Am. J. Trop. Med. Hyg., 97(5), 2017, pp. 1489–1497
doi:10.4269/ajtmh.16-0887
Copyright © 2017 by The American Society of Tropical Medicine and Hygiene

Variability of Cutaneous Leishmaniasis Lesions Is Not Associated with Genetic Diversity of *Leishmania tropica* in Khyber Pakhtunkhwa Province of Pakistan

Nazma Habib Khan,[1,2]* Martin S. Llewellyn,[3] Gabriele Schönian,[4] and Colin J. Sutherland[1]

[1]*Department of Immunology & Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom;* [2]*Department of Zoology, University of Peshawar, Peshawar, Khyber Pakhtunkhwa, Pakistan;* [3]*Department of Pathogen Molecular Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom;* [4]*Institute of Microbiology and Hygiene, Charitè-University Medicine Berlin, Hindenburgdamm, Berlin, Germany*

Abstract. *Leishmania tropica* is the causative agent of cutaneous leishmaniasis in Pakistan. Here, intraspecific diversity of *L. tropica* from northern Pakistan was investigated using multilocus microsatellite typing. Fourteen polymorphic microsatellite markers were typed in 34 recently collected *L. tropica* isolates from Pakistan along with 158 archival strains of diverse Afro-Eurasian origins. Previously published profiles for 145 strains of *L. tropica* originating from different regions of Africa, Central Asia, Iran, and Middle East were included for comparison. Six consistently well-supported genetic groups were resolved: 1) Asia, 2) Morroco A, 3) Namibia and Kenya A, 4) Kenya B/Tunisia and Galilee, 5) Morocco B, and 6) Middle East. Strains from northern Pakistan were assigned to Asian cluster except for three that were placed in a geographically distant genetic group; Morocco A. Lesion variability among these Pakistani strains was not associated with specific *L. tropica* genetic profile. Pakistani strains showed little genetic differentiation from strains of Iraq, Afghanistan, and Syria (F_{ST} = 0.00–0.06); displayed evidence of modest genetic flow with India (F_{ST} = 0.14). Furthermore, genetic structuring within these isolates was not geographically defined. Pak–Afghan cluster was in significant linkage disequilibrium (I_A = 1.43), had low genetic diversity, and displayed comparatively higher heterozygosity (F_{IS} = −0.62). Patterns of genetic diversity observed suggest dominance of a minimally diverse clonal lineage within northern Pakistan. This is surprising as a wide clinical spectrum was observed in patients, suggesting the importance of host and other factors. Further genotyping studies of *L. tropica* isolates displaying different clinical phenotypes are required to validate this potentially important observation.

INTRODUCTION

Parasites of genus *Leishmania* multiply within host macrophages and result in a range of clinical manifestations in different vertebrate hosts.[1,2] Globally, an estimated 350 million people live at risk of leishmaniasis, and annually, more than 0.7 million cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis cases are reported.[3]

Intraspecific heterogeneity in *Leishmania tropica* has been demonstrated previously through serological and molecular typing.[4–7] Clinical manifestation of the species ranges from typical and atypical cutaneous forms to complicated visceral and viscerotropic infections.[8–10] Within its documented geographical range, 35 zymodemes of *L. tropica* have been described so far.[11] However, any association between the parasite's genetic diversity and clinical presentation remains uncertain because variable clinical outcomes are reported from regions of both high and low iso-enzymatic polymorphism.[11] Indeed, previous studies of *L. tropica* genotypic diversity could not convincingly relate this to clinical manifestations of the disease or geographical distribution.[12,13] Using multilocus microsatellite typing (MLMT), Schwenkenbecher et al.[14] defined 10 genetic structural units within *L. tropica* from 12 Asian and North African countries and identified Asia as the most heterogeneous cluster. However, South Asian strains, particularly those from Pakistan and Afghanistan, remain inadequately characterized genetically.

Approximately 21,000 to 35,000 cases of CL are reported annually in Pakistan.[3] Both anthroponotic (ACL) and zoonotic forms of CL are prevalent in the country. ACL infections are generally attributed to *L. tropica* and have been commonly described from urban centers of Punjab, Baluchistan, Azad Jammu Kashmir, and Khyber Pakhtunkhwa (KP) and its surrounding tribal belt.[15–17] Frequent reports of atypical presentations of CL from Pakistan could possibly be a consequence of infections on atypical sites and/or idiosyncrasies of host immune response.[18] Commonly reported atypical forms include lupoid, keloidal, psoriasiform, erysipeloid, verrucous, zosteriform, paronychial, whitlow, eczematoid, and acneform CL.[19–22] Molecular epidemiology of *Leishmania* in northern Pakistan (KP), a principal focus of CL, is largely unexplored. Although multiple clinical variants are known in the region, there rarely has been a characterization of the causative pathogen[18] or description of any particular genetic profiles for them.[8,19–24] Improved understanding of Pakistani *L. tropica* genetic diversity in its regional context can thus shed light on current or historic importation routes of this species into Pakistan.

Hypervariable and codominant markers such as microsatellites have been successfully used in investigating intraspecific profiling and heterogeneity of other *Leishmania* species.[25–29] We compared recent *L. tropica* isolates from northern Pakistan with those from other Asian and African localities, using polymorphic loci across the genome to investigate intraspecific diversity. Genetic variability of *L. tropica* and the variety of CL's clinical manifestation seen in the Northern KP province were tested for any associations. Genetic affiliations between *L. tropica* isolates from KP and elsewhere in the region were also explored.

MATERIALS AND METHODS

Patients and sampling method. Clinical data and biological samples were collected from patients suspected of CL visiting the dermatology outpatient units in three major hospitals of Peshawar, the capital of the KP province of

*Address correspondence to Nazma Habib Khan, Department of Zoology, University of Peshawar, Jamrud Road, Peshawar, Khyber Pakhtunkhwa 25120, Pakistan. E-mail: nazma@upesh.edu.pk

Pakistan, namely Kuwait Teaching Hospital (KWH), Khyber Teaching hospital, and Combined Military Hospital (CMH) as previously described.[18] The sampling was carried out from May to September 2010. Briefly, selected patient lesions were photographed to obtain standard clinical descriptions from dermatologists that basically included identifying the lesion as dry, wet, or mixed, whether ulcerated lesions were typical or atypical, and finally designating atypical morphologies. Later, lesions were punctured with sterile lancets and exudates were dispensed into biphasic culture medium containing rabbit blood agar and M199 medium (Sigma, Steinheim, Germany) supplemented with 10% heat-inactivated fetal calf serum. Cultures positive for *Leishmania* promastigotes were propagated and subjected to MLMT. Ethics Committees for London School of Hygiene and Tropical Medicine (LSHTM) (ref. #5677, March 2010) and University of Peshawar (ref. #28/Pharm, May 2010) approved the study methods. Informed written consent to participate in the study was obtained from each patient (or their parent or guardian in case of minors/children under the age of 16 years) before his/her inclusion in the study.

In addition to the polymerase chain reaction (PCR)-confirmed *L. tropica* cultures obtained from KP (N = 34),[18] strains from other geographical regions obtained from cryobank repository at LSHTM were also genotyped by MLMT, comprising *L. tropica* (N = 155) and an out-group of *Leishmania donovani* (N = 2) and *Leishmania major* (N = 1) (Table 1, Supplemental Table 1).

Genotyping. Cultures were spun at 2,000 rpm at 4°C for 10 minutes. A commercial "QIAGEN DNeasy Blood and Tissue Kit" (QIAGEN, Hilden, Germany) was used to extract DNA from pellets following manufacturer's instructions.

All the samples presently attained from Pakistan were preidentified to *Leishmania* species level using minicircle kinetoplast DNA nested PCR as described by Noyes et al.[30] (Methods described in Khan et al.[18]). Strains identified as *L. tropica* were subjected to MLMT. At LSHTM, for the present study, 14 polymorphic markers (GA1, GA2, GA3, GA6, GA9, LIST7010, LIST7011, LIST027, LIST7033, LIST7039, LIST7040, 4GTG, 27GTG, and GT4) selected from Schwenkenbecher et al.[14]

TABLE 1

Country-wise distribution of *L. tropica* strains (typed at the LSHTM and from published data)

Country	Number of isolates	Year of isolation
Pakistan*	38	1987–2010
Afghanistan	40	1982–2004
Syria	87	2007
Israel	35	1949–2009
Palestine	40	2000–2004
Turkey	34	1995–1998
India	15	1971–2008
Iraq	9	1966–2000
Morocco	9	1988–1990
Azerbaijan	5	1958–1980
Kenya	7	1981–1985
Iran	4	1984–2000
Namibia	4	1976–1984
Saudi Arabia	2	1992–2006
Kuwait	2	1988–1989
Tunisia	2	1980–1988
Jordan	1	1999
Egypt	1	1990
Out-groups	3	1967–1980
Total	338	–

LSHTM = The London School of Hygiene and Tropical Medicine.
* Among these, 34 strains were collected in the present study.

and by Jamjoom et al.[27] were tested and optimized across a set of *Leishmania* species reference strains. Fluorescence-labeled forward primers were used (Supplemental Table 2) for the amplification of microsatellites and size of the amplicons determined by capillary electrophoresis with an ABI 3730 sequencer (Applied Biosystem, Foster City, CA) using GeneMapper v3.7. In each run, *L. tropica* reference strain MHOM/PS/2001/ISL590 was included as control (fragment sizes and length of regions flanking the microsatellite repeats known) (Supplemental Table 2).

Microsatellite profiles previously described for 145 strains by Schwenkenbecher et al.,[14] and Krayter et al.[31,32] were included for comparison (Table 1, Supplemental Table 1). The GT4 locus was only used for the isolates typed at LSHTM whereas 27GTG was not used by Schwenkenbecher et al.[14] Data for two loci, GT4 and 27GTG, were not available for all isolates in the extended set and were treated as missing data in those cases. Using the reference strain, profiles from published work were rendered compatible with the new data generated in this project. Several studies have used MLMT profiles of previously published strains as backdrops for assessing the genetic diversity of freshly obtained strains.[25,31–34]

Data analysis. To define genetic clustering among strains, three population analysis methods were deployed. These were discriminant analysis of principal components (DAPC);[35] a multivariate method that defines the structure of genetic clusters without assuming Hardy–Weinberg equilibrium (HWE) within population allelic frequencies, STRUCTURE;[36] and Bayesian analysis of population structure (BAPS).[37,38] Outputs from each analysis were compared and contrasted to reach a consensus view of patterns of genetic structure in the *L. tropica* populations investigated.

The DAPC was carried out in the adegenet package of R (2.15.0). Bayesian information criterion (BIC) values were obtained for five consecutive runs at 10^9 iterations. Mean of the values were plotted against K (number of clusters), and the optimum number of clusters (K) was chosen at the "elbow" of the BIC curve.[35]

BAPS software v5.2 assumes a Bayesian stochastic approach for determining the optimum number of partitions in a dataset by performing the analysis at two levels. It infers the number of possible partitions in an analysis called mixture analysis. It then calculates presence of significant gene flow (Admixture) between the inferred subpopulations or clusters in an analysis called the Admixture analysis.[37,38] Different maximum values of K were used in mixture analysis (maximum K was allowed to vary between 10 and 25) followed by an admixture analysis run at 100 iterations.[37,38] BAPS, however, has been shown to overestimate the number of clusters with a reduced number of markers.[39]

STRUCTURE V2.3.1 assumes a Bayesian model-based clustering method for inferring population structure using the Markov chain Monte Carlo scheme. Isolates are assigned probabilistically to one population or more (in case of admixed genotypes) based on a set of allele frequencies characteristic for each population.[40] K (the number of clusters) may be predefined or calculated by the software. The number of iterations were set to 100,000 with an additional 500,000 iterations for calculating K, which was allowed to vary between 1 and 20 for 10 independent runs. The mean of LnP (D) (estimated log likelihood values ln Pr(K/X), where X is the number of genotypes) for each K was then plotted against the number of clusters (K). The optimum value of K is chosen where the graph

ceases to follow a Gaussian distribution.[36] Bayesian approaches such as the BAPS and STRUCTURE operate under the assumption of HWE among populations and linkage equilibrium among loci tested.[39]

Expected and observed heterozygosity (H_e and H_o, respectively) and inbreeding coefficient (F_{is}) were estimated using Arlequin v3.5.[41] In addition, loci were tested individually for deviations from HWE proportions in Arlequin v3.5, and significance was assessed after a sequential Bonferroni correction.[42] Linkage disequilibrium (LD) was measured using Maynard-Smith's (1993) index of association (I_A) in MULTILOCUS v1.3.[42,43] The degree of genetic differentiation and gene flow among geographically defined populations was assessed by calculating linearized F_{ST} values (in Arlequin v3.5). Population differentiation based on linearized F_{ST} values was visualized via principal coordinates analysis (PCoA) using GenAlEx 6.5.[44,45] Calculation of proportions and tests for significance of associations between clinical forms and genotype in the current Pakistani isolates were carried out in the statistical package STATA v.13.

RESULTS

In total, 12 different MLMT profiles were obtained for 13 microsatellite markers among 38 Pakistani *L. tropica* strains tested (34 isolated for this study, four from the LSHTM repository). Nine profiles were unique to individual strains, and three were shared by more than one strain. Twenty-four strains shared a single common profile. Interestingly, three 1991 Islamabad strains (MHOM/PK/1991/BOMBER, MHOM/PK/1991/NAMRO, and MHOM/PK/1991/CHERAMAT) presented a unique profile. For Pakistani *L. tropica* strains analyzed, LIST027, LIST011, and LIST010 were the most polymorphic markers, each presenting four alleles, followed by three alleles produced from LIST039 and LIST040. GT4, GA1, GA9, and 27GTG produced two alleles each, whereas 4GTG, 27GTG, 39GTG, and 45GTG were monomorphic (Supplemental Table 3).

Genetic clustering analysis with reference to placement of Pakistani strains. The DAPC, BAPS, and STRUCTURE approaches were used to resolve the genetic structure of *L. tropica* datasets so as to assess the comparative applicability and robustness of these methods. The DAPC identified 11 principal components that accounted for approximately 80% of the total genetic variability, and the optimal number clusters (K) was estimated at 14. Six super clusters (A–F) could be visually assigned to the 14 clusters inferred by DAPC (Figure 1). Mixture analysis in BAPS software revealed 18 clusters within the data, whereas STRUCTURE estimated the

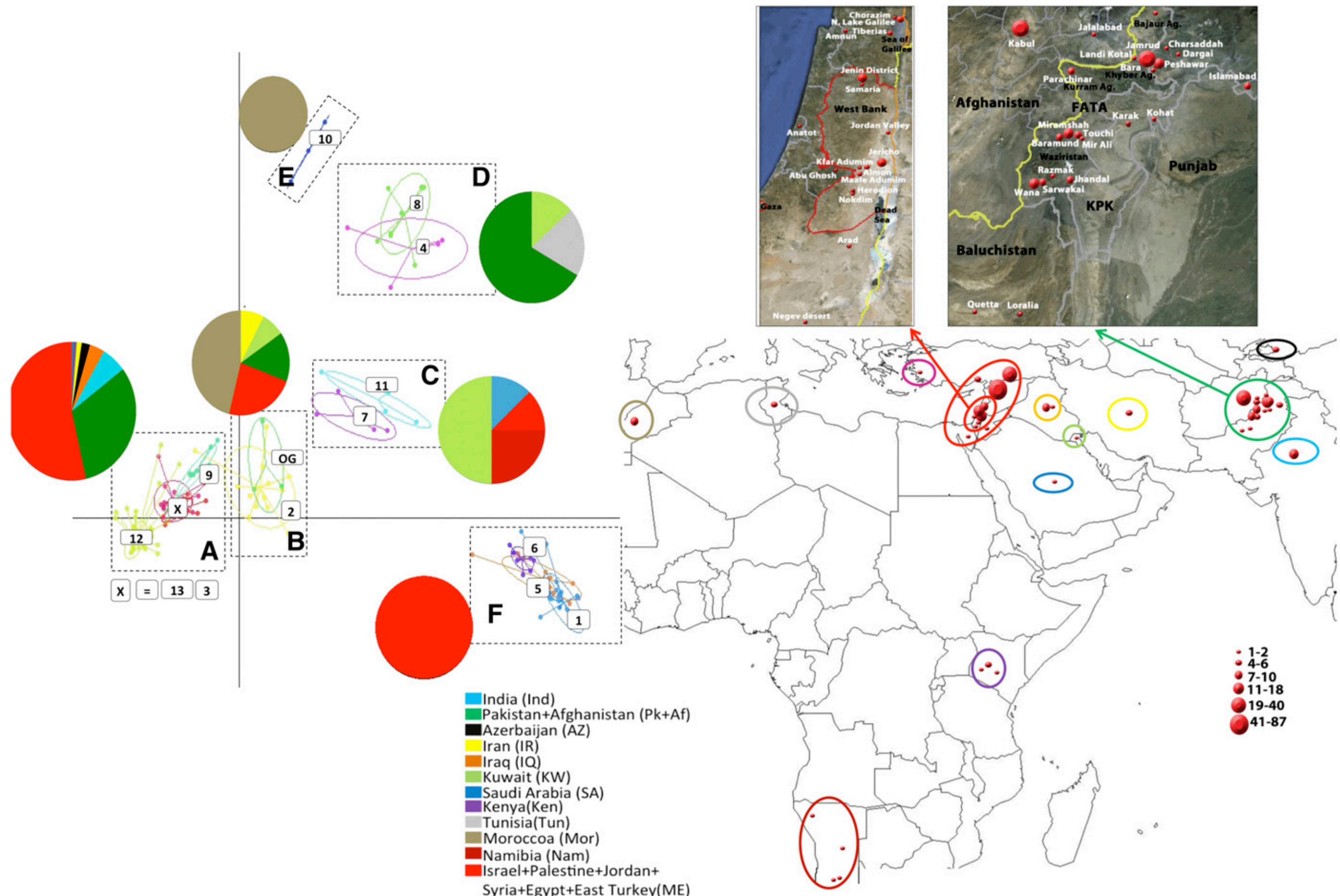

FIGURE 1. Correspondence between a priori geographical and discriminant analysis of principal components (DAPC) genetic clustering of *L. tropica*. (**A**) Scatter plot based on the DAPC for 16 clusters via K-means clustering across 14 microsatellite markers. Squares represent super-clusters (**A–F**) encompassing clusters depicted by DAPC. Spheres next to each super-cluster represent the number of strains from one or more corresponding geographical clusters identified by colors given in the legend. (**B**) Global distribution of the geographically assigned clusters. Colored ellipses encircle these geographical clusters (identified by colors given in the legend). A red dot on the map represents the isolation site of one or more strains. The size of the dot is proportional to number of strains represented by it. This figure appears in color at www.ajtmh.org.

value of K to level off at 4, and thus showed a more condensed genetic structure of the dataset. Percentage congruence for the population assignment between DAPC and BAPS was 89.3% (Figure 2). The composition of five broad genetic groups that presented consensus across all three clustering algorithms are as follows: 1) Asia: Corresponding to super-cluster A, 2) Morroco A: corresponding to super-cluster B, 3) Namibia and Kenya A: Corresponding to super-cluster C, 4) KenyaB/Tunisia and Galilee: Corresponding to super-cluster D, 5) Morocco B: corresponding to super-cluster E, and 6) Middle East (ME): Corresponding to super-cluster F. Composition of subclusters within each group is elaborated in Figure 2.

The isolates from northern Pakistan were distributed through three clusters and two super-clusters by the DAPC. CMH013 (north Waziristan) and KWH002 (Khyber agency) were a part of Morocco A (super-cluster B). Three isolates, KWH001 (Peshawar), KWH010 (Khyber agency), and CMH061 (south Waziristan), were allocated to AzMix of super-cluster A i.e., Asia. Rest of the isolates including the older ones from Islamabad (1987–1991) were assigned to the Asiamix cluster of super-cluster A. Clustering among Pakistani strains, however, did not relate to any particular location within KP northern Pakistan (Figures 1 and 2).

Genetic associations between Pakistani *L. tropica* diversity and clinical presentation. Of all the strains in the present study (N = 34), photographs of only 32 lesions were of sufficient quality to be appropriately described by dermatologists. It was observed that no genetic cluster of the MLMT profile of Pakistani strains (i.e., AsiaMix, AzMix, and MorAMix) could be linked to dry, wet, or mixed presentation of the lesions among these 32 infected individuals (Table 2, Supplemental Table 4).

Only 22 of the total described lesions were ulcerated, two of which had typical CL presentation (central crust with raised indurated edges).[46] The remaining 20 fell into one of the four atypical groups. These atypical presentations could not be related to a genetic cluster or profile of Pakistani strains (Table 2, Supplemental Table 4). Some atypical forms, for example Keloidal-type infection (CMH061), was produced by one isolate only, and thus, no conclusive inferences could be drawn for these specific clinical types. More widely, it was noticed that ten of the eleven non-Pakistani viscerotropic strains in the study sample (two from Kenya, four from India, one from Iraq and Israel each, and one from Saudi Arabia) were assigned to the Asian super-cluster A. MHOM/IR/89/ARD-L2, isolated from an Iranian patient with leishmanial lymphadenitis, was in Morocco A.

Genetic diversity, inbreeding indices, and linkage disequilibrium. A priori cluster assignment, based on the MLMT data, generated 12 geographical *L. tropica* clusters (Figure 1). A few of these clusters could be discretely identified by genetic structure analysis as depicted by DAPC in Figure 1. For instance, the ME cluster apparently represented a genotypically and geographically diverse group. Genetic diversity, inbreeding indices, and LD values were calculated for clusters with ≥ 7 isolates (PkAF = Pakistani–Afghan, India, IQ = Iraq,

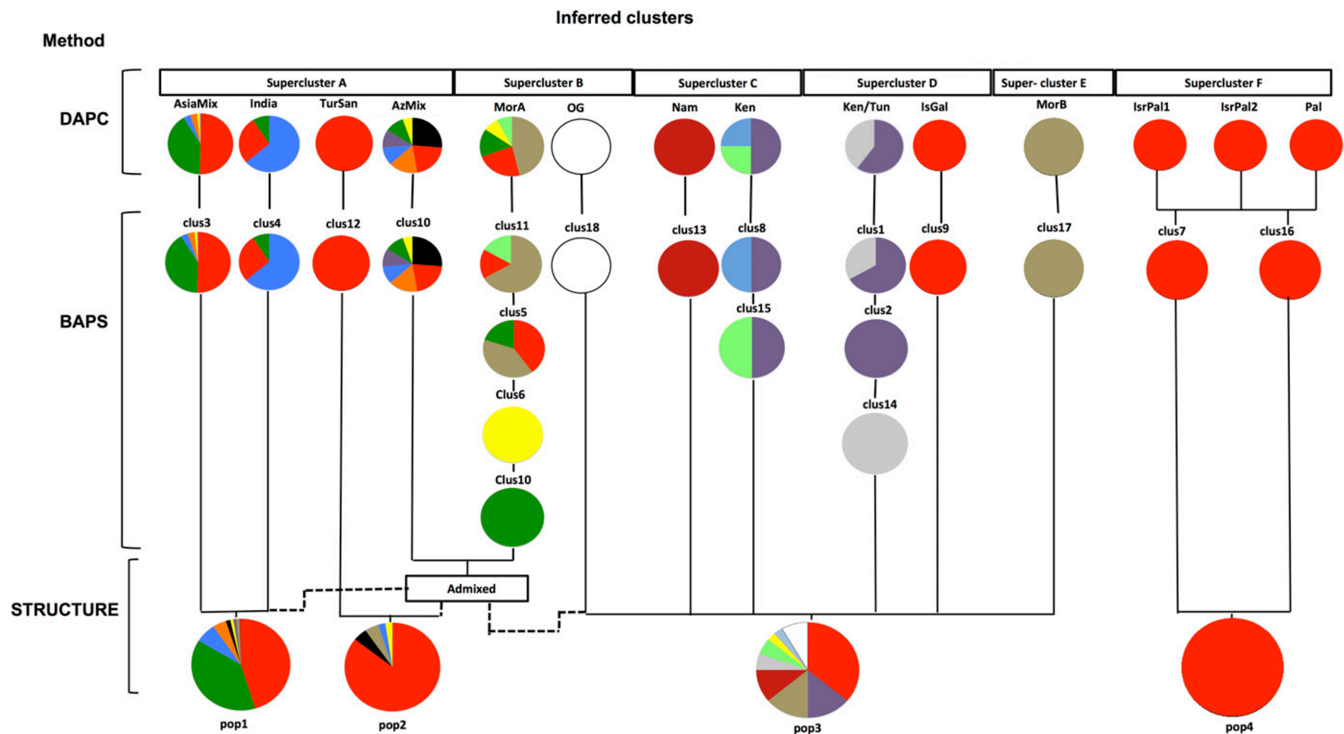

FIGURE 2. A schematic summarizing the clustering inferred by discriminant analysis of principal components (DAPC), BAPS, and STRUCTURE for the *L. tropica* dataset. Spheres represent the number of strains from one or more corresponding geographical clusters using the color scheme given in Figure 1. Composition of genetic clusters i.e., given cluster name and country of origin (number of isolates) are as follows: AsiaMix = Syria (86), Afghanistan (39), Pakistan (33), India (6), Iraq (6), Iran (2), Israel (1), Jordan (1), Saudi Arabia (1); India = India (7), Palestine (3), Afghanistan (1); TurSan = Turkey (31), Iran (1), Syria (1), Israel (1); AzMix = Azerbaijan (5), Pakistan (3), Iraq (3), Kenya (2), India (2), Israel (2), Palestine (1), Turkey (1); MorA = Morroco (6), Pakistan (2), Turkey (2), Israel (1), Iran (1), Kuwait (1); OG = *L. donovani* (2), *L. major* (1); Nam = Namibia (4); Ken = Kenya (2), Saudi Arabia (1), Kuwait (1); Ken/Tun = Kenya (3), Tunisia (2); IsGal = Israel North Galilee (10); MorB = Morocco (3); Isr/Pal1 = Israel/Palestine (34); Isr/Pal2 = Israel/Palestine (11); Pal = Israel/Palestine (11), Egypt (1). This figure appears in color at www.ajtmh.org.

TABLE 2
Types of lesions in relation to genetic clusters of *Leishmania tropica* in Khyber Pakhtunkhwa

Dry/Mixed/Wet type	Genetic cluster (%)			*P* value*
	AsiaMix	AzMix	MorAMix	
Dry (*N* = 13)	11 (40.7)	2 (66.7)	0	0.261
Mixed (*N* = 15)	13 (48.2)	0	2 (100)	
Wet (*N* = 4)	3 (11.1)	1 (33.3)	0	
Clinical description of ulcerated lesions				
Typical ulcerated lesion (*N* = 2)	2 (100)	0	0	0.186
Psoriasiform (*N* = 11)	10 (90.9)	1 (9.1)	0	
Cellulitis-like (*N* = 5)	3 (60)	1 (20)	1 (20)	
Verruciform (Wart-like) (*N* = 3)	3 (100)	0	0	
Keloidal (*N* = 1)	0	1 (100)	0	

* Calculated for Pearson chi-squared test.

ME = Middle East, Ken = Kenya and Mor = Morrocco) (Table 3). Nei's genetic diversity (H_E) suggested that Ken (H_E = 0.60) followed by Mor (H_E = 0.58) were the most diverse groups genetically, whereas PkAF (H_E = 0.33) was the least diverse. In heterozygosity analysis, H_O was higher than H_E in PkAF, IQ, and marginally in India. The remaining groups were in heterozygote deficit ($H_E > H_O$). PkAF showed excess heterozygosity, producing a significantly negative F_{IS} value ($F_{IS} = -0.62$; $P > 0.95$). LD analysis provided clear evidence of significant allelic disequilibrium in all populations (PkAF, India, Ken, ME, and Mor) except IQ ($P < 0.05$) (Table 3).

Pairwise gene flow and genetic differentiation across countries. An F_{ST} matrix for selected country-wise clusters was produced (Table 4). The Pakistani cluster exhibited a moderate degree of gene flow with India (F_{ST} = 0.14) and negligible genetic differentiation from strains from Iraq (F_{ST} = 0.06), Afghanistan (F_{ST} = 0.00), and Syria (F_{ST} = 0.00) (Table 4, Figure 3). Strains from Morocco, Kenya, and Israel/Palestine showed moderate to high differentiation from South Asian and Middle Eastern isolates including those from Pakistan, Afghanistan, India, Iraq, and Syria (F_{ST} = 0.11–0.73). Notably, strains from Turkey were strongly differentiated (F_{ST} = −0.92–1.02) from the other country clusters studied (Table 3). These genetic distances were also evident from the principal component analysis of pairwise F_{ST} values (Figure 3).

DISCUSSION

This study was an attempt to understand the contribution of genetic diversity of *L. tropica* to clinical disease in the northern KP province of Pakistan and to explore relatedness with other regional parasite populations.

Genetic structure of *L. tropica* in northern Pakistan. Thirty-four strains from the present collection were broadly partitioned through two super clusters, namely, Asia and Morocco A. When compared with some other countries, *L. tropica* population seemed more or less homogenous in this region of Pakistan. For instance, strains from Azerbaijan, Iran, Israel, Afghanistan, and Iraq which are diverse at zymodeme level,[11,47] also showed heterogeneous placements in the resolved genetic structure of the species in our study. However, many of these strains were collected at different times and provided no information about the site of isolation within countries, thus influence of temporal and spatial isolation on genetic partition cannot be overlooked.[48] A study during an ACL out-break of 1997 in the Timargarha Afghan refugee camp in KP province presented homogeneity of *L. tropica* within the camp[30] suggesting the out-break was probably contributed by a specific genotypic strain of *L. tropica*. Thus, the homogeneity we described in northern Pakistan is perhaps not surprising, given all isolates were sampled contemporaneously. The northern Pakistan strains were not without diversity, however, and the minor fragmentation of this diversity across genetic groups that we observed is worth mentioning. It points to multiple origins of strains presenting to hospitals of this region in Pakistan. This might also suggest presence of unreported zymodemes within Pakistani strains that possess isoenzyme profiles that are probably the same or similar to those observed among other countries of Asia (such as MON-75 and MON-60) and Morocco A (such as MON-109 and MON-112).[11] Moreover, presence of apparently distinct populations in the same geographical area may also point toward coexistence of different vectors, reservoir hosts, and/or eco-epidemiological conditions.[49,50]

The minimal genetic structuring observed among Pakistani isolates in our study was not related to any specific geographic locations within the region (KP province). Although we did not formally test in our data set, true isolation by distance (IBD) in human infecting parasites such as *Leishmania* is a very rare phenomenon giving the capacity for dispersal with highly mobile hosts. This has been shown in several human infecting

TABLE 3
Population diversity and linkage disequilibrium indices of selected geographical clusters

Cluster	N*	PL†	H_O‡	H_OSD§	H_E‖	H_ESD	% Loci in HD¶	% Loci in HE¶	F_{IS} (*P* value)**	I_A (*P* value)††	% Loci in LD‡‡	% LD physical‖‖
Pk-Afg	78	10	0.57	0.49	0.33	0.23	30	60	−0.62 (1.000)	1.43 (< 0.001)	42.9	0
India	15	11	0.43	0.39	0.41	0.19	18.2	18.2	−0.01 (0.553)	2.42 (< 0.001)	50	14.3
Iraq	9	7	0.66	0.34	0.51	0.1	14.3	14.3	−0.12 (0.844)	0.1 (0.332)	35.7	0
Kenya	7	11	0.13	0.2	0.6	0.22	63.6	0	0.79 (0.000)	2.14 (< 0.001)	21.4	0
Middle East	198	13	0.33	0.37	0.5	0.27	76.9	23.1	0.51 (0.000)	4.49 (< 0.001)	64.3	14.3
Morocco	9	10	0.08	0.12	0.58	0.14	90	0	0.87 (0.000)	4.68 (< 0.001)	28.6	0

Pk–Afg = Pak–Afghan; LD = linkage disequilibrium.
* Number of samples per cluster.
† Total number of polymorphic loci tested.
‡ H_O = mean observed heterozygosity.
§ Standard deviation.
‖ H_E = mean unbiased (sample corrected) expected heterozygosity[76].
¶ Proportion of loci in HD (Heterozygote deficit) & HE (Heterozygote excess).
** Inbreeding coefficient (*P* value generated over 16,000 permutations).
†† Index of association (*P* value calculated by comparison to 1,000 randomizations of null distribution).
‡‡ Percentage of loci in significant linkage disequilibrium by comparison to 10,000 randomizations of null distribution.
‖‖ Proportion of statistically linked loci physically linked on the same chromosome.

TABLE 4
Pairwise F_{ST} generated for selected country-wise comparisons of gene flow

	India	IQ	IsrPal	Ken	Mor	Af	Pak	Sy	Tur
India									
IQ	0.09								
IsrPal	0.90	1.03							
Ken	0.37	0.45	0.45						
Mor	0.57	0.63	0.71	0.32					
Af	0.14	0.07	1.20	0.87	0.94				
Pak	0.14	0.06	1.09	0.73	0.75	0.00			
Sy	0.17	0.08	1.34	0.97	1.02	0.00	0.00		
Tur	1.02	0.92	1.18	1.03	0.92	1.10	0.90	1.02	

Af = Afghanistan; IsrPal = Israel–Palestine; IQ = Iraq; Ken=Kenya; Mor = Morrocco; Pk = Pakistan; Sy = Syria; Tur = Turkey. Gray highlighted cells represent F_{ST} values > 0.25 indicating substantial differentiation (only limited gene flow) as described by Hartl and Clark[77].

pathogens, such as *Plasmodium falciparum, Schistosoma mansoni*, and *Candida albicans*, thus suggesting that rapid human migration patterns may reduce spatial structure within these pathogen populations.[51–53] The historical samples from elsewhere in Pakistan had slightly variable genotypes and were allocated to the Asia super cluster (Figure 2), that also contained all 2010 KP isolates. This suggests long-term stability of Pakistani genotypes within this cluster rather than recent introductions. These minor variants could have arisen via mutation, human migration, or possibly because of coexisting zoonotic cycles.[11] However, so far there are no indications for zoonotic transmission of *L. tropica* in KP or in Pakistan. The most widespread genotype of Pakistani *L. tropica* was also shared by most isolates from neighboring India, Afghanistan, Iran, and Syria, suggesting substantial connectivity and human-driven dispersal between these countries.

Genotypic characterization of clinical phenotypes by *L. tropica*. The clinical presentation of CL could not be associated with the genetic variability observed among Pakistani *L. tropica* strains. These observations have been corroborated by molecular level studies on *L. tropica* elsewhere and from other clinically diverse *Leishmania* species.[12,54] The precise contribution of a parasite genotype to its clinical outcome remains unknown, and studies frequently fail to associate specific genotypes to exceedingly differentiated clinical forms of CL. For example, *L. tropica* causing leishmaniasis recidivans, a chronic form of CL characterized by complex lesions, could not be differentiated serologically from those isolates causing simple sores.[55] On the other hand, different species can produce morphologically similar lesions.[56–58] Studies have also failed to identify exclusive zymodemes or genetic affiliations for visceralizing *L. tropica* and *Leishmania infantum* isolates.[10,59–62]

These findings support the view that clinical appearance of CL is not only determined by *L. tropica* genetic variation, but is also strongly influenced by a range of other factors including host immune status and response, size of inocula, site of the sandfly bite, duration of the lesions, secondary infections, or—more likely—a combination of all these factors.[63] However, occasionally, studies do demonstrate genetic predisposition for clinical variability seen in *Leishmania* species that possibly operate through polymorphic genes.[64,65]

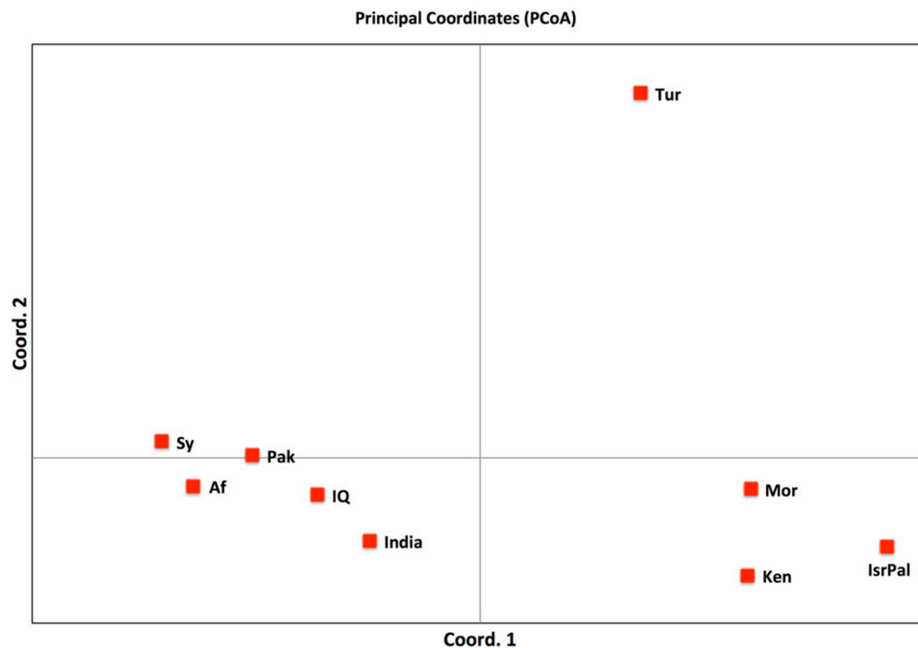

FIGURE 3. Principal coordinates analysis plot for selected countries derived from linearized pairwise F_{ST}. This figure appears in color at www.ajtmh.org.

Gene flow and recombination. Heterozygosity is one of the key descriptors of genetic variation in asexual diploid organisms.[66] The Pak–Afghan group in our study was in heterozygote excess and significant LD, potentially demonstrating a predominant clonal lineage of this cluster which is well ascertained for *L. tropica*.[67,68] It is assumed that sexual reproduction in such protozoans may possibly be underestimated because of insufficient sampling, poor genetic models used, and poor discriminatory power of markers analyzed.[69] There is, however, medical relevance to long term clonal reproduction as such lineages may tend to accumulate mutations for genes that control key medical characteristics such as drug resistance or strain pathogenicity.[70] Several studies back the widespread drug resistance to traditional antimonial treatments in South Asia and the study area in particular.[71,72] However, heterozygosity observed in the Pak–Afghan cluster may be explained either by heterozygosity at individual loci or by the presence of mixtures of strains because isolates in the present study were not cloned.[28,34] It would, therefore, be interesting to analyze clones of Pakistani strains that were allocated to different populations, subpopulations, or clusters although studies suggest that, in nature, complex infections with mixed parasite genotypes in mammalian hosts are probably rare especially in hypoendemic foci.[73]

Based on F_{ST}, there was low level of differentiation among isolates from Pakistan, Afghanistan, Iraq, India, and Syria, and these five countries shared genotypes displaying significantly high levels of inbreeding. The observed gene flow among these parasite populations may be a consequence of human migration, displacement of refugees, and frequent travel (for labor, trade etc.) among these countries,[74,75] leading to apparent homogenization of gene pools. The observed gene flow pattern does not provide any conclusive inferences about the probable routes of import of *L. tropica* in to Asia and its subsequent spread from western to eastern regions of the continent. There is a possibility of more than one instance of introduction of this species into Asia. In our study, strains from Morocco and Kenya were placed in genetically diverse African clusters and grouping of these two clusters in PCoA with Israeli/Palestinian strains suggest an import from the genetically rich Morocco and Kenya to Israel/Palestine, probably through the North African tract of Egypt (Sinai Peninsula). However, no definitive conclusions can be made because there is geographic discontinuity in the present data (no isolates from countries adjacent to the Africa or from Arabian Gulf states were analyzed e.g., Yemen, Oman, Somalia, and Ethiopia). The Pak–Afghan cluster harbored the least diverse parasites of all regions in Asia. Conclusions derived from the diversity indices and country-wise F_{ST} suggest a hypothetical route of import from western borders (Iran) to this region. The presence of some minor genetic partition in Pakistan may indicate import routes other than the west, from north, and north west (Tajikistan or China), for instance. However, these hypotheses need further validation.

CONCLUSION

This study provides the first detailed investigation of the population structure and genetic diversity of *L. tropica* in northern Pakistan and the surrounding region. No intraspecific (i.e., within *L. tropica*) clinical variations within northern Pakistan could be explicitly assigned to any genetic cluster. Further sampling of different clinical forms and analysis of diversity using an appropriate array of polymorphic nuclear targets, mitochondrial genes or possibly whole genomes should be pursued to further test the validity of our findings. A clearer picture of the history of import and circulation of *L. tropica* within Asia could also be elucidated through continuing such studies across the Afro–Eurasian range of *L. tropica.*

Received November 9, 2016. Accepted for publication June 30, 2017.

Published online September 25, 2017.

Note: Supplemental tables appear at www.ajtmh.org.

Acknowledgments: We thank the staff and field workers from the Department of Zoology, University of Peshawar and the London School of Hygiene and Tropical Medicine for their cooperation and support during the study. We are also grateful to the staff at the Kuwait Teaching Hospital, Khyber Teaching Hospital, and Combined Military Hospital, Peshawar for their extended assistance.

Financial support: This work was supported by "Faculty Development Program (FDP)" scholarship awarded by Higher Education Commission, Pakistan to NK.

Authors' addresses: Nazma Habib Khan, Department of Zoology, University of Peshawar, Peshawar, Khyber Pakhtunkhwa, Pakistan, E-mail: nazma@upesh.edu.pk. Martin S. Llewellyn, Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, United Kingdom, E-mail: martin.llewellyn@glasgow.ac.uk. Gabriele Schönian, Institute of Microbiology and Hygiene, Chariteì-University Medicine Berlin, Hindenburgdamm, Berlin, Germany, E-mail: gabriele.schoenian@t-online.de. Colin J. Sutherland, Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom, E-mail: colin.sutherland@lshtm.ac.uk.

REFERENCES

1. Nagill R, Kaur S, 2011. Vaccine candidates for leishmaniasis: a review. *Int Immunopharmacol 11:* 1464–1488.
2. Seifert K, Croft SL, 2006. In vitro and in vivo interactions between miltefosine and other antileishmanial drugs. *Antimicrob Agents Chemother 50:* 73–79.
3. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One 7:* e35671.
4. Azmi K, Schnur L, Schonian G, Nasereddin A, Pratlong F, El Baidouri F, Ravel C, Dedet JP, Ereqat S, Abdeen Z, 2012. Genetic, serological and biochemical characterization of *Leishmania tropica* from foci in northern Palestine and discovery of zymodeme MON-307. *Parasit Vectors 5:* 121.
5. Le Blancq SM, Peters W, 1986. *Leishmania* in the old World: 2. Heterogeneity among *L. tropica* zymodemes. *Trans R Soc Trop Med Hyg 80:* 113–119.
6. Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J, 1990. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. *Ann Parasitol Hum Comp 65:* 111–125.
7. Schnur LF, et al., 2004. Multifarious characterization of *Leishmania tropica* from a Judean desert focus, exposing intraspecific diversity and incriminating *Phlebotomus sergenti* as its vector. *Am J Trop Med Hyg 70:* 364–372.
8. Bari AU, 2012. Clinical spectrum of cutaneous leishmaniasis: an overview from Pakistan. *Dermatol Online J 18:* 4.
9. Khanra S, Datta S, Mondal D, Saha P, Bandopadhyay SK, Roy S, Manna M, 2012. RFLPs of ITS, ITS1 and hsp70 amplicons and sequencing of ITS1 of recent clinical isolates of Kala-azar from India and Bangladesh confirms the association of *L. tropica* with the disease. *Acta Trop 124:* 229–234.
10. Magill AJ, Grogl M, Gasser RA Jr, Sun W, Oster CN, 1993. Visceral infection caused by *Leishmania tropica* in veterans of Operation Desert Storm. *N Engl J Med 328:* 1383–1387.
11. Pratlong F, Dereure J, Ravel C, Lami P, Balard Y, Serres G, Lanotte G, Rioux JA, Dedet JP, 2009. Geographical distribution and

epidemiological features of old World cutaneous leishmaniasis foci, based on the isoenzyme analysis of 1048 strains. *Trop Med Int Health 14:* 1071–1085.
12. Schonian G, Schnur L, el Fari M, Oskam L, Kolesnikov AA, Sokolowska-Kohler W, Presber W, 2001. Genetic heterogeneity in the species *Leishmania tropica* revealed by different PCR-based methods. *Trans R Soc Trop Med Hyg 95:* 217–224.
13. Schwenkenbecher JM, Frohlich C, Gehre F, Schnur LF, Schonian G, 2004. Evolution and conservation of microsatellite markers for *Leishmania tropica*. *Infect Genet Evol 4:* 99–105.
14. Schwenkenbecher JM, Wirth T, Schnur LF, Jaffe CL, Schallig H, Al-Jawabreh A, Hamarsheh O, Azmi K, Pratlong F, Schonian G, 2006. Microsatellite analysis reveals genetic structure of *Leishmania tropica*. *Int J Parasitol 36:* 237–246.
15. Rowland M, Munir A, Durrani N, Noyes H, Reyburn H, 1999. An outbreak of cutaneous leishmaniasis in an Afghan refugee settlement in north-west Pakistan. *Trans R Soc Trop Med Hyg 93:* 133–136.
16. Noor SM, Hussain D, 2004. Cutaneous leishmaniasis in Sadda, Kurram agency, Pakistan. *J Pak Assoc Dermatol 14:* 114–117.
17. Marco JD, et al., 2006. Multilocus enzyme electrophoresis and cytochrome B gene sequencing-based identification of *Leishmania* isolates from different foci of cutaneous leishmaniasis in Pakistan. *Am J Trop Med Hyg 75:* 261–266.
18. Khan NH, Bari AU, Hashim R, Khan I, Muneer A, Shah A, Wahid S, Yardley V, O'Neill B, Sutherland C, 2016. Cutaneous leishmaniasis in Khyber Pakhtunkhwa province of Pakistan: clinical diversity and species-level diagnosis. *Am J Trop Med Hyg 95:* 1106–1114.
19. Bari AU, Rahman SB, 2008. Many faces of cutaneous leishmaniasis. *Indian J Dermatol Venereol Leprol 74:* 23–27.
20. Bari AU, Raza N, 2010. Lupoid cutaneous leishmanisis: a report of of 16 cases. *Indian J Dermatol Venereol 76:* 85.
21. Raja KM, Khan AA, Hameed A, Rahman SB, 1998. Unusual clinical variants of cutaneous leishmaniasis in Pakistan. *Br J Dermatol 139:* 111–113.
22. Shamsuddin S, Mengal JA, Gazozai S, Mandokhail ZK, Kasi M, Muhammad N, 2006. Atypical presentation of cutaneous leishmaniasis in native population of Baluchistan. *J Pak Assoc Dermatol 16:* 196–200.
23. Bari AU, 2006. Epidemiology of cutaneous leishmaniasis. *J Pak Assoc Dermatol 16:* 156–162.
24. Rahman S, Abdullah FH, Khan JA, 2009. The frequency of old world cutaneous leishmaniasis in skin ulcers in Peshawar. *J Ayub Med Coll Abbottabad 21:* 72–75.
25. Alam MZ, Kuhls K, Schweynoch C, Sundar S, Rijal S, Shamsuzzaman AK, Raju BV, Salotra P, Dujardin JC, Schonian G, 2009. Multilocus microsatellite typing (MLMT) reveals genetic homogeneity of *Leishmania donovani* strains in the Indian subcontinent. *Infect Genet Evol 9:* 24–31.
26. Bulle B, et al., 2002. Practical approach for typing strains of *Leishmania infantum* by microsatellite analysis. *J Clin Microbiol 40:* 3391–3397.
27. Jamjoom MB, Ashford RW, Bates PA, Kemp SJ, Noyes HA, 2002. Towards a standard battery of microsatellite markers for the analysis of the *Leishmania donovani* complex. *Ann Trop Med Parasitol 96:* 265–270.
28. Ochsenreither S, Kuhls K, Schaar M, Presber W, Schonian G, 2006. Multilocus microsatellite typing as a new tool for discrimination of *Leishmania infantum* MON-1 strains. *J Clin Microbiol 44:* 495–503.
29. Rougeron V, et al., 2009. Extreme inbreeding in *Leishmania braziliensis*. *Proc Natl Acad Sci USA 106:* 10224–10229.
30. Noyes HA, Reyburn H, Bailey JW, Smith D, 1998. A nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. *J Clin Microbiol 36:* 2877–2881.
31. Krayter L, Alam MZ, Rhajaoui M, Schnur LF, Schonian G, 2014a. Multilocus microsatellite typing reveals intra-focal genetic diversity among strains of *Leishmania tropica* in Chichaoua province, Morocco. *Infect Genet Evol 28:* 233–239.
32. Krayter L, Bumb RA, Azmi K, Wuttke J, Malik MD, Schnur LF, Salotra P, Schonian G, 2014b. Multilocus microsatellite typing reveals a genetic relationship but, also, genetic differences between Indian strains of *Leishmania tropica* causing cutaneous leishmaniasis and those causing visceral leishmaniasis. *Parasit Vectors 7:* 123.
33. Alam MZ, Bhutto AM, Soomro FR, Baloch JH, Nakao R, Kato H, Schonian G, Uezato H, Hashiguchi Y, Katakura K, 2014. Population genetics of *Leishmania* (*Leishmania*) *major* DNA isolated from cutaneous leishmaniasis patients in Pakistan based on multilocus microsatellite typing. *Parasit Vectors 7:* 332.
34. Gelanew T, Kuhls K, Hurissa Z, Weldegebreal T, Hailu W, Kassahun A, Abebe T, Hailu A, Schonian G, 2010. Inference of population structure of *Leishmania donovani* strains isolated from different Ethiopian visceral leishmaniasis endemic areas. *PLoS Negl Trop Dis 4:* e889.
35. Jombart T, Devillard S, Balloux F, 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genet 11:* 94.
36. Pritchard JK, Wen X, Falush D, 2009. *Documentation for Structure Software: Version 2.3.* Available at: http://pritch.bsd.uchicago.edu/structure.html. Accessed April 14, 2012.
37. Corander J, Marttinen P, 2006. Bayesian identification of admixture events using multilocus molecular markers. *Mol Ecol 15:* 2833–2843.
38. Corander J, Marttinen P, Siren J, Tang J, 2008. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics 9:* 539.
39. Rodriguez-Ramilo ST, Toro MA, Fernandez J, 2009. Assessing population genetic structure via the maximisation of genetic distance. *Genet Sel Evol 41:* 49.
40. Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multilocus genotype data. *Genetics 155:* 945–959.
41. Excoffier L, Lischer HEL, 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour 10:* 564–567.
42. Agapow PM, Burt A, 2001. Indices of multilocus linkage disequilibrium. *Mol Ecol Notes 1:* 101–102.
43. Maynard Smith J, Smith NH, O'Rourke M, Spratt BG, 1993. How clonal are bacteria? *Proc Natl Acad Sci USA 90:* 4384–4388.
44. Peakall R, Smouse PE, 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research–an update. *Bioinformatics 28:* 2537–2539.
45. Peakall R, Smouse PE, 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes 6:* 288–295.
46. Uzun S, Durdu M, Culha G, Allahverdiyev AM, Memisoglu HR, 2004. Clinical features, epidemiology, and efficacy and safety of intralesional antimony treatment of cutaneous leishmaniasis: recent experience in Turkey. *J Parasitol 90:* 853–859.
47. Plourde M, Coelho A, Keynan Y, Larios OE, Ndao M, Ruest A, Roy G, Rubinstein E, Ouellette M, 2012. Genetic polymorphisms and drug susceptibility in four isolates of *Leishmania tropica* obtained from Canadian soldiers returning from Afghanistan. *PLoS Negl Trop Dis 6:* e1463.
48. Rougeron V, De Meeus T, Hide M, Le Falher G, Bucheton B, Dereure J, El-Safi SH, Dessein A, Banuls AL, 2011. Multifaceted population structure and reproductive strategy in *Leishmania donovani* complex in one Sudanese village. *PLoS Negl Trop Dis 5:* e1448.
49 Githure JL, Hendricks LD, Schmur LF, Kilu G, Perkins PV, 1986. *Characterization of Crithidia-Like Organism Isolated from Man, Animals, and Sandflies in Leishmaniasis Endemic Foci in Kenya*. Proceedings of the Seventh Annual Medical Scientific Conference. Nairobi, Kenya: Kenya Medical Research Institute, 243–247.
50. Hamarsheh O, Presber W, Abdeen Z, Sawalha S, Al-Lahem A, Schonian G, 2007. Genetic structure of Mediterranean populations of the sandfly *Phlebotomus papatasi* by mitochondrial cytochrome b haplotype analysis. *Med Vet Entomol 21:* 270–277.
51. Fundyga RE, Lott TJ, Arnold J, 2002. Population structure of *Candida albicans*, a member of the human flora, as determined by microsatellite loci. *Infect Genet Evol 2:* 57–68.
52. Machado RL, Povoa MM, Calvosa VS, Ferreira MU, Rossit AR, dos Santos EJ, Conway DJ, 2004. Genetic structure of *Plasmodium falciparum* populations in the Brazilian Amazon region. *J Infect Dis 190:* 1547–1555.

53. Thiele EA, Sorensen RE, Gazzinelli A, Minchella DJ, 2008. Genetic diversity and population structuring of *Schistosoma mansoni* in a Brazilian village. *Int J Parasitol 38:* 389–399.
54. Myint CK, et al., 2008. Polymorphisms of cytochrome b gene in *Leishmania* parasites and their relation to types of cutaneous leishmaniasis lesions in Pakistan. *J Dermatol 35:* 76–85.
55. Bray RS, Rahim GA, 1969. Studies on the immunology and serology of leishmaniasis: VII. Serotypes of *Leishmania tropica*. *Trans R Soc Trop Med Hyg 63:* 383–387.
56. Dedet JP, Pratlong F, 2009. Leishmaniasis. Cook GC, Zumla AI, eds. *Manson's Tropical Diseases*. Philadelphia, PA: Saunders/Elsevier Science Limited, 1341–1365.
57. Schonian G, Akuffo H, Lewin S, Maasho K, Nylen S, Pratlong F, Eisenberger CL, Schnur LF, Presber W, 2000. Genetic variability within the species *Leishmania aethiopica* does not correlate with clinical variations of cutaneous leishmaniasis. *Mol Biochem Parasitol 106:* 239–248.
58. Kuhls K, Alam MZ, Cupolillo E, Ferreira GE, Mauricio IL, Oddone R, Feliciangeli MD, Wirth T, Miles MA, Schonian G, 2011. Comparative microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. *PLoS Negl Trop Dis 5:* e1155.
59. Alborzi A, Rasouli M, Shamsizadeh A, 2006. *Leishmania tropica*-isolated patient with visceral leishmaniasis in southern Iran. *Am J Trop Med Hyg 74:* 306–307.
60. Magill AJ, Grogl M, Johnson SC, Gasser RA Jr, 1994. Visceral infection due to *Leishmania tropica* in a veteran of Operation Desert Storm who presented 2 years after leaving Saudi Arabia. *Clin Infect Dis 19:* 805–806.
61. Oren R, Schnur LF, Ben Yehuda D, Mayner V, Okon E, Rachmilewitz EA, 1991. Visceral leishmaniasis: a difficult diagnosis and unusual causative agent. *J Infect Dis 164:* 746–749.
62. Pratlong F, Rioux JA, Marty P, Faraut-Gambarelli F, Dereure J, Lanotte G, Dedet JP, 2004. Isoenzymatic analysis of 712 strains of *Leishmania infantum* in the south of France and relationship of enzymatic polymorphism to clinical and epidemiological features. *J Clin Microbiol 42:* 4077–4082.
63. Murray HW, Berman JD, Davies CR, Saravia NG, 2005. Advances in leishmaniasis. *Lancet 366:* 1561–1577.
64. McCall LI, Zhang WW, Matlashewski G, 2013. Determinants for the development of visceral leishmaniasis disease. *PLoS Pathog 9:* e1003053.
65. Romano A, et al., 2014. Cross-species genetic exchange between visceral and cutaneous strains of *Leishmania* in the sand fly vector. *Proc Natl Acad Sci USA 111:* 16808–16813.
66. Bougnoux ME, Diogo D, Francois N, Sendid B, Veirmeire S, Colombel JF, Bouchier C, Van Kruiningen H, d'Enfert C, Poulain D, 2006. Multilocus sequence typing reveals intrafamilial transmission and microevolutions of *Candida albicans* isolates from the human digestive tract. *J Clin Microbiol 44:* 1810–1820.
67. Tibayrenc M, Kjellberg F, Ayala FJ, 1990. A clonal theory of parasitic protozoa: the population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. *Proc Natl Acad Sci USA 87:* 2414–2418.
68. Tibayrenc M, Ayala FJ, 2002. The clonal theory of parasitic protozoa: 12 years on. *Trends Parasitol 18:* 405–410.
69. Ramirez JD, Llewellyn MS, 2014. Reproductive clonality in protozoan pathogens—truth or artefact? *Mol Ecol 23:* 4195–4202.
70. Tibayrenc M, 1998. Genetic epidemiology of parasitic protozoa and other infectious agents: the need for an integrated approach. *Int J Parasitol 28:* 85–104.
71. Hadighi R, Boucher P, Khamesipour A, Meamar AR, Roy G, Ouellette M, Mohebali M, 2007. Glucantime-resistant *Leishmania tropica* isolated from Iranian patients with cutaneous leishmaniasis are sensitive to alternative antileishmania drugs. *Parasitol Res 101:* 1319–1322.
72. Jamal Q, Khan NH, Wahid S, Awan MM, Sutherland C, Shah A, 2015. In-vitro sensitivity of Pakistani *Leishmania tropica* field isolate against buparvaquone in comparison to standard anti-leishmanial drugs. *Exp Parasitol 154:* 93–97.
73. Bastien P, Blaineau C, Pages M, 1992. *Leishmania*: sex, lies and karyotype. *Parasitol Today 8:* 174–177.
74. Aagaard-Hansen J, Nombela N, Alvar J, 2010. Population movement: a key factor in the epidemiology of neglected tropical diseases. *Trop Med Int Health 15:* 1281–1288.
75. Saroufim M, Charafeddine K, Issa G, Khalifeh H, Habib RH, Berry A, Ghosn N, Rady A, Khalifeh I, 2014. Ongoing epidemic of cutaneous leishmaniasis among Syrian refugees, Lebanon. *Emerg Infect Dis 20:* 1712–1715.
76. Nei M, 1973. Analysis of gene diversity in subdivided populations. *Proc Natl Acad Sci USA 70:* 3321–3323.
77. Hartl DL, Clark AG, 1997. *Principles of Population Genetics.* Sunderland, MA: Sinauer Associates Inc.