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Microsatellite analysis reveals genetic structure of *Leishmania tropica*

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Abstract

The current rapid spread of leishmaniases caused by *Leishmania tropica* and the complexity of its clinical spectrum call for this parasite's epidemiological and evolutionary investigation. Evaluation of its population structure by isoenzyme electrophoresis and previous molecular biological analysis has proved difficult. In this study, we used 21 microsatellite loci to type 117 strains from different African and Asian locations. Eighty-one different genotypes were found. A genetic bottleneck supported by a gradient in the number of alleles and consistent with the geographical structure of the Middle East suggests an African origin of this species. A Bayesian approach identified 10 genetic clusters that correlated predominantly with geographical origin. The strains in the 'Asia' cluster form a very heterogeneous sub-population, with a varied but inter-related genotype that is geographically very widely dispersed and consistent with anthroponotic transmission of the parasite. The other nine clusters were more homogenous. The propagation of *L. tropica* appears to be predominantly clonal. In Africa and the Middle East, anthroponotic and zoonotic systems of distribution may contribute to the development of overlapping, genetically distinct populations of *L. tropica*.

Keywords: Leishmania tropica; Microsatellite markers; Population structure; Life cycle; Geographical distribution

1. Introduction

Leishmania tropica is a particularly interesting and vexing parasitic species. It is very heterogeneous, displaying serological (Jaffe et al., 1990; Jacobson et al., 2003; Schnur et al., 2004), biochemical (Le Blancq and Peters, 1986; Rioux et al., 1990; Pratlong et al., 1991; Mebrahtu et al., 1992) and genetic (Schönian et al., 2001) heterogeneity. It is also associated with a broad and complex clinical spectrum of disease represented by numerous cases of simple cutaneous leishmaniasis (CL) manifesting as either single or multiple lesions, relatively rare cases of leishmaniasis recidivans (LR), rarer cases of visceral leishmaniasis (VL), including some that have also displayed post kala azar dermal leishmaniasis (PKDL) after treatment, and even an occasional case of oro-nasal and naso-pharyneal leishmaniasis (OL) (Schnur et al., 1981; Magill et al., 1994; Sacks et al., 1995; Schnur and Greenblatt, 1995). It occurs in tropical and sub-tropical regions of the Old World where its geographical distribution is very wide. *Leishmania tropica* is transmitted by at least three phlebotomine species of sand fly: *Phlebotomus (Paraphlebotomus) sergenti* in Saudi Arabia (Al-Zahrani et al., 1988), Morocco (Guilvard et al., 1991), Afghanistan (Killick-Kendrick et al., 1995) and Israel (Schnur et al., 2004); *Phlebotomus (Adlerius) arabicus* in Israel (Jacobson et al., 2003) and *Phlebotomus guggisbergi* in Kenya (Lawyer et al., 1991). Another, putative, vector for *L. tropica* in Kenya is *Phlebotomus aculeatus* (Johnson et al., 1993).

The epidemiology of leishmaniases caused by *L. tropica* has not been elucidated fully. Disease is often described as being urban and anthroponotic and most infections occur in and around quite densely populated cities. However, the paucity of cases in some foci and sudden occurrence of small outbreaks of

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disease in semi-rural locations suggest that disease may be zoonotic in some cases (Mebrahtu et al., 1992; Klaus et al., 1994; Sang et al.; Kamhawi et al., 1995; Guessous-Idrissi et al., 1997; Jacobson et al., 2003; Schnur et al., 2004). Strains of *L. tropica* have been isolated from dogs in Morocco (Dereure et al., 1991) and in Kenya hyraxes are a putative animal reservoir host (Sang et al., 1994). Further, a study using a PCR of the internal transcribed spacer region 1 carried out on tissues from three hyraxes caught in Israel just north of the Sea of Galilee yielded DNA products characteristic for *L. tropica* (Jacobson et al., 2003) and a strain of *L. tropica* was cultured from a fourth hyrax (Jaffe et al., 2004).

The high degree of heterogeneity of *L. tropica* was suggested to be associated with genetic exchange (Pratlong et al., 1991). It is of medical and economic importance to know if its propagation is strictly clonal or panmictic. Recently, there has been an upsurge of cases of CL caused by *L. tropica*, including the emergence of several new foci in Morocco (Rhajaoui et al., 2004), Kenya (Johnson et al., 1999), Ethiopia (Gebre-Michael et al., 2004), Israel (Jacobson et al., 2003; Schnur et al., 2004), The Palestinian Authority (Al-Jawabreh et al., 2004), Jordan (Kamhawi et al., 1995), Iran (Yaghoobi-Ershadi et al., 2002) and Afghanistan (Reithinger et al., 2003). The rapid spreading of the parasite with increasing rates of infection begs epidemiological investigation. It is of interest to clarify the situation of strains from Namibia and some of those from Tunisia with characteristics similar to *L. tropica*, which have been proffered as new species (Lanotte G., Rioux J. A., Serres E., 1986. Approche cladistique du genre *Leishmania* Ross, 1903. A propos de 192 souches originaires de l'Ancien Monde. Analyse numerique de 50 zymodemes identifies par 15 enzymes et 56 isoenzymes. Pp. 269–288 in: *Leishmania*. Taxonomie et Phylogenese. Applications eco-epidemiologiques. (Colloque International CNRS/INSERM/OMS 2-6 Juillet 1984), Montpellier: IMEEE; Rioux, J. A., Lanotte G., Pratlong F., 1986. *Leishmania killicki* n.sp. (*Kinetoplastidae, Trypanosomatidae*). Pp. 139–142 in: *Leishmania*. Taxonomie et Phylogenese. Applications eco-epidemiologiques. (Colloque International CNRS/INSERM/OMS 2–6 Juillet 1984), Montpellier: IMEEE; Van Eys et al., 1989; Rioux et al., 1990).

None of the methods revealing the heterogenic character of *L. tropica* have shown a clear idea of the population composition for this species. Revealing the genetic composition and population structure through genetic analysis of this species based on co-dominant genetic markers like microsatellites should help to give clues to its heterogeneity and propagation. Microsatellite markers have been used in numerous population studies (Russell et al., 1999; Chambers and MacAvoy, 2000; Sunnucks, 2000; Toth et al., 2000; Wirth and Bernatchez, 2001; Bulle et al., 2002) and have been shown to be a useful tool in determining genetic distances between individuals and groups of individuals. Development of microsatellite markers suitable for the epidemiological and genetic investigations of *L. tropica* was recently described (Schwenkenbecher et al., 2004), and a further number of markers is available from a set that was developed for *Leishmania donovani* (Jamjoom et al., 2002, 2004). Here, 117 strains of *L. tropica* from different geographical regions were genotyped using microsatellite markers. The data set was analysed with the aim of unravelling the population structure of the parasite and the genetic relationships of the strains.

2. Material and methods

2.1. Source of parasites

Thirty-three strains of *L. tropica* used in this study were from Turkey, 27 from The Palestinian Authority, 21 from Israel, nine from Morocco, seven from Kenya, five from Azerbaijan, four from Namibia, four from India, three from Iraq, two from Tunisia and one each from Jordan and Egypt (Table S1). The strains were obtained from: the Department of Biomedical Research, the Royal Tropical Institute, Amsterdam, The Netherlands; the WHO's Reference Centre for the Leishmaniases, the Hebrew University, Jerusalem, Israel; the Centre National de Référence des *Leishmania*, Université de Montpellier I, France; the Department of Molecular Biology, the Lomonossov State University, Moscow, Russia; The Islah Charitable Social Society, Jericho, The Palestinian Authority and the Department of Biochemistry, Al-Quds University, Abu Dis, The Palestinian Authority. The strain *Leishmania major* MHOM/IL/1980/Friedlin used as an out-group was also obtained from the WHO's Reference Centre for the Leishmaniases, Jerusalem.

2.2. DNA extraction and microsatellite genotyping

DNA was extracted as described by Schönian et al. (1996). Microsatellite analysis was performed using 21 microsatellite markers developed and optimized for *L. tropica* (Schwenkenbecher et al., 2004) and *L. donovani* (Jamjoom et al., 2002). Microsatellite amplification and fragment size analysis by either polyacrylamide electrophoresis or fluorescence detection in an automated sequencing system (Beckman Coulter) was done as described by Schwenkenbecher et al. (2004).

2.3. Genetic distance and genotyping

The entire dataset was clone-corrected for all analyses except for the generation of the Neighbour-Joining tree.

Population structure was investigated using the Bayesian model-based clustering approach implemented in STRUCTURE (Pritchard et al., 2000). On the basis of multilocus data, this approach places individuals into K populations. Individuals can be assigned to multiple clusters with the membership coefficients of all the clusters summing up to one. A burnin period of 20,000 iterations was set and probability estimates were obtained, using 200,000 Markov Chain Monte Carlo iterations. The most appropriate number of populations was determined by comparing log-likelihoods (ln $\Pr(K/X)$, where X is the number of genotypes) for values of K between 1 and 15. On comparing the values graphically, the resulting curve had a Gaussian distribution. At the maximum, the plateau, the value of K encompassed the main structure embodied in the data.

Beyond this point, the graph ceased to follow a Gaussian distribution curve. To identify ancestral source populations, STRUCTURE analyses were performed with increasing K values.

A distance matrix based on the proportion of shared alleles, a method implemented in MICROSAT 1.5d (http://hpgl.stanford.edu/projects/microsat), was generated. The distance matrix was then converted into a Nexus file and entered into the PAUP version 4.0b8 (Swofford, D. L., 2000. PAUP. Phylogenetic Analysis Using Parsimony (and Other Methods). Version 4,0b8. Sinauer Associates, Sunderland, Massachusettes) to generate a phylogenetic tree using the neighbour-joining method. The strain *L. major* MHOM/IL/80/Friedlin was used to root the tree. Tree topology and branch support was assessed by bootstrapping (1000 iterations).

To detect discrete genetic substructure at the population and individual levels, a factorial correspondence analysis (FCA) was performed, using Genetix (Dawson and Belkhir, 2001), which graphically projects the individuals on the factor space defined by the similarity of their allelic state.

The software FSTAT (http://www.unil.ch/izea/softwares/fstat.html) was used to assess the allelic diversity (A). The expected (H_E) and observed (H_O) heterozygosity and the inbreeding coefficient F_{is} were estimated using GENEPOP 3.4 (Rousset and Raymond, 1995). Disequilibrium measures were used to compare the observed genotypic frequencies with those expected from the allelic frequencies. Linkage between all pairs of loci in each population was tested using ARLEQUIN (Schneider S., Roessli D., Excoffier L., 2000. Arlequin ver. 2000: A software for population genetics analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland).

Wright's F statistics (Wright, 1969) was calculated in GENEPOP 3.4 to assess how genetic variation was partitioned between populations. The genetic differentiation between populations based on the stepwise mutation model (R_{ST}) was estimated. According to Wright (1978), values >0.25 indicate strong genetic differentiation. This program was also used to do a Mantel test to see if genetic isolation coincides with increasing geographical distance, using R_{ST} values.

To compare allelic abundance in the different geographical regions, the strains of *L. tropica* were sorted according to their main geographical origin: Africa; the Middle East, including Israel, The Palestinian Authority, Jordan and Egypt; and Asia, with strains from India, Iraq, Turkey and Azerbaijan. In order to compare groups of identical size, the groups comprising higher numbers of strains were reduced to the size of the group 'Asia', which was the smallest group. The groups of larger size were re-sampled and the number of alleles in each group was estimated using 100 replicates of the re-sampled data.

3. Results

3.1. Analysis of microsatellite variation

Eighty-one different genotypes were detected among the 117 strains of *L. tropica* analysed, of which 73 were unique to individual strains and eight were shared by more than one strain. The identical genotypes were in: a group of 27 strains from Sanliurfa, Turkey; eight strains falling into three groups, two containing three strains each and one containing two strains, all of which came from the region encompassed by Israel and The Palestinian Authority; a further group of three strains from a focus just north of the Sea of Galilee, which is also in Israel; and three groups of two strains each all from Asia. The 'strains' MHOM/AZ/1958/OD and MHOM/AZ/1958/NLB305 are two lines of the same Azerbaijani strain, each of which had been kept in a different cryobank for many years, and were identical in all their microsatellites except one, differing in size by one repeat.

Analysis of the 81 genotypes revealed a number of allelic variants (A) that varied between two (GACA4 and Mix9) and 15 (LIST7039), with an average of 5.7 per locus (Table 1). The observed heterozygosity (H_o) ranged from sero to 0.32, indicating the presence of both homozygotic and heterozygotic loci in the population. The expected heterozygosity (H_e) as a measure for genetic diversity ranged from 0.064 to 0.5 and was generally higher than the mean observed. The inbreeding coefficients per locus revealed more positive than negative values, indicating a larger number of homozygotes in the population.

Locus LIST7040 failed to amplify the DNA from four Namibian strains and the Kenyan strain MHOM/KE/1981/NLB162. This can be explained by mutated flanking sites preventing the annealing of the primers rather than by missing alleles.

The number of alleles varied for the different regions investigated. It was high in the group 'Africa' with 88 different alleles (± 5.5), compared to the group 'Asia', with only 51

Table 1
Genetic characteristics and variation of the 21 microsatellite loci detected in the populations of *Leishmania tropica*

Nr.	Locus	A	H_o	H_e	F_{is}
1	GA1	3	0.067	0.049	−0.366
2	GA2	8	0.120	0.366	0.672
3	GA3	8	0.009	0.220	0.960
4	GA6	3	0.000	0.097	1.000
5	GA9	4	0.169	0.298	0.432
6	GA10	3	0.130	0.242	0.462
7	GA11	4	0.000	0.217	1.000
8	4GTG	3	0.000	0.111	1.000
9	GTG1	3	0.214	0.116	−0.851
10	GTG3	5	0.084	0.174	0.517
11	GT4	6	0.067	0.155	0.569
12	LIST7011	8	0.004	0.272	0.985
13	LIST7027	10	0.159	0.453	0.650
14	LIST7010	9	0.065	0.556	0.883
15	LIST7036	7	0.212	0.353	0.398
16	Mix9	2	0.067	0.049	−0.362
17	LIST7039	15	0.181	0.520	0.652
18	LIST7033	5	0.000	0.149	1.000
19	GACA4	2	0.000	0.062	1.000
20	LIST7040	7	0.121	0.127	0.053
21	GM2	4	0.000	0.195	1.000
	Overall	5.7	0.079	0.228	0.651

A, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{is}, inbreeding coefficient.

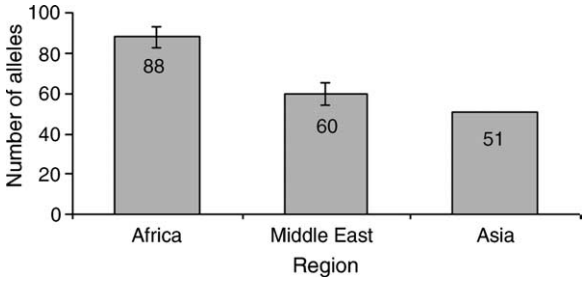

Fig. 1. Allele frequency distribution of the 21 microsatellite loci in different geographical areas. The error bars refer to the re-sampled data in the groups Africa and Middle East.

different alleles. The number of alleles in the group 'Middle East' was 60 (±8.2). Even if the last value had a high deviation, a decreasing number of alleles was seen when moving from Africa via the Middle East to Asia (Fig. 1).

3.2. Population structure

The population structure was determined by analysing the data with the program STRUCTURE. The number of clusters, K, was allowed to vary from one to 15 in independent runs of the algorithm. At $K=10$, the likelihood of the data reached a plateau and individuals had their least partial memberships in multiple clusters (Fig. 2). The distribution of the strains in the clusters corresponded to their geographical origin (Table 2). Both numbers and geographical designations are used in referring to the populations.

After demonstrating that the populations of *L. tropica* were formed by distinct genetic units; the broader genetic relationships among the populations were determined by applying STRUCTURE to the entire set of data, using values of K increasing from two to 10 (Fig. 2). At $K=2$, one cluster was anchored by the populations 'Asia', 'Adana' and 'Morocco A', whereas the other cluster consisted of the populations I, IV and the majority of the African strains. The Namibian strains shared membership with both clusters. At $K=3$, population I separated from the 'African' cluster, which also included the strains in population IV that came from just north of the Sea of Galilee. During the next few steps of the analysis, the smaller sub-populations split off from each other, and populations V and III, 'Morocco A' and 'Adana', respectively, separated from the 'Asia' population II. At $K=6$, the 'Namibia' cluster split from the rest of the African strains and at $K=10$ all the strains were distributed among separate geographical groups with the main percentage of their membership coefficient values in one of the 10 clusters mentioned above.

A neighbour-joining tree constructed using the PAUP software resulted in a similar distribution of the strains (Fig. 3). Three major clades were generated: the first comprising populations II, III and V; the second comprising the populations I, IV, VI, VII, VIII and IX; and the third consisting solely of the Namibian strains. The only deviation from the STRUCTURE results was that strain MHOM/KE/1984/NLB248 of population 'Kenya B' clustered with the strains of 'Kenya A'. Strain MHOM/KE/1981/NLB162 did not fall into any of the ten populations but was closest to, although very distant from, the Namibian strains and the strain *L. major* MHOM/IL/80/Friedlin, that served as the out-group.

A three-dimensional factorial correspondence analysis (FCA) confirmed the distribution of the strains with emphasis of the distinct genetic isolation of the populations 'Middle East', 'Galilee' and 'Morocco B' (Fig. S2). The validity of the estimated population structure of *L. tropica* was further supported by high R_{ST} values. For all pairwise comparisons between the sub-populations, all estimates had higher values than 0.25, indicating strong genetic differentiation (Table S2).

3.3. Genetic diversity and allelic associations

The 'Asia' population was the most heterogeneous of the populations studied, indicated by its having the highest number of alleles per locus, its high values of observed (H_o) and expected (H_e) heterozygosity and its negative inbreeding coefficient (Table 2). Mainly homozygous allele combinations were found at 14 of the 21 loci. Seven loci were heterozygous in all the strains except for the strains from India and the Jenin District that were homozygous at four of these loci. This fixed heterozygosity was accompanied by the over-representation of a particular allelic combination in all of these loci. The other populations were characterized by homozygous microsatellite patterns, indicated by low H_o-values and large inbreeding coefficients.

In this analysis, only loci with one or two different alleles were found, which was compatible with the concept of strains of *L. tropica* being diploid. However, the ploidy of leishmanial organisms is still controversial, and the presence of two alleles at any given locus could be owing to mixtures of genotypes in a host, which seems to be very frequent in parasitic protozoa. Therefore, linkage disequilibrium analysis was favoured for examining associations among loci. Pairwise analyses revealed highly significant linkage disequilibrium for many pairs of polymorphic loci in the populations 'Asia', 'Galilee', 'Morocco A' and 'Namibia' (Table 2). The percentage of linked locus pairs in the population 'Middle East' was lower than in the other populations. Linked locus pairs were not observed in the populations 'Kenya A' and 'Morocco B' that each consisted of only three strains.

3.4. Isolation by distance

The genetic differentiation coincides only slightly with geographical distance ($P<0.05$) owing to the large distances between populations like populations 'Tunisia' and 'Namibia'. Since genetically different populations are geographically very close and even overlap each other, one can assume that geographical distance plays only a minor role in the development of genetic distances (Fig. 4).

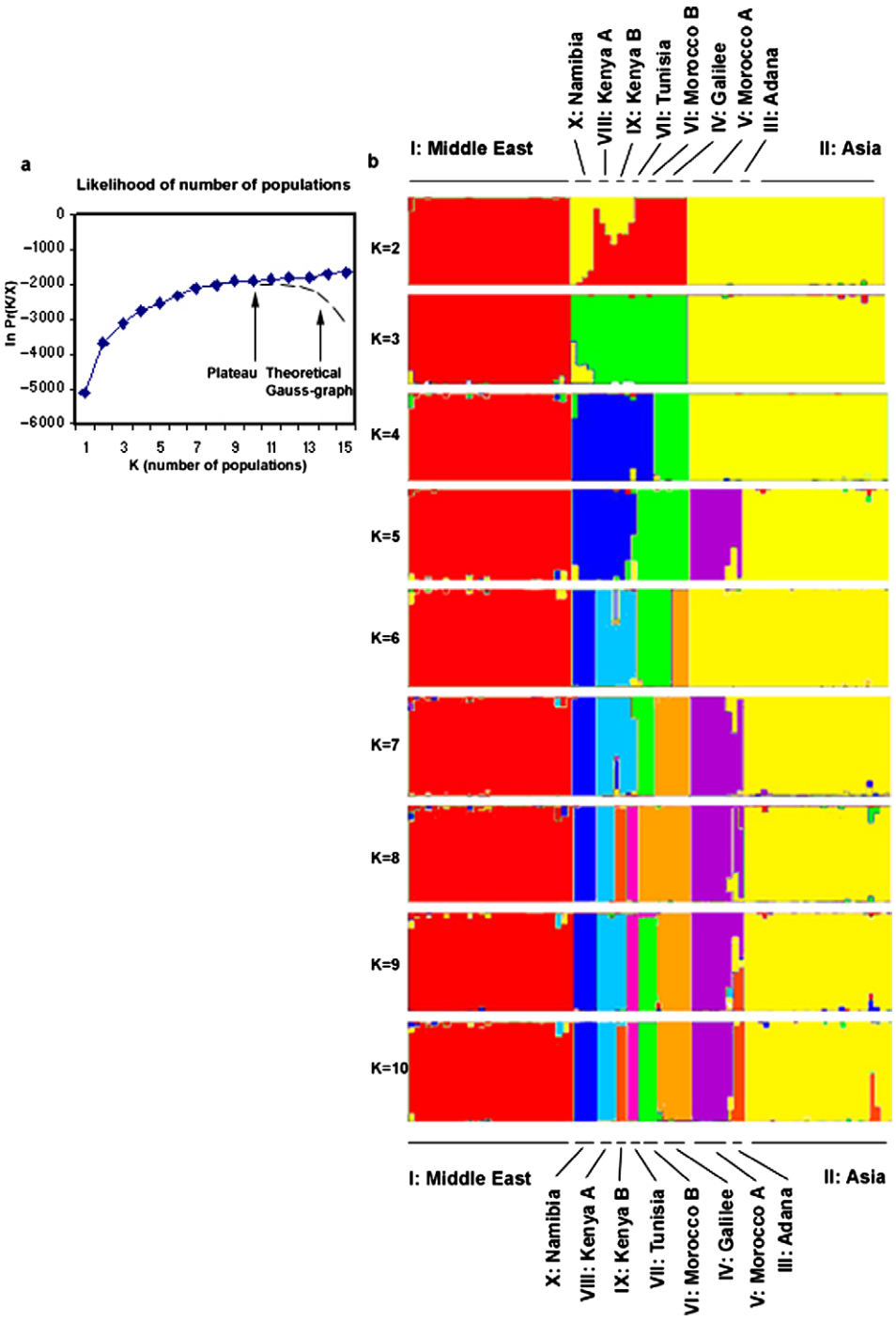

Fig. 2. Population structure of the 117 strains of *Leishmania tropica* determined by STRUCTURE: (a) estimation of the most appropriate population number, (b) averages of 10 STRUCTURE runs at each value of *K* where each strain is represented by a single vertical line divided into *K* colours, *K* being the assumed number of clusters, with each colour representing one cluster and the length of the coloured segment indicating the individual strain's estimated degree of kinship to that cluster. The different colours correspond to the population designations given at the top and the bottom of the depiction.

4. Discussion

The strains of *L. tropica* investigated were from various Old World foci, and their microsatellite diversity suggested distinct genetic composition. However, Schönian et al. (2001) were unable to distinguish geographical populations among some of these strains examined here with older molecular biological methods and isoenzyme analysis except for the Namibian cluster.

The programme STRUCTURE was favoured for analysing the population structure of *L. tropica* because this Bayesian model-based algorithm attempts to identify genetically distinct subgroups on the basis of allelic frequencies (Pritchard et al., 2000; Falush et al., 2003a) without considering prior information like

Table 2
Characterization of the populations of *Leishmania tropica*

Group	Name (C in %)	*N* (S)	Origin	A	H_o	H_e	F_{is}	LD (%)
I	Middle East (100)	33 (28)	Israel, Palestinian Authority	2.14	0.026	0.150	0.830	18
II	Asia (96)	53 (25)	India, Iraq, Israel, Palestinian Authority, Soviet Union, Turkey, Kenya	2.57	0.320	0.260	−0.240	31
III	Adana (100)	2 (2)	Turkey (Adana)	1.19	0.075	0.150	0.500	–
IV	Galilee (100)	8 (6)	Israel (Galilee)	1.57	0.024	0.214	0.880	49
V	Morocco A (87,5)	7 (7)	Morocco	1.67	0.062	0.200	0.685	25
VI	Morocco B (100)	3 (3)	Morocco	1.14	0.032	0.064	0.500	–
VII	Tunisia (100)	2 (2)	Tunisia	1.52	0.025	0.200	0.875	–
VIII	Kenya A (100)	3 (3)	Kenya	1.86	0.174	0.440	0.604	–
IX	Kenya B (100)	2 (2)	Kenya	1.24	0.050	0.500	0.900	–
X	Namibia (100)	4 (4)	Namibia	1.3	0.000	0.183	1.000	40

C, correlation of genetic with geographic clustering; *N*, samples per population, *S*, number of unique genotypes; *A*, mean number of alleles per locus; H_o, mean observed heterozygosity; H_e, mean expected heterozygosity; F_{is}, within population coefficient of inbreeding; LD, Linkage disequilibrium (Percentage of linked locus pairs among polymorphic loci), $P > 0.05$.

geographical details. Furthermore, STRUCTURE uses individual genotypic data more efficiently than programmes based on genetic distance matrices that summarize all information on two populations into a single number (Pritchard et al., 2000; Rosenberg et al., 2001, 2002).

In this study, the number of genetic clusters to which strains were assigned with the highest likelihood was congruent with groups pre-defined by their genetic distances and geographical origins. The validity of the population structure was supported by the Neighbour-Joining tree based on genetic distances calculated from the variation in microsatellite repeat numbers (Fig. 3). Significant differences in heterozygosity among the ten populations might affect the magnitude of genetic differentiation between them. However, consistent results were obtained by integrating Wright's and Slatkin's statistics with Bayesian clustering algorithms.

Although it can be concluded that the genetic differentiation of *L. tropica* follows a geographical pattern, the role of isolation by distance in the evolution of genetically different populations was not overestimated. Distinct populations were found in neighbouring regions like within the Middle East and within Morocco. Considering the quick mutation rate of microsatellite markers in comparison to other genomic markers, time rather than distance is more likely to lead to genetic drift and changes in allelic frequency, thus generating linkage disequilibrium.

The population Asia comprised samples from western Turkey through to and including India that were isolated over a period of 55 years. Although past molecular investigation of strains from this extensive geographical area have revealed a heterogeneous cluster (Schönian et al., 2001), the overall pattern of microsatellite variation appears to have remained relatively stable within the given space and time frame. This and a high level of fixed heterozygosity provide evidence of clonal propagation. Variations of the fixed heterozygote genotypes in some of the heterozygous loci found in a few strains from India, the Jenin District and Turkey can be explained by a loss of alleles. The grouping of two Kenyan strains in the population 'Asia' suggests transfer of the disease by recent human activities.

This microsatellite analysis could not correlate occasional visceralization of *L. tropica* in patients in Israel, India and Iraq (Schnur et al., 1981; Magill et al., 1994; Sacks et al., 1995) with a particular genotype. Notably, all eight of the visceralizing strains of *L. tropica*, a species generally considered to be dermatotropic, fell into the heterologous population Asia. Three of these strains were found in the same branch, three other viscerotropic strains from India did, however, group elsewhere in the Asia population. Three of them, MHOM/KE/1981/NLB029B and MHOM/KE/1981/NLB030B from Kenya and the Iraqi strain MHOM/IQ/1979/Ldj1, grouped together tightly in one branch as did two of the Indian ones, MHOM/IN/1984/C87 and MHOM/IN/1997/K26, in another branch while the other three, MHOM/IL/1949/LRC-L43 from Israel and MHOM/IN/1979/DD7 and MHOM/IN/1991/K112 from India, fitted in independently (Fig. 3).

As mentioned, the 'strains' MHOM/AZ/1958/OD and MHOM/AZ/1958/NLB305 are two lines of the same Azerbaijani strain of *L. tropica*. It was interesting to note that a change had occurred between these two lines in their microsatellite composition during their years of separation. This was in just one of the 21 microsatellite loci, that differed by the expected stepwise mutation. None of the other strains were received in duplicate from different donors of the strains studied. Many laboratories possess different lines of the same strains and comparing these different lines might give insights into the rates of genetic change of leishmanial parasites.

All the other populations appeared more homogeneous and exhibited low heterozygosity. In the Middle East, human CL caused by *L. tropica* has increased markedly over recent years in, both, numbers of cases and the exposure of new endemic foci (Kamhawi et al., 1995; Klaus and Frankenburg,

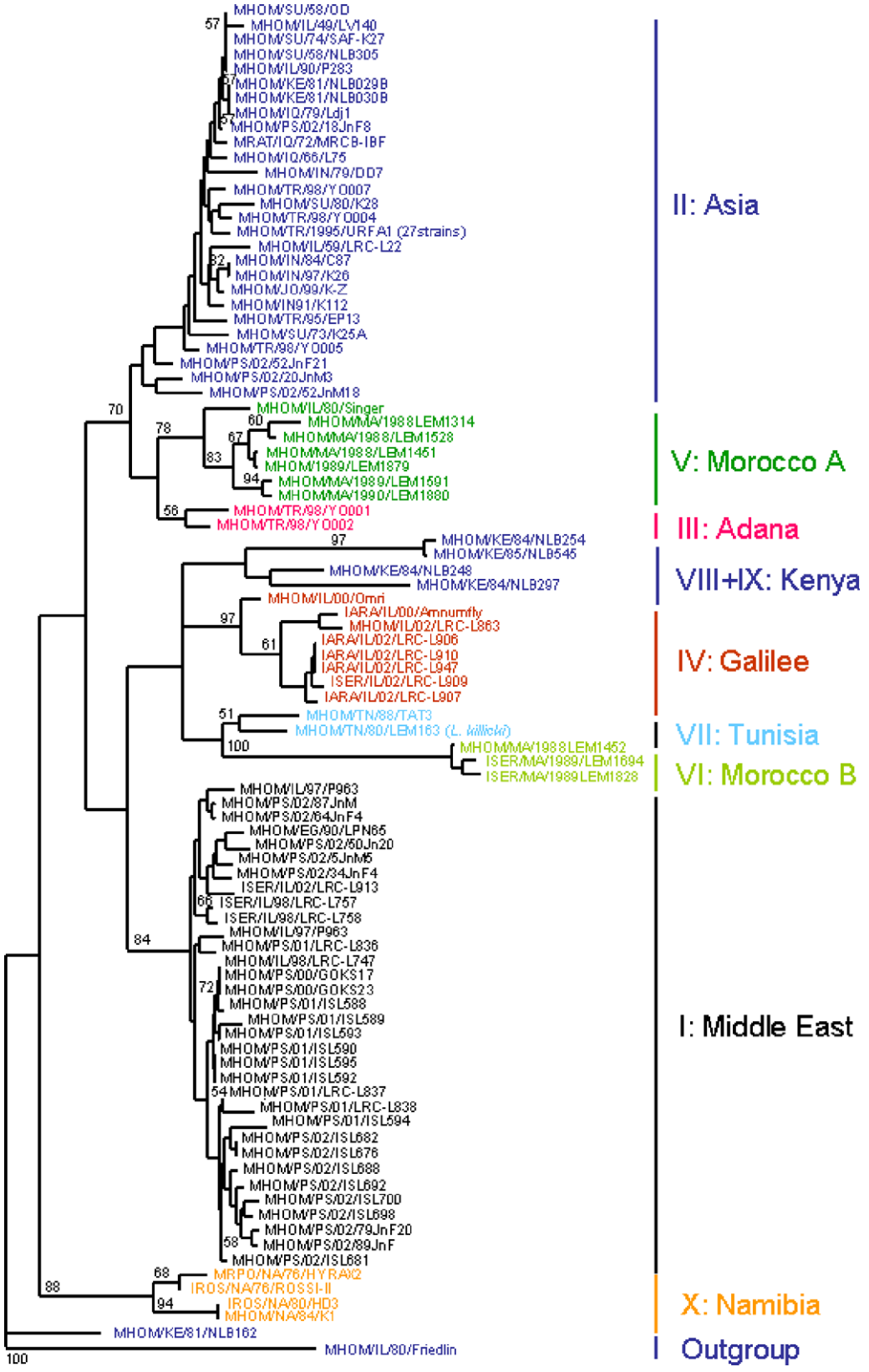

Fig. 3. A neighbour-joining tree of the 117 strains of *Leishmania tropica*, derived from genetic distances based on the proportion of shared alleles among the 21 microsatellites. The numbers on the branches indicate the percentages with which a branch is supported in 1000 bootstrap replications. Only bootstrap values above 50 are shown. For details of the strains see Table S1. For the coloured version see Fig. S1.

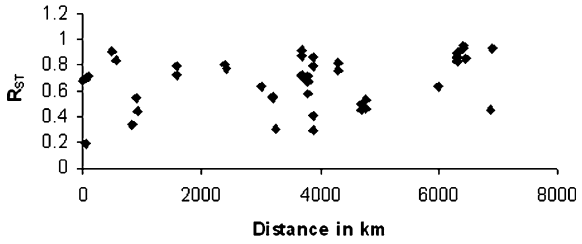

Fig. 4. Relationship between pairwise R_{ST} and geographical distance among the populations of *Leishmania tropica*.

1999; Jacobson et al., 2003; Al-Jawabreh et al., 2004; Schnur et al., 2004). Strains collected there belonged to three different populations. Those isolated between 1949 and 1990, which included four recently isolated from cases domiciled in the Jenin District, clustered in the population 'Asia'; whereas the strains comprising the population 'Middle East', all of which where either from Israel or The Palestinian Authority except for one strain from the Sinai Peninsular, and the population 'Galilee' were all isolated within the last 15 years. Strains from the Jenin District are scattered in both the populations 'Asia' and 'Middle East'. This suggests that new genotypes have spread recently and rapidly in the range of an established population. The emergence of two new separate populations could be owing to two separate founder effects. The new focus of CL in the Galilee is, for example, distinct from all other foci of *L. tropica* described to date. There, *Ph. arabicus* is the main vector, which transmits parasites that are antigenetically, biochemically and molecular biologically different from the other types of strains of *L. tropica* (Jacobson et al., 2003; Schnur et al., 2004) and the hyrax *Procavia capensis* is the putative wild animal reservoir host (Jaffe et al., 2004).

Overlapping anthroponotic and zoonotic life cycles can be anticipated for the different populations in the Middle East as rock hyraces have been incriminated as reservoir hosts of *L. tropica* in the focus just north of the Sea of Galilee (Jacobson et al., 2003; Jaffe et al., 2004). New zoonotic situations might also be occurring in other endemic areas like the vicinity of Jericho, an old well-established focus of human CL caused by *L. major* but where many cases of human CL caused by *L. tropica* were very recently diagnosed (Al-Jawabreh et al., 2004). A similar situation occurs in Morocco, where the charaterization of strains from sand fly vectors, humans and dogs suggested that both zoonotic and anthroponotic transmission cycles are operating in the same area in Morocco (Pratlong et al., 1991; Dereure et al., 1991). Microsatellite analysis revealed that two genetically very distinct populations of *L. tropica* co-exist within the same focus. This matches the considerable isoenzyme variant diversity reported for this region that showed distinct sub-groups of strains which were, in turn, composed of 'small variant' zymodemes (Pratlong et al., 1991). Pratlong et al. (1991) speculated that this old focus was colonized by strains of different geographical origin and that these strains diversified into lesser variants apparently by recent mutation. Our results support this hypothesis as population 'Morocco A' is related to population 'Asia', whereas population 'Morocco B' is genetically closer to the other African populations. The population 'Morocco A' could be the result of the importation of a strain of *L. tropica* from a distant Asian location into Morocco and its establishment and dispersal in the Moroccan focus from which these six human cases came. This is further supported by the clustering of the Israeli strain MHOM/IL/1980/Singer with the six Moroccan strains in population 'Morocco A'. In previous studies, depending on the criteria used to characterize and classify it, this strain grouped with an Indian strain (Schönian et al., 2001), or had been found to be a separate entity (Le Blancq and Peters, 1986; Pratlong F., Lanotte G., Ashford R.W., Rioux J.A., 1986. Le complexe *L. tropica*. A propos de l'analyse numerique de 29 souches identifees par la methode enzymatique. Pp. 129–137 in: *Leishmania*. Taxonomie et Phylogenese. Applications eco-epidemiologiques. (Colloque International CNRS/INSERM/OMS 2-6 Juillet 1984), Montpellier: IMEEE; Van Eys et al., 1989). The neighbour-joining tree reveals that this strain, although genetically close to the six strains in population 'Morocco A', is, in fact, separate from them.

Genetically different strains falling into different populations were also exposed among the strains from the vicinity of Adana, Turkey. Two of them fell into the population 'Asia', forming a micro-cluster together with the strains from Sanliurfa, Turkey. However, the other two strains, MHOM/TR/1998/YO001 and MHOM/TR/1998/YO002, formed the distinct population 'Adana'. This population bridged the populations 'Asia' and 'Morocco A', as is well illustrated by the three-dimensional analysis (Fig. S2). The Turkish strain MHOM/TR/1998/YO007 listed as coming from Izmir, a large city in western Turkey, also fell into the population 'Asia'. This might indicate that the person from whom strain MHOM/TR/1998/YO007 was isolated migrated to Izmir from somewhere further east in Turkey.

The 27 genetically identical strains from Sanliurfa, Turkey, were isolated during an outbreak in 1995. This demonstrates a more practical value of species-specific and strain-specific microsatellite markers in determining autochthonous epidemic outbreaks and distinguishing them from the importation of large numbers of cases into a foreign focus.

Isolated populations might quickly accumulate distinctive allelic repeats as exemplified by the Namibian strains. These strains have been considered a distinct taxonomic group related to *L. tropica* (Rioux et al., 1990). Jacobson (2003) called for a brave taxonomist to define them as a new species. For parasitic microbes, the delimitation of a species is often a matter of convenience, and phenotypic criteria such as epidemiology, clinical symptoms and pathogenicity are used rather than genetics. Our data confirm the results of Schönian et al. (2001) derived genetically and those of Rioux et al. (1990) derived through enzyme electrophoretic variant analysis, that the Namibian strains form a distinct cluster. Linkage disequilibrium and low genetic variation suggest that the Namibian population was affected by strong genetic drift. The STRUCTURE analysis indicated relationship to the African populations 'Kenya A' and 'Kenya B', 'Tunisia' and 'Morocco B'.

Schwenkenbecher et al. (2004) showed that the microsatellite markers used here were specific for *L. tropica*. All but one of them were able to amplify DNA from the Namibian strains, indicating that these were strains of *L. tropica*.

In their taxonomic studies based on isoenzyme analysis, Rioux et al. (1990) grouped Namibian strains together with a Tunisian strain of *Leishmania killicki*, a separate species also belonging to the *L. tropica* complex (Rioux, J. A., Lanotte G., Pratlong F., 1986. *Leishmania killicki* n.sp. (*Kinetoplastidae, Trypanosomatidae*). Pp. 139–142 in: *Leishmania*. Taxonomie et Phylogenese. Applications eco-epidemiologiques. (Colloque International CNRS/INSERM/OMS 2–6 Juillet 1984), Montpellier: IMEEE). Our results are congruent with those of Schönian et al. (2001), who queried whether *L. killicki* is indeed a separate species. The two Tunisian strains examined in this study formed a separate cluster that was genetically quite distant to the other clusters. However, the STRUCTURE results suggested linkage to the other African strains, especially those of population Morocco B.

The high diversity of the Kenyan strains might be related to the age of the focus. The strain *L. tropica* MHOM/KE/1981/NLB162 was somewhat anomalous. Although it formed a genetic cluster with strain MHOM/KE/1984/NLB248 in STRUCTURE, in the neighbour-joining tree it was closest to, but quite distant from, the Namibian strains of *L. tropica* and the strain *L. major* MHOM/IL/80/Friedlin serving as the outgroup. The fact that Mebrahtu et al. (1992) listed the strain MHOM/KE/1981/NLB162 as *L. tropica* sensu lato indicates that, by their enzyme electrophoretic variant analysis, this strain fitted their general description of a strain of *L. tropica* but there was some uncertainty. The species-specificity of the microsatellite-markers used in this study (Schwenkenbecher et al., 2004) support that this strain belongs to the species *L. tropica*. Analysis of more strains is needed to understand the population dynamics of *L. tropica* in Kenya better.

The dispersal of human pathogens has been attributed to human migratory fluxes (Falush et al., 2003b; Wirth et al., 2004). A genetic bottleneck supported by the geography of the Middle Eastern region is compatible with the gradient in the number of alleles. This is suggesting that *L. tropica* spread out from Africa. The current spread of a fixed heterozygous genotype in Asia might indicate that its origin was associated with a hybridization event of two *L. tropica* parasites, which had a strong founder effect and gave rise to a successful variant of *L. tropica*. Neighbour-joining showed paraphyletic grouping of most of the African strains, possibly, because the ancestral population either had not been included in our analysis or had disappeared.

Species-specific microsatellite markers were very effective in discerning variation among strains of *L. tropica* that is correlated with geographical origin and time of isolation. Propagation of *L. tropica* is predominantly clonal and variation is, most probably, driven by simple genetic drift. The analysis described here should be extended by examinating more strains, including more isolates, especially from sand fly vectors and reservoir animals, where they exist, as they are more geographically focalised. This would clarify the extent to which the observed population structure is owing to geographical, ecological and biological barriers.

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Appendix. Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2005.09.010

References

Al-Jawabreh, A., Schnur, L.F., Nasereddin, A., Schwenkenbecher, J.M., Abdeen, Z., Barghuthy, F., Khanfar, H., Presber, W., Schönian, G., 2004. The recent emergence of *Leishmania tropica* in Jericho and its environs, a classical focus of *L. major*. Trop. Med. Int. Health 9, 1–5.

Al-Zahrani, M.A., Peters, W., Evans, D.A., Chin, C., Smith, V., Lane, R.P., 1988. *Phlebotomus sergenti*, a vector of *Leishmania tropica* in Saudi Arabia. Trans. R. Soc. Trop. Med. Hyg. 82, 416.

Bulle, B., Millon, L., Bart, J.M., Gallego, M., Gambarelli, F., Portus, M., Schnur, L., Jaffe, C.L., Fernandez-Barredo, S., Allunda, J.M., Piarroux, R., 2002. Practical approach for typing strains of *Leishmania infantum* by microsatellite analysis. J. Clin. Microbiol. 40, 3391–3397.

Chambers, G.K., MacAvoy, E.S., 2000. Microsatellites: consensus and controversy. Comp. Biochem. Physiol. B. 126, 455–476.

Dawson, K.J., Belkhir, K., 2001. A Bayesian approach to the identification of panmictic populations and the assignment of individuals. Genet. Res. 78, 59–77.

Dereure, J., Rioux, J.A., Gallego, M., Perieres, J., Pratlong, F., Mahjour, J., Saddiki, H., 1991. *Leishmania tropica* in Morocco: infection in dogs. Trans. R. Soc. Trop. Med. Hyg. 85, 595.

Falush, D., Stephens, M., Pritchard, J.K., 2003a. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164, 1567–1587.

Falush, D., Wirth, T., Linz, B., Pritchard, J.K., Stephens, M., Kidd, M., Blaser, M.J., Graham, D.Y., Vacher, S., Perez-Perez, G.I., Yamaoka, Y., Megraud, F., Otto, K., Reichard, U., Katzowitsch, E., Wang, X., Achtmann, M., Suerbaum, S., 2003b. Traces of human migrations in *Helicobacter pylori* populations. Science 299, 1582–1585.

Gebre-Michael, T., Balkew, M., Ali, A., Ludovisi, A., Gramiccia, M., 2004. The isolation of *Leishmania tropica* and *L. aethiopica* from *Phlebotomus (Paraphlebotomus)* species (Diptera: Psychodidae) in the Awash Valley, northeastern Ethiopia. Trans. R. Soc. Trop. Med. Hyg. 98, 64–70.

Guessous-Idrissi, N., Chiheb, S., Hamdani, A., Riyad, M., Bichichi, M., Hamdani, S., Krimech, A., 1997. Cutaneous leishmaniasis: an emerging epidemic focus of *Leishmania tropica* in north Morocco. Trans. R. Soc. Trop. Med. Hyg. 91, 660–663.

Guilvard, E., Rioux, J.A., Gallego, M., Pratlong, F., Mahjour, J., Martinez-Ortega, E., Dereure, J., Saddiki, A., Martini, A., 1991. *Leishmania tropica* in Morocco. III—The vector of *Phlebotomus sergenti*. Apropos of 89 isolates. Ann. Parasitol. Hum. Comp. 66, 96–99.

Jacobson, R.L., 2003. *Leishmania tropica* (Kinetoplastida: Trypanosomatidae)—a perplexing parasite. Folia Parasitol. (Praha) 50, 241–250.

Jacobson, R.L., Eisenberger, C.L., Svobodova, M., Baneth, G., Sztern, J., Carvalho, J., Nasereddin, A., El Fari, M., Shalom, U., Volf, P., Votypka, J.,

Dedet, J.P., Pratlong, F., Schoenian, G., Schnur, L.F., Jaffe, C.L., Warburg, A., 2003. Outbreak of cutaneous leishmaniasis in northern Israel. J. Infect. Dis. 188, 1065–1073.

Jaffe, C.L., Perez, M.L., Sarfstein, R., 1990. *Leishmania tropica*: characterization of a lipophosphoglycan-like antigen recognized by species-specific monoclonal antibodies. Exp. Parasitol. 70, 12–24.

Jaffe, C.L., Baneth, G., Abdeen, Z.A., Schlein, Y., Warburg, A., 2004. Leishmaniasis in Israel and the Palestinian Authority. Trends Parasitol. 20, 328–332.

Jamjoom, M.B., Ashford, R.W., Bates, P.A., Kemp, S.J., Noyes, H.A., 2002. Towards a standard battery of microsatellite markers for the analysis of the *Leishmania donovani* complex. Ann. Trop. Med. Parasitol. 96, 265–270.

Jamjoom, M.B., Ashford, R.W., Bates, P.A., Chance, M.L., Kemp, S.J., Watts, P.C., Noyes, H.A., 2004. *Leishmania donovani* is the only cause of visceral leishmaniasis in East Africa; previous descriptions of *L. infantum* and *L. archibaldi* from this region are a consequence of convergent evolution in isoenzyme data. Parasitology 129, 399–409.

Johnson, R.N., Ngumbi, P.M., Robert, L.L., Anjili, C.O., Killick-Kendrik, R., Meredith, S.E.O., 1993. Phlebotomine sandflies of Kenya (Diptera: Psychodidae) II. *Phlebotomus aculeatus* as a probable vector of *Leishmania tropica* s.l.. Ann. Trop. Med. Parasitol. 87, 541–544.

Johnson, R.N., Lawyer, P.G., Ngumbi, P.M., Menrahtu, Y.B., Mwanyumba, J.P., Mosonik, N.C., Makasa, S.J., Githure, J.I., Roberts, C.R., 1999. Phlebotomine sand fly (Diptera: Psychodidae) seaonal distribution and infection rates in a defined focus of *Leishmania tropica*. Am. J. Trop. Med. Hyg. 60, 854–858.

Kamhawi, S., Abdel-Hafez, S.K., Arbagi, A., 1995. A new focus of cutaneous leishmaniasis caused by *Leishmania tropica* in northern Jordan. Trans. R. Soc. Trop. Med. Hyg. 89, 255–257.

Killick-Kendrick, R., Killick-Kendrick, M., Tang, Y., 1995. Anthroponotic cutaneous leishmaniasis in Afghanistan: The high susceptibility of *Phlebotomus sergenti* to *Leishmania tropica*. Trans. R. Soc. Trop. Med. Hyg. 89, 477.

Klaus, S., Frankenburg, S., 1999. Cutaneous leishmaniasis in the Middle East. Clin. Dermatol. 17, 137–141.

Klaus, S., Axelrod, O., Jonas, F., Frankenburg, S., 1994. Changing patterns of cutaneous leishmaniasis in Israel and neighbouring territories. Trans. R. Soc. Trop. Med. Hyg. 88, 649–650.

Lawyer, P.G., Mebrahtu, Y.B., Ngumbi, P.M., Mwanyumba, P., Mbugua, J., Kiilu, G., Kipkoech, D., Nzovu, J., Anjili, C.O., 1991. *Phlebotomus guggisbergi* (Diptera: Psychodidae), a vector of *Leishmania tropica* in Kenya. Am. J. Trop. Med. Hyg. 44, 290–298.

Le Blancq, S.M., Peters, W., 1986. *Leishmania* in the Old World: heterogeneity among *L. tropica* zymodemes. Trans. R. Soc. Trop. Med. Hyg. 80, 113–119.

Magill, A.J., Grogl, M., Johnson, S.C., Gasser Jr.., R.A., 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.

Mebrahtu, Y.B., Lawyer, P.G., Pamba, H., Koech, D., Perkins, P.V., Roberts, C.R., Were, J.B., Hendricks, L.D., 1992. Biochemical characterization and zymodeme classification of *Leishmania* isolates from patients, vectors, and reservoir hosts in Kenya. Am. J. Trop. Med. Hyg. 47, 852–892.

Pratlong, F., Rioux, J.A., Dereure, J., Mahjour, J., Gallego, M., Guilvard, E., Lanotte, G., Perieres, J., Martini, A., Saddiki, A., 1991. *Leishmania tropica* in Morocco. IV–Intrafocal enzyme diversity. Ann. Parasitol. Hum. Comp. 66, 100–104.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

Reithinger, R., Mohsen, M., Aadil, K., Sidiqi, M., Erasmus, P., Coleman, P.G., 2003. Anthroponotic Cutaneous Leishmaniasis, Kabul, Afghanistan. Em. Inf. Dis. 9, 727–729.

Rhajaoui, M., Fellah, H., Pratlong, F., Dedet, J.P., Lyagoubi, M., 2004. Leishmaniasis due to *Leishmania tropica* MON-102 in a new Moroccan focus. Trans. R. Soc. Trop. Med. Hyg. 98, 299–301.

Rioux, J.A., 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.

Rosenberg, N.A., Burke, T., Elo, K., Feldman, M.W., Freidlin, P.J., Groenen, M.A., Hillel, J., Maki-Tanila, A., Tixier-Boichard, M., Vignal, A., Wimmers, K., Weigend, D., 2001. Empirical evaluation of genetic clustering methods using multilocus genotypes from 20 chicken breeds. Genetics 159, 699–713.

Rosenberg, N.A., Pritchard, J.K., Weber, J.L., Cann, H.M., Kidd, K.K., Zhivotovsky, L.A., Feldman, M.W., 2002. Genetic structure of human populations. Science 298, 2381–2385.

Rousset, F., Raymond, M., 1995. Testing heterozygote excess and deficiency. Genetics 140, 1413–1419.

Russell, R., Pilar Iribar, M., Lambson, B., Brewster, S., Blackwell, J.M., Dye, C., Ajioka, J.W., 1999. Intra and inter-specific microsatellite variation in the *Leishmania* subgenus Viannia. Mol. Biochem. Parasitol. 103, 71–77.

Sacks, D.L., Kenney, R.T., Kreutzer, R.D., Jaffe, C.L., Gupta, A.K., Sharma, M.C., Sinha, S.P., Neva, F.A., Saran, R., 1995. Indian kala-azar caused by *Leishmania tropica*. Lancet 345, 959–961.

Sang, D.K., Njeru, W.K., Ashford, R.W., 1994. A zoonotic focus of cutaneous leishmaniasis due to *Leishmania tropica* at Utut, Rift Valley Province, Kenya. Trans. R. Soc. Trop. Med. Hyg. 88, 35–37.

Schnur, L.F., Greenblatt, C.L., 1995. Leishmania. In: Kreier, J.P. (Ed.), Parasitic Protozoa. Academic Press, San Diego, pp. 1–160.

Schnur, L.F., Chance, M.L., Ebert, F., Thomas, S.C., Peters, W., 1981. The biochemical and serological taxonomy of visceralizing *Leishmania*. Ann. Trop. Med. Parasitol. 75, 131–144.

Schnur, L.F., Nasereddin, A., Eisenberger, C.L., Jaffe, C.L., El Fari, M., Azmi, K., Anders, G., Killick-Kendrick, M., Killick-Kendrick, R., Dedet, J.P., Pratlong, F., Kanaan, M., Grossmann, T., Jacobson, R.L., Schoenian, G., Warburg, A., 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.

Schönian, G., Schweynoch, C., Zlateva, K., Oskam, L., Kroon, N., Gräser, Y., Presber, W., 1996. Identification and determination of the relationships of species and strains within the genus *Leishmania* using single primers in the polymerase chain reaction. Mol. Biochem. Parasitol. 77, 19–29.

Schönian, G., Schnur, L.F., El Fari, M., Oskam, L., Kolesnikov, A.A., Sokolowska-Köhler, 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.

Schwenkenbecher, J.M., Fröhlich, C., Gehre, F., Schnur, L.F., Schönian, G., 2004. Evolution and Conservation of microsatellite markers for *Leishmania tropica*. Infect. Genet. Evol. 4, 99–105.

Sunnucks, P., 2000. Efficient genetic markers for population biology. Trends Ecol. Evol. 15, 199–203.

Toth, G., Gaspari, Z., Jurka, J., 2000. Microsatellites in different eukaryotic genomes: survey and analysis. Genome Res. 10, 967–981.

Van Eys, G.J., Schoone, G.J., Ligthart, G.S., Alvar, J., Evans, D.A., Terpstra, W.J., 1989. Identification of 'Old World' *Leishmania* by DNA recombinant probes. Mol. Biochem. Parasitol. 34, 53–62.

Wirth, T., Bernatchez, L., 2001. Genetic evidence against panmixia in the European eel. Nature 409, 1037–1040.

Wirth, T., Wang, X., Linz, B., Novick, R.P., Lum, J.K., Blaser, M., Morelli, G., Falush, D., Achtman, M., 2004. Distinguishing human ethnic groups by means of sequences from *Helicobacter pylori*: lessons from Ladakh. Proc. Natl Acad. Sci. USA 101, 4746–4751.

Wright, S., 1969. Evolution and the Genetics of Natural Populations, vol. 2. The University of Chicago Press, Chicago.

Wright, S., 1978. Evolution and the genetics of populations. Variability within and among Natural Populations, vol. 4. The University of Chicago Press, Chicago.

Yaghoobi-Ershadi, M.R., Hanafi-Bojd, A.A., Javadian, E., Jafari, R., Zahraei-Ramazani, A.R., Mohebali, M., 2002. A new focus of cutaneous leishmaniasis caused by *Leishmania tropica*. Saudi Med. J. 23, 291–294.