

The origin and evolution of the *Leishmania donovani* complex as inferred from a mitochondrial cytochrome oxidase II gene sequence[☆]

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Received 15 January 2001; received in revised form 2 April 2001; accepted 24 April 2001

Abstract

Members of the *Leishmania donovani* complex are parasites of the reticulo-endothelial system that are often associated with serious epidemics of a life threatening disease known as visceral leishmaniasis or kala-azar. Twenty-two *Leishmania* isolates representative of the geographical range of the parasite were analysed for sequence variations in their cytochrome oxidase II gene. In performing phylogenetic analysis, the maximum parsimonious, neighbour joining and maximum likelihood trees were congruent and produced a tree that differentiated between two clades conforming to the current classification of the species complex into two species: *Leishmania donovani* and *Leishmania infantum*. Furthermore, the molecular haplotypes were concordant, in general, with the isoenzyme data of the complex. The *donovani* isolates from the Sudan that possessed the most ancestral sequence were of a single haplotype that significantly resembled the sequence of *Leishmania major*. Our sequence data tallied with a general neutral model of sequence evolution with manifestations of weak selection. The data allowed an approximate dating of the origin of the complex to a period contemporary to or predating the spread of modern humans out of Africa. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Leishmania*; Cytochrome oxidase; Phylogeny; Evolution

1. Introduction

The leishmaniasis are a group of vector born diseases caused by parasites of the genus *Leishmania*. They are obligatory dwellers in their mammalian host and if they do not cause fatal disease may remain infecting their host for life. The visceral, often fatal, form of leishmaniasis known as kala-azar is caused by parasites of the *Leishmania donovani* species complex, which includes *L. donovani* and *Leishmania infantum* in the Old World and *Leishmania chagasi* in the New World. During the eighties and nineties kala-azar has made a resurgence in countries like India and Sudan, claiming thousands of human lives. The outbreaks in Sudan have been particularly severe and parasites involved in these outbreaks have received considerable investigation (Ashford et al., 1992; Ibrahim et al., 1995; Andresen et al., 1996). Both RAPD (Andresen et al., 1996) and isoenzyme (Ashford et al., 1992; Ibrahim et al., 1995) analysis provided evidence

for a close genetic relationship of the *L. donovani* parasite and thus of a recent expansion of the particular genotypes involved in the Sudanese outbreaks. Evidence of immunogenetic factors that predispose human populations to the disease is not yet conclusive, but population studies in Brazil and elsewhere are underway (Blackwell et al., 1997).

Several studies have addressed the evolution and phylogenetic relationships between the *Kinetoplastidae*, the group that includes among other parasites those of the genus *Trypanosoma* and *Leishmania* (Lake et al., 1988; Landweber and Gilbert, 1994; Maslov et al., 1994; Croan et al., 1997; Noyes et al., 1997; Brewster and Barker, 1999; Mauricio et al., 1999; Tibayrenc and Ayala, 1999; Stevens et al., 1999).

Sequence information on the mitochondrial genome of the *Kinetoplastidae* is accumulating largely from studies on the editing phenomenon, a post-transcriptional process known to take place among the *Kinetoplastidae* (Stuart, 1989; Simpson and Shaw, 1989; Benne, 1989). Based on this sequence information, we designed PCR primers and analysed 540 bp of the COII gene of *L. donovani* in an attempt to re-construct the genetic history and evolution of this important group of parasites.

[☆] Sequences used in this study are deposited in Genbank accession numbers M10126, AF151632 and AF287688-AF287696.

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2. Materials and methods

Eleven parasite strains representing the geographical range of distribution of *L. donovani* worldwide and their natural host profiles were studied, in addition, 11 *L. donovani* isolates from Sudan (four from southern Sudan and seven from the eastern Sudan) were grown in culture or DNA directly obtained from Dr. Keld Andresen (CMP, Copenhagen) or Dr. Bronwen Lambson (University of Cambridge). Following DNA extraction according to the method of Morel et al. (1980), kinetoplast DNA was used in PCR assays. The design of our primers was based on the alignment of *Leishmania tarentolae* and *Trypanosoma equiperdum* COII gene sequences from the Genbank databases. For amplification of the cytochrome oxidase gene the following pair of primers were used:

Forward 5'-ATGGCTTTTATATTATCA-3'
Reverse 5'-GGCATAAATCCATGTAAG-3'

The PCR conditions were 35 cycles of denaturing at 95°C for 15 s annealing at 50°C for 45 s, extension at 72°C for 1 min. The PCR products were purified using gel extraction kits (Quieten, UK) and sequenced using the ABI PRISM Dye Terminator sequencing kit. Reaction products were run on ABI 373A automated sequencer (Applied Biosystems).

The same pair of primers was used to sequence the PCR product from both directions and mutations were confirmed by examining overlapping sequences.

Sequences were manipulated using the computer program DNASTAR. Alignment was performed using the program PILEUP or by hand, formatted to the GCG format and analysed using the PHYLIP (Phylogeny Inference Package), Version 3.5 (Felsenstein, 1993).

Trees were constructed using the programs DNAML and DNAPAR using the methods of maximum parsimony and neighbour joining. To generate a consensus tree, 100 alignment pseudoreplicates were created with the bootstrap option Seqboot and Consense of the PHYLIP package. Another haplotype tree was constructed using the program TCS 1.0 alpha developed by Mark Clement Posada and Keith Crandall based on a form of statistical parsimony originally developed by Templeton et al. (1992) to resolve sequences that differ by few parsimonious sites.

3. Results

3.1. Sequence analysis of the COII gene

A total of 540 nucleotide sites were considered for the analysis out of the entire length of the COII gene which is ~630 nucleotides in *L. tarentolae*. As expected the terminal 3'- and 5'-ends are highly conserved, particularly the 3'-end where the edited domain lies. We sequenced a total of 22 *L. donovani* isolates, 2 *L. major* and compared those to a single *L. tarentolae* sequence retrieved from the

Genbank databases. Sequences were aligned using the program PILEUP of the GCG package and by hand. The novel sequences are deposited in Genbank under the accession numbers M10126, AF151632 and AF287688-AF287696.

3.2. Test of neutrality

To evaluate the conformity of our data to a neutral model of sequence evolution (Kimura, 1983) we utilised the approach of Templeton (1996) which employs a simple contingency test to evaluate evolutionary trends and to test for neutrality.

We started by evaluating the extent of nucleotide bias in the different lineages sketched in Fig. 1 on the basis of determining the fixed mutations on each species sequence. In general there was no significant difference in the nucleotide content between the three species, however, a difference in the direction of substitutions was noted between the two main evolutionary lineages (the *tarentolae* to *major/donovani* and the *tarentolae/major* to *donovani*) drawn in Fig. 1. The difference was in the form of a nucleotide bias and was most pronounced in the transition from *major/tarentolae* to *donovani*. A general bias of A to G and T to C in this lineage versus G to A and C to T on the *tarentolae* to *major* lineage (Table 1) was significant by a one tailed Fisher exact test (FET) probability (AG, GA, $P = 0.038$; TC, CT, $P = 0.007$).

Table 2 gives the distribution of two types of substitutions (transitions and transversions) on the first, second and third codon positions along the various evolutionary lines identified in Fig. 1. The results indicate a general transition

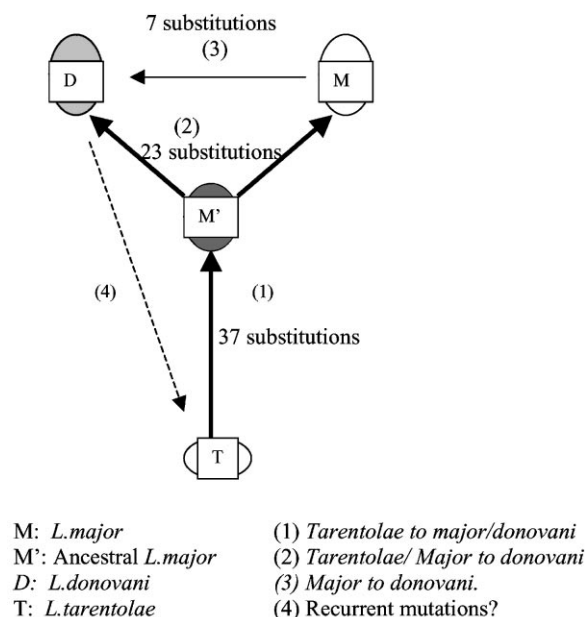


Fig. 1. Schematic figure of the three hypothetical evolutionary lineages under study, the number of substitutions in each lineage and possible recurrent mutations.

Table 1

The number and direction of substitutions (transitions or transversions) from one nucleotide to another along the three main evolutionary lineages identified in Fig. 1^a

	<i>tarentolae, major</i> → <i>donovani</i>	<i>tarentolae</i> → <i>major, donovani</i>	<i>donovani tarentolae</i> → <i>major</i>
A → T	1	9	2
A → G	11	4	3
A → C	1	1	0
T → A	1	6	1
T → G	1	1	0
T → C	5	0	1
G → A	1	3	0
G → T	0	2	0
G → C	0	0	0
C → A	0	1	0
C → T	1	8	1
C → G	0	0	0
Total	22	35	8

^a The shaded rows indicate where the putative trends are observed (AG, GA, $P = 0.038$; TC, CT, $P = 0.007$).

Table 2

The type of substitutions in the three main evolutionary lineages and recurrent substitutions classified by their position in codon and as transversions or transitions (only two polymorphic changes in codon third position were noted)

Direction of sequence evolution	Position in codon					
	1		2		3	
	N	V	N	V	N	V
T → MD	3	2		2	7	19
TM → D	2	0	1	0	15	4
TD → M	0	0	1	0	6	3
M → D	2	0	0	0	5	0

bias and a rate of evolution of first, second and third codon position $k_2 < k_1 < k_3$. Table 3 shows the total number of silent versus replacement substitutions and transitions versus transversions in the three evolutionary lineages and the contingency analysis of the differences in these parameters between the three lineages. The null hypothesis for homogeneity was rejected only at the *tarentolae* to *major/donovani* lineage (FET, $P = 0.0001$).

Table 3

Number of silent vs. replacement substitutions and transition vs. transversions in the three main evolutionary lineages^a

	Silent	Replacement	FET	Transition	Transversion	FET
T → MD	26	9	0.289	12	23	0.0001
TM → D	19	3	0.36	18	4	0.407
M → D	5	2		7	0	

^a The contingency analysis of difference in mutational changes between lineages is shown in bold (calculated by FET).

3.3. Phylogenetic inference and trees of the *donovani* complex under different assumptions

The three trees constructed by maximum parsimony, maximum likelihood and neighbour joining did not differ in any significant manner in their topology. The position of the node in Fig. 2 leading to the *L. infantum* group is a replacement mutation altering the amino acid number 28 from valine to isoleucine. However, in the tree drawn by the program Consense of the PHYLIP package shown in Fig. 2 (branch lengths not proportional to distance), the bootstrap values did not support this node. In contrast, there was strong support in the consensus tree towards the root of the tree. This might be due to the limited number of parsimony sites. As mentioned earlier, the A + T/G + C ratios are not significantly different between the three evolutionary lineages of the genus *Leishmania* studied here, thus it has no bearing on the parsimony of the consensus tree drawn for *donovani* and *major*. All trees were rooted using the species *Leishmania major* as an out-group except for the haplotype tree constructed by the program TCS 1.0, where the program was left to position the root, placing it at the haplotype from the Sudan (Fig. 3). The algorithm used in this program is based on a parsimony statistic developed by Templeton (1996) for the resolution of sequences that differ by fewer parsimony sites.

The *L. major* haplotype fell into a branch that included parasites of the *L. donovani* species. In fact, the sequence of *L. major* showed particular similarity to the *donovani* haplotype from Sudan. The first split in the tree was of this haplotype (Sudan). It was followed by the split of the lineage containing the isolates from India, Kenya and Saudi Arabia and then the split of the *infantum* group. The *tangil* isolate from Bangladesh had an intermediate haplotype displaying characters of both *L. donovani* and *L. infantum*. The *infantum* “clade” included isolates from Europe and Asia as well as isolates from Ethiopia and Kenya.

The divergence time for the main lineages was estimated. The universal mutation rate (μ) for mitochondria DNA has been estimated as 2% per site per million years or 2×10^{-8} per site per year. Using this high mutation rate we calculated the time of coalescence of the *donovani* with the *major* lineage to be around 500 kya and the separation of the *infantum* lineage from the *donovani* to be around 250 kya.

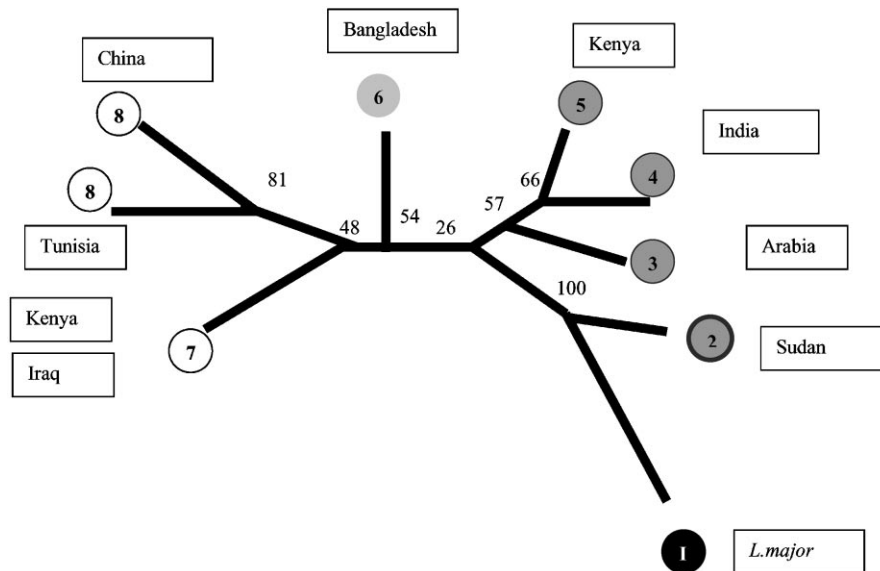


Fig. 2. Phylogenetic tree based on the COII sequence of *L. donovani* parasites from various geographical areas representing the natural global range of the parasite in addition to a *L. major* strain originally taken as an out-group. The tree is rooted using the same *L. major* sequence. The various haplotypes are designated numbers from 1 to 8. The different grades in shading indicate the biological and isoenzyme assignment of the parasites into *L. donovani* (dark grey) and *L. infantum* (white). The intermediate haplotype of the strain Tangil from Bangladesh is in (light grey) and the parasite *L. major* in black. The numbers at the forks indicate the number of times the groups at the top of that fork occurred out of 100 trees.

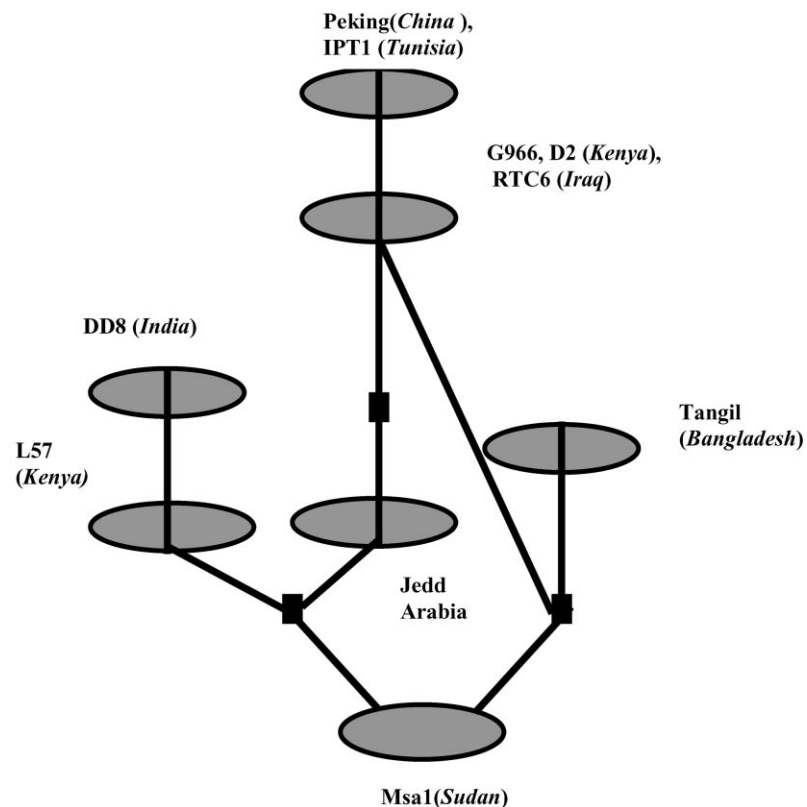


Fig. 3. Haplotype tree constructed using the program TCS 1.0. Sequence file was fed for analysis and constructing of a parsimonious tree. Tree branches based on haplotype distance data were supported at 95% confidence limits.

Table 4

The specific code of 10 of the isolates used in the study representing the geographical range of the *L. donovani* parasites, their natural host profile, their isoenzyme code as designated by the London School of Tropical Medicine and their mitochondrial haplotype obtained in this study designated Latin numbers^a

Code	Mitochondrial haplotype	Subspecies	Zymodeme ^b	Subspecies	Origin
HOM/IN/80/DD8	IV	<i>donovani</i>	LON41	<i>donovani</i>	India
HOM/BD/93/Tangil	VI	<i>infantum</i>	LON41	<i>donovani</i>	Bangladesh
HOM/SA/81/JEDDAH	V	<i>donovani</i>	LON42	<i>donovani</i>	Arabia
HOM/IQ/00/RTC-6	VIII	<i>infantum</i>	LON43	<i>infantum</i>	Iraq
IMAR/KE/62/LRC-L57	III	<i>donovani</i>	LON44	<i>donovani</i>	Kenya
CAN/KE/00/D2	VIII	<i>infantum</i>	LON45	<i>infantum</i>	Kenya
HOM/SD/95/MSA ^c	II	<i>donovani</i>	LON48	<i>donovani</i>	Sudan
HOM/TN/80/IPT1	VII	<i>infantum</i>	LON49	<i>infantum</i>	Tunisia
HOM/CN/54/PEKING	VII	<i>infantum</i>	LON49	<i>infantum</i>	China
HOM/KE/79/GG966	VIII	<i>infantum</i>	LON49	<i>infantum</i>	Kenya

^a The species classification of each strain as deduced from isoenzymes or mtDNA haplotype is shown for each method. The code refers to the host from which the parasite is isolated, the country, year and the special numbers or letters used in the original identification of the isolate. The country or area from which the parasite was isolated is indicated as "origin".

^b The zymodeme data are from Le Blancq and Peters (1986) and other references.

^c Other strains from Sudan as in Ibrahim et al. (1995) were included, as well as freshly obtained isolates.

Assuming a similar rate for the separation of the *major* lineage from the *tarentolae* we estimated the divergence time for the *tarentolae* sequence (ignoring the possible difference in the rate of evolution between the parasites infecting reptiles and mammals) to be around 5.5 mya. Under an alternative hypothetical low mutation rate of 10^{-8} the figures were brought to 250 and 120 kya for the *donovani*–*major* and *infantum*–*donovani* divergence, respectively.

3.4. Concordance between the isoenzyme patterns and DNA haplotypes

The relation of the isoenzyme assignment of the isolates studied, to their mitochondrial haplotypes is shown in Table 4. Concordance was pronounced at the level of the species but not for each isolate separately (i.e. all the *infantum* zymodemes fell under one or another of the relevant mitochondria haplotypes and vice versa). There were a total of eight isoenzyme profiles and six corresponding haplotypes.

4. Discussion

We employed a sequence of the cytochrome oxidase II gene one of the genes commonly used in phylogenetic studies from three *Leishmania* species, *L. donovani*, *L. major* and *L. tarentolae*. The last two species were employed as out-groups and as an aid in understanding the sequence evolution of the *donovani* complex, the main subject of our study. *L. major* is evolutionary close to *L. donovani* as shown by isoenzymes (Le Blancq and Peters, 1986; Rioux et al., 1990) and DNA markers. *L. tarentolae*, by contrast, has been considered by some investigators to belong to a separate genus, *Sauroleishmania* (see Lainson and Shaw, 1987).

Although there is no standard value by which a species pairs should differ, the amount of sequence difference

between *donovani* and *tarentolae* (11%) should probably place those two at the threshold of the genus boundaries. Some authors gave estimates of an average of 0.2–0.5% in mitochondrial DNA (Klicka and Zink, 1997) for recently diverging species to differ in their sequence.

4.1. Phylogenetic analysis and test of neutrality

One way of evaluating the reliability of our phylogenetic inference is to test whether the evolution of the sequence we study conforms to the neutral model of sequence evolution. Performance of a neutrality test is becoming mandatory to define the evolutionary questions at stake (Williams et al., 1995; Wayne and Simonsen, 1998). In addition to measuring the extent of nucleotide bias and the direction of evolution in each lineage we calculated the probability values for the contingency analysis of the various types of substitutions between the *tarentolae* and *major* and *donovani*. Our data tallied with a general neutral model of sequence evolution in observing a transition bias and an increasing rate of evolution of second, first and third codon positions. We employed simple tests of neutrality based on the contingency analysis of neutrality proposed by Templeton (1996) comparing the transition: transversion ratios along the three main evolutionary lineages and the degree of deviation from the neutral model assumptions.

The rejection of these assumptions on one lineage (*tarentolae* to *major/donovani*) the oldest of the three lineages may well be due to saturation of third codon positions and factors pertaining to the relatively large evolutionary distance separating *tarentolae* from the other two species. Interestingly, we observed a bias in the codon usage towards GC rich sequence when comparing the type of substitutions along the various evolutionary lineages. Such a feature, indicating a subtle evolutionary trend towards CG rich mitochondrial genes could be discussed within a model of

weak selection (Wayne and Simonsen, 1998) rather than an outright rejection of neutrality.

The phylogenetic inference was robust as depicted in the topology of the different trees. The problem of the limited parsimony sites which was reflected in weak bootstrap values in a couple of nodes was overcome by employing a parsimony statistic designed to address such problems (Templeton, 1996) and shown in the haplotype tree which was concordant with the previous analysis.

4.2. Concordance of isoenzyme and biology with mitochondrial haplotypes

Though our mitochondria tree received marked support from previous isoenzyme data in which the separation between *donovani* and *infantum* has been reaffirmed, a direct concordance between isoenzymes and mitochondrial DNA variation was not always possible. It is possible that that isoenzyme loci are evolving faster than the mitochondrial COII sequence as suggested by isolates from Sudan, which possess a single mitochondrial haplotype but show more than one isoenzyme pattern.

A striking feature shown in the sequence alignment and now in the trees is the difference of the *donovani* haplotype from Sudan from other *donovani* haplotypes and its resemblance to *L. major*. The sequence distance between the Sudanese *donovani* and the *donovani* from elsewhere even seems to justify the several attempts to give the former a separate nomenclature based on biological and epidemiological characteristics as suggested by Lainson and Shaw (1987). This might support the assignment of some *donovani* from Sudan to a subspecies category known as "*Leishmania donovani archibaldi*". Rioux et al. (1990), however, assigns only one group of parasites to this category, while in our study we find all the parasites isolated in the Sudan to possess a single mitochondrial haplotype, which is significantly different from all other members of the *donovani* complex.

"Cladistic" analysis of the biology of these parasites lends further supports to the division of the *donovani* complex between the forest related *donovani* of the Sudan, the dog reservoir associated *infantum* and the anthroponotic Indian *donovani*, which is also faithfully depicted in the haplotype tree in Fig. 2.

4.3. How did *Leishmania* spread?

Several authors (Le Blancq and Peters, 1986; Mauricio et al., 1999) have shown the presence of a phylogeographic pattern in the distribution of the *L. donovani* complex. Based on the phylogeny by Rioux et al. (1990), Ashford et al. (1992) made an intuitive interpretation suggesting a radiation from a root stock in Sudan for a group of parasites, by way of a canine reservoir to become *L. infantum*, while another radiated in Asia via other parts of east Africa becoming *L. donovani*, this hypothesis, now seems to receives

strong support from our data. Along the same line, Seaman et al. (1992) suggested a recent transport of *L. donovani* from East Africa to India, even as recent as the 19th century. Though our molecular haplotype data do not support the later hypothesis in particular (the very recent introduction of leishmaniasis to the Indian subcontinent), one cannot rule out the possibility that the Indian subcontinent has witnessed more than one introduction of *L. donovani* into the human population. Ancestral parasites of the *L. donovani* reference strain DD8 may have arrived with early migrations through Arabia from Africa as the isolate from Arabia (Jeddah) shows an intermediate haplotype between the Indian and the Sudanese. The other parasite from Bangladesh looks more similar to the *infantum* group almost showing an intermediate sequence between the *infantum* and the *donovani* clades. This parasite may well be a relic of the original *infantum* sequence that has followed a similar root of migration or alternatively may have arrived more recently with the Indo-European migration to the Indian subcontinent.

The similarity between the European and Mediterranean *infantum* (represented by IPTI) and the Chinese *infantum* (Peking) suggests a very recent common origin either through a recent trade relationship through the Silk Road or even before that during the Indo-European expansion. Likewise the *infantum* like parasites (e.g. GG996) may have re-entered Africa relatively recently accompanying the migration into Africa of the Neolithic farmers which has been suggested to have taken place ca. 10,000 B.C. (Cavalli-Sforza et al., 1994) or even more recently through recent trade contacts including the slave trade as the presence of identical haplotypes in Iraq and East Africa might suggest. An interesting feature is the comparable number of haplotypes of COII in *Leishmania* and humans (Ruvolo et al., 1994), which are not entirely surprising when we consider the fact that *Leishmania*, is a well-adapted intracellular parasite of the monocyte/macrophage cell lineage.

Taking these sequence features into consideration we are tempted to propose that the transition from a *L. major*-like *donovani* ancestor took place in the *Acacia* forests of East Africa before or concurrent with the emergence and spread of modern man out of Africa. The original zoonosis may have taken place much earlier in an ancestral *L. major*-like parasite that has adapted to a carnivore or Canid species and acquired an ability to visceralise. *L. major* is primarily a parasite of rodents but could infect man causing a skin disease. The dispersion of *L. major* occurred in its mammalian hosts and the ranges of its gerbil hosts in Africa and Asia are known to have contracted and expanded during glacial and interglacial cycles of the Pleistocene and probably earlier in the Pliocene (Essegir et al., 1997).

At least one microorganism, the lymphotropic virus HTLV1 (Vandamme et al., 1998), is now traced to a co-migration in an "out of Africa scenario". It has also been shown recently that the notorious malaria parasite *Plasmodium falciparum* has spread in a more recent migration out of Africa (Rich and Ayala, 2000). With the availability of

the vast sequence information in the databases it is almost certain that other parasites and pathogens will follow suit.

4.4. Dating the events

We have chosen to ignore the possible variation in evolutionary rates between the main *Leishmania* lineages, which have recently been shown in Trypanosomes (Stevens et al., 1999). Therefore, our results on the divergence of the genus *Leishmania* must be taken as tentative pending the acquisition of more sequence data from a wider species profile of the genus. On the other hand our estimates for coalescence of *L. donovani* under two hypothesised rates of mutation low (10^{-8}) and high (2×10^{-8}), gave the dates of 500 and 250 kya, respectively, for the separation of the *donovani* complex from *major* and *donovani* from *infantum*. Using the low mutation rate, the figures were brought to 250 and 120 kya, respectively. It is possible to match both dates roughly with the figures of 100–200 kya given for the emergence of modern humans in Africa and the Middle East by archaeological and genetic data (Cann et al., 1987; Foley and Lahr, 1997; Cavalli-Sforza, 1998).

Underhill et al. (2000) recently proposed a more recent common ancestor to the human Y chromosome and of a more recent emergence of modern *Homo sapiens* out of Africa. Interestingly the populations shown to harbour the most ancestral alleles in the Y chromosomes are those inhabiting the areas where visceral leishmaniasis continues to threaten human lives.

In conclusion, we propose a model for the origin and distribution of members of the *L. donovani* complex, based on close co-habitation and co-evolution with its human host. Several aspects, however, of this relationship should be verified. A plausible explanation for the virulence of the disease (VL) in the northern part of east Africa (Sudan and Ethiopia) where it presumably originated is required. Tentatively we speculate that the parasites and disease may have passed through short periods of contraction and expansion in their native habitat, occasionally affecting adjacent human populations. This may have resulted in a stability of the parasites population structure, but rather a fragile host parasite relationship.

Acknowledgements

The authors acknowledge the kind assistance and comments of Mark Seielstad and A.M. El Hassan. This work has been supported by grant from the Wellcome Trust and in part by the MRC (UK).

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