslav Yurchenko:** Resources, Writing - review & editing, Supervision, Funding acquisition. **Alexei Yu. Kostygov:** Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2021.105888.

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