

Evolution of the genus *Leishmania* revealed by comparison of DNA and RNA polymerase gene sequences¹

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Abstract

Previous hypotheses of *Leishmania* evolution are undermined by limitations in the phylogenetic reconstruction method employed or due to the omission of key parasites. In this experiment, sequences of the gene encoding the DNA polymerase α catalytic polypeptide (POLA) were analysed phylogenetically in combination with those encoding the RNA polymerase II largest subunit gene (RPOIILS) to infer a comprehensive phylogeny of *Leishmania*. Nineteen species of parasites were studied, comprising representatives of each *Leishmania* species-complex (*Leishmania Leishmania tropica*, *Leishmania Leishmania donovani*, *Leishmania Leishmania mexicana*, *Leishmania Leishmania hertigi* and *Leishmania Viannia braziliensis*), as well as parasites of questionable taxonomy (*Leishmania herreri*, *Sauroleishmania adleri*, *Sauroleishmania deanei*, *Sauroleishmania gymnodactyli* and *Sauroleishmania tarentolae*). The analyses presented here provide strong support for the hypothesis that the *Leishmania* that infect reptiles (also known as *Sauroleishmania*) evolved from mammalian *Leishmania*. One implication of this finding is that the taxonomic definition of *Leishmania* should be broadened to encompass characteristics of the reptilian parasites. However, this taxonomic revision is complicated in that *Leishmania (L.) hertigi*, *Leishmania (L.) deanei* and *Leishmania herreri*, which exhibit some biological properties of *Leishmania*, are more closely related to *Endotrypanum* on the basis of these sequence comparisons. Consequently, the taxonomic discrimination between *Leishmania* that infect mammals, *Leishmania* that infect reptiles and *Endotrypanum* may be more problematic than has been previously thought. Since our resulting phylogenetic hypothesis is supported by the analyses of two different genes, we speculate on the origin and evolutionary expansion of this lineage of kinetoplastid protozoa. © 1997 Elsevier Science B.V.

Abbreviations: PCR, polymerase chain reaction; SSU rRNA, small subunit ribosomal RNA; NS, not sequenced.

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¹ *Note:* Nucleotide sequence data reported in this paper have been submitted to the GenBank™ data base with the accession numbers: POLA/RPOIILS (AF009134/AF009153, *Leishmania adleri*); (AF009135/NS, *Leishmania aethiopica*); (AF009136/AF009154, *Leishmania amazonensis*); (AF009137/NS, *Leishmania arabica*); (AF009138/AF009155, *Leishmania braziliensis*); (AF009139/NS, *Leishmania chagasi*); (AF009140/AF009156, *Leishmania deanei*); (AF009141/AF009157, *Leishmania donovani*); (AF009142/AF009158, *Endotrypanum monterogeei*); (AF009143/AF009159, *Leishmania gymnodactyli*); (AF009144/AF009160, *Leishmania herreri*); (AF009145/AF009161, *Leishmania hertigi*); (AF009146/AF009162, *Leishmania hoogstraali*); (AF009147/NS, *Leishmania infantum*); (AF009148/AF009163, *Leishmania major*); (AF009149/AF009164, *Leishmania mexicana*); (AF009150/AF009165, *Leishmania panamensis*); (AF009151/AF009166, *Leishmania tarentolae*); (AF009152/AF009167, *Leishmania tropica*).

Keywords: Leishmania; Sauroleishmania; DNA polymerase α ; RNA polymerase II; Evolution; Phylogeny

1. Introduction

The genus *Leishmania* comprises some 30 species of morphologically similar kinetoplastid protozoa, including 21 species which are responsible for a spectrum of human diseases ranging from mild to fatal infections [1,2]. A combination of biological [3], immunological [4,5], biochemical [6–8] and molecular criteria [9–12] have been used to identify and classify *Leishmania* species. Five assemblages of *Leishmania* (species-complexes) have been distinguished, which in the most recent classification are assigned to one of two primary lineages (subgenus *Leishmania Leishmania* and subgenus *Leishmania Viannia*). In this classification scheme the enigmatic species *Leishmania herreri*, *Leishmania (L.) hertigi* and *Leishmania (L.) deanei* are retained in the genus, while the *Leishmania*-like parasites of reptiles are recognised as a separate genus *Sauroleishmania* [3].

Historically, *Leishmania* have proven refractory to systematic studies. SSU rRNA gene sequence comparisons have been used successfully to infer phylogenetic relationships within other genera of the Kinetoplastida [13–15]. However, the small inter-specific variability among these sequences of *Leishmania* prevents any reliable phylogenetic inferences to be made [16]. Construction of molecular phylogenetic trees of *Leishmania* have therefore relied upon other sequence comparisons [17–19], or alternative methods for generating comparative data [20,21]. Of these, no single method has proven to be useful in studying relationships among all *Leishmania* species-complexes, and consequently a comprehensive phylogeny of the genus has not emerged.

Since comparisons based on any single molecular sequence may be misleading, phylogenetic inferences should reflect concordant results from different sequences [22]. Ongoing studies in our laboratory have focussed on identifying genes which exhibit a tempo of evolution suitable for resolving the relationships among *Leishmania*

[23,24]. Here we present a phylogenetic analysis of sequences derived from the genes encoding the DNA polymerase α catalytic subunit (POLA) and RNA polymerase II largest subunit (RPOIILS) polypeptides from parasites representing each of the species-complexes of *Leishmania* as well as some of dubious taxonomic position. In addition to establishing the POLA and RPOIILS genes as useful tools for exploring phylogenetic relationships among *Leishmania*, the current taxonomy of *Leishmania* is evaluated and their biogeographical evolution discussed.

2. Materials and methods

2.1. Parasite Strains

The 19 species of parasites used in this study are shown in Table 1. This table also lists the geographical distribution of each species and pathology of human infection. *Leishmania* promastigotes were cultured axenically in HO-MEM [25] supplemented with 5–10% fetal calf serum at 25°C. DNA was extracted as described previously [26].

2.2. PCR, cloning and DNA sequencing

PCR primer pairs DPO1/DPO2 (5'-CTTGA-CACGCTTCTCCGA-3' for/5'-GCCGAGGCA-GCCATACAT-3' rev) and RPOF1/RPOR1 (5'-GACACAGCCGTCAAGAC-3' for/5'-GCAGC-CGCACAATGCGCT-3' rev) were based on regions of nucleotide sequence from within the POLA and RPOIILS genes conserved between *Trypanosoma brucei* (GenBank™ accession numbers J04841 and X60951, respectively) and *Leishmania (L.) major* (to be described elsewhere). PCR amplifications, cloning and DNA sequencing were performed in triplicate to identify errors introduced by Taq polymerase. Reaction conditions for the amplification of POLA are as described

Table 1
Parasite strains studied

Species	Strain	Hosts	Pathology ^a
<i>L. (L.) donovani</i> complex			
<i>L. donovani</i>	MHOM/IN/80/DD8*	Human	VL
<i>L. infantum</i>	MHOM/TN/80/IPT1*	Canidae	VL
<i>L. chagasi</i>	MHOM/BR/74/PP75*	Canidae	VL
<i>L. (L.) tropica</i> complex			
<i>L. tropica</i>	MHOM/SU/58/Strain-OD*	Canidae, Mustelidae	CL
<i>L. major</i>	MHOM/SU/73/5-ASKH*	Rodentia	CL
<i>L. arabica</i>	MPSM/SA/83/JISH220	Gerbellinae	N
<i>L. aethiopica</i>	MHOM/ET/72/L100*	Procaviidae, Canidae	CL
<i>L. (L.) mexicana</i> complex			
<i>L. mexicana</i>	MNYC/BZ/62/M379*	Rodentia	CL
<i>L. amazonensis</i>	MHOM/BR/73/LV78	Rodentia, Marsupialia	CL
<i>L. (V.) braziliensis</i> complex			
<i>L. braziliensis</i>	MHOM/VE/XX/LbV	Rodentia, Edentata	CL, MC
<i>L. panamensis</i>	MHOM/PA/XX/CIDEP004	Edentata	CL
<i>L. (L.) hertigi</i> complex			
<i>L. hertigi</i>	MCOE/PA/65/C-8, LV42*	<i>Choendou</i> spp.	N
<i>L. deanei</i>	MCOE/BR/XX/LV402/M2909	<i>Choendou</i> spp.	N
<i>L. herreri</i>	MCHO/CR/74/LV344/Ch-97	Edentata	N
<i>L. hoogstraali</i>	RLIZ/SD/XX/LV31	<i>Hemidactylus</i> spp.	N
<i>L. adleri</i>	RLIZ/KE/XX/LV30	<i>Latastia</i> spp.	N
<i>L. gymnodactyli</i>	RGEC/SU/XX/LV247	<i>Agamae</i> spp.	N
<i>L. tarentolae</i>	RTAR/DZ/39/LV414	<i>Tarentola</i> spp.	N
<i>E. monterogeii</i>	MCHO/CR/62/LV88, A9	Edentata	N

VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; MC, mucocutaneous leishmaniasis; N, no recorded human infection

^a Characteristic human disease pathology.

* Indicates WHO reference strains.

previously for RPOIILS sequences [24]. POLA fragments amplified with DPO1/DPO2 ranged from 957–970 bp while RPOIILS fragments amplified with RPOF1/RPOR1 were 1306 bp. Amplified fragments were cloned into the pGEM-T vector (Promega) and DNA sequences obtained by use of a Li-Cor 4000L DNA sequencer using SequiTherm DNA polymerase (Epicentre Technologies).

2.3. Sequence alignment and phylogenetic analyses

POLA and POLA/RPOIILS nucleotide sequences were aligned using the ClustalW multiple sequence alignment program using default values

[27]. Phylogenetic analyses were performed using the PHYLIP package (version 3.5) unless otherwise stated [28]. Trees were inferred using three methods: (1) maximum likelihood (ML) [29] with the global rearrangement option in effect; (2) neighbor joining (NJ) [30] for which distance matrices were calculated under the Kimura 2-parameter model; (3) weighted parsimony using the program PAUP 3.1.1 [31]. The exhaustive search option of PAUP was employed for alignments containing fewer than 12 sequences, and the branch and bound option used on other sequence alignments. The average observed transition:transversion (ti/tv) ratio among sequences of closely related species was ≈ 5 , as revealed by pairwise comparisons. Transversions were, there-

fore, weighted five times that of transitions. The reliability of the trees were assessed by the bootstrap method ($n = 100$). Alternative tree topologies were evaluated by the Kishino-Hasegawa test under ML [32].

3. Results

3.1. Analysis of the POLA data set

Following the removal of primer sequences and a 5 amino acid insertion present in the *Endotrypanum monterogeii* and *L. herreri* sequences, the POLA alignment contained 924 nucleotide positions. Single amino acid insertions or deletions (indels) located in sequences of *L. (V.) braziliensis*, *L. (V.) panamensis* (position 535–537 in the *L. (L.) donovani* sequence) and those of the *L. (L.) tropica* complex parasites (position 523–525 in the *L. (L.) donovani* sequence) were retained.

The optimal tree resulting from the ML, NJ and parsimony analyses is shown in Fig. 1. Old World *Leishmania* of the *L. (L.) tropica* and *L. (L.) donovani* complexes constituted the crown taxa of the POLA tree, an arrangement which was strongly supported by bootstrap values of 91 (ML), 99 (NJ) and 92% (parsimony). A single amino acid deletion (nucleotide positions 523–525 in the *L. (L.) donovani* sequence) unique to all *L. (L.) tropica* complex sequences studied is consistent with the close affiliation of the constituent species. The monophyly of the human pathogenic *L. (Leishmania)* clade (*L. (L.) tropica*, *L. (L.) donovani* and *L. (L.) mexicana* complexes) was supported by bootstrap values of 51 (ML), 90 (NJ) and 80% (parsimony). Contrary to one current taxonomic hypothesis which distinguishes the mammalian and saurian *Leishmania* as separate genera [3], none of the phylogenetic methods revealed support for a compatible molecular tree. Furthermore, the emergence of the *Leishmania* which infect reptiles succeeds the separation of *L. (Viannia)* from the primary leishmanial lineage. The position of the *L. (V.) braziliensis* complex as the earliest diverging of the human parasites was fully supported by the bootstrap analyses.

In comparison to the relatively short internodal distances which separated most *Leishmania* clades on the POLA tree, the *L. (L.) hertigi* complex and *L. herreri* were both defined by long branch lengths and, by this criterion, are genetically quite distinct from other *Leishmania*. Moreover, pairwise sequence comparisons revealed that the *L. herreri* POLA sequence was considerably more similar to *E. monterogeii* than to the sequences of other *Leishmania*, while the *L. (L.) hertigi* / *L. (L.) deanei* sequences were intermediate in their similarity to sequences of parasites from these genera (data not shown).

One consistent feature of the POLA tree was the strong statistical support for the grouping of *Leishmania* species of the same complex. However, most intra-complex relationships were not well supported by the bootstrap analyses and, therefore, could not be reconstructed on the basis of POLA sequence comparisons.

3.2. Analysis of the combined POLA/RPOIILS data set

To further analyse relationships among the *Leishmania* species-complexes, the POLA alignment was combined with a 1268 bp RPOIILS alignment and analysed phylogenetically. Since neither POLA, RPOIILS nor combined POLA/RPOIILS sequence analyses can confidently resolve the intra-complex branching order (data not shown), we utilised species-complex consensus sequences to investigate the inter-complex, subgenus and genus relationships of these parasites and to reduce the computational burden. The standard IUPAC ambiguity codes were used for those nucleotide positions with more than one possible character state in a consensus sequence. The combined POLA/RPOIILS alignment therefore comprised seven sequences of 2188 characters, 232 of which were considered phylogenetically informative as defined by parsimony criteria. The single optimal tree topology obtained by ML, NJ and parsimony analysis was identical to the POLA tree (Fig. 1) in branching order among the major taxonomic groups. With the *E. monterogeii* sequence selected as the outgroup root, the assignment of the *L. (L.) hertigi* complex sequence to

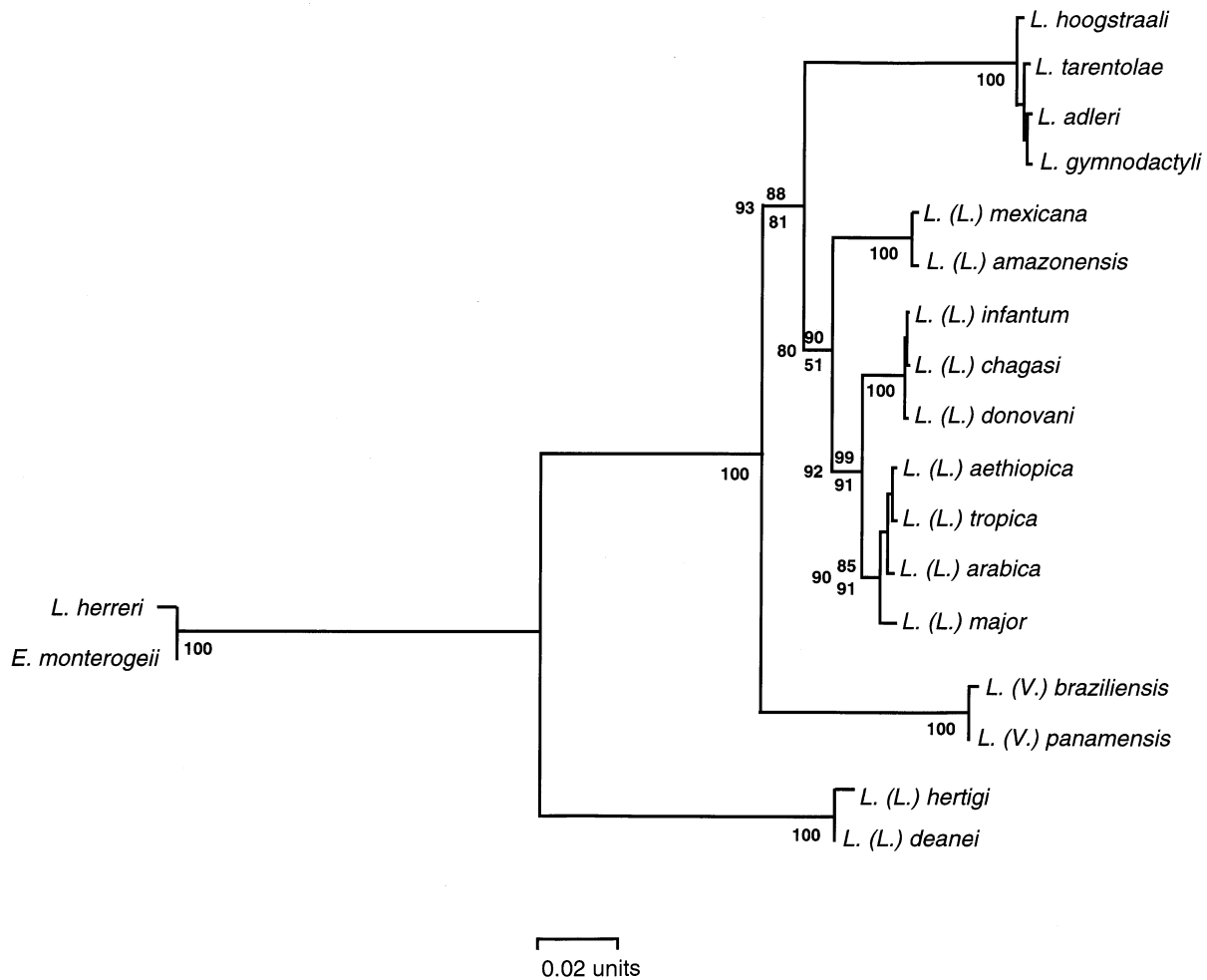


Fig. 1. Phylogenetic relationships among POLA sequences from *Leishmania*, based on a 924-nucleotide sequence alignment. An unrooted NJ tree is shown with branch lengths drawn proportional to the distances calculated under the Kimura 2-parameter model. The bar corresponds to 0.02 substitutions per nucleotide site. Numbers at nodes show bootstrap support for that node. NJ, ML and parsimony analyses yielded identical tree topologies, although the bootstrap values obtained differed among the different tree building methods used: the upper numbers represent NJ bootstrap values, lower numbers show ML bootstrap values and numbers to the left of the nodes are values obtained under parsimony.

either the *Leishmania* or *Endotrypanum* lineage was ambiguous.

In order to test the robustness of the POLA/RPOIILS phylogeny, nine alternatives to the optimal tree, each requiring alternate evolutionary models of these parasites, were tested for significant conflict with the optimal ML tree [32]. Three alternate trees were not rejectable under ML when a 95% confidence interval was adopted. The consensus of the first two non-re-

jectable tree topologies resulted in a trichotomy of the *L. (L.) tropica*, *L. (L.) mexicana*, *L. (L.) donovani* complexes, while the third tree placed the divergence of the saurian *Leishmania* immediately prior to that of the *L. (L.) donovani* and *L. (L.) tropica* complexes (data not shown). The respective positions of *L. herreri*, the *L. (L.) hertigi* complex and the *L. (V.) braziliensis* complex were retained in all non-rejectable trees.

Preliminary analyses on the extent of nucleotide substitutions at third codon positions in the POLA and RPOIILS gene sequences derived from *Leishmania*, *Endotrypanum*, *Crithidia* and *Leptomonas* showed them to be saturated. This observation suggested that nucleotide sequence comparisons based on POLA and RPOIILS may be misleading at this taxonomic level. Consequently, further analyses were performed in order to substantiate our observations as well as to provide support for the root of the tree. In the first instance a sequence alignment containing *Leishmania* and *Endotrypanum* POLA/RPOIILS nucleotide sequences was analysed using the DNAMLK algorithm of the PHYLIP package, since this algorithm assumes a molecular clock and does not require an outgroup to be specified (Fig. 2). Subsequently, the inferred protein sequences of both POLA and RPOIILS were aligned and analysed by parsimony using *Leptomonas seymouri*, *Crithidia luciliae* and *Trypanosoma brucei* sequences as a specified outgroup (not shown). Both of these analyses identify *Endotrypanum* as the sister group to part of *Leishmania*, confirming that *L. herreri* and *L. hertigi* are descendants of the *Endotrypanum* lineage, and provide strong statistical support for the nodes shown.

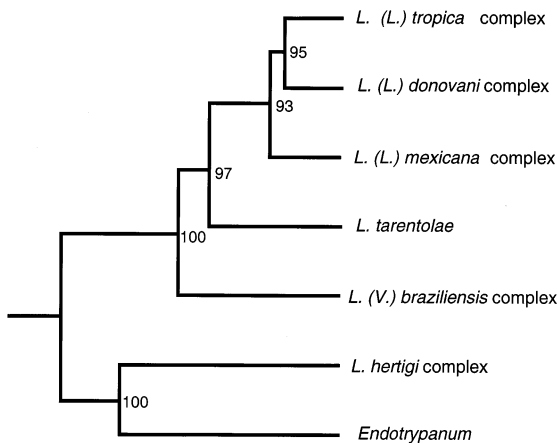


Fig. 2. Rooted phylogenetic tree depicting the evolutionary relationships of the *Leishmania*/*Endotrypanum* lineage based on DNAMLK analysis which assumes a molecular clock. Bootstrap values are shown to the right of each node.

The phylogenetic analysis of Fig. 1 shows the branch to the clade containing the saurian *Leishmania* as being longer than the branches to the other *Leishmania* species. As the NJ tree building method used in this analysis reflects inequalities in rates of evolution between sequences as inequalities in branch lengths, one may postulate that the saurian *Leishmania* sequences are evolving at a faster rate than the sequences from the other *Leishmania* species. To verify this hypothesis, POLA/RPOIILS phylogenetic trees obtained by the ML and MLK algorithms were compared statistically [32], and were found to differ significantly at the 95% confidence interval. It therefore appears that a molecular clock assumption was not warranted for the seven taxon data set. However, a molecular clock assumption was valid if the saurian *Leishmania* consensus sequence was excluded from the analysis. If we accept that the majority of sequences are evolving in a clock-like manner, then *Endotrypanum* represents the sister taxon to the *Leishmania* and the root position is as shown in Fig. 2.

4. Discussion

Several works have established the utility of RPOIILS for investigating the phylogenetic relationships among taxa that have evolved relatively recently (i.e. over the last 80 million years or so) [23,24,33,34]. Like RPOIILS, we anticipated that POLA would be similarly useful in systematic studies of *Leishmania*. Consequently, we analysed the phylogenetic relationships among the major lineages of *Leishmania* using sequences derived from these two different genes (RPOIILS and POLA). Since there is no evidence to imply genetic linkage between the DNA polymerase α catalytic polypeptide and the RNA polymerase II largest subunit polypeptide genes, it appears they can be treated as two independently evolving gene loci.

Optimal phylogenetic trees of *Leishmania* based on POLA and POLA/RPOIILS sequences were identical in topology. Fig. 3 shows a schematic tree which summarises this information. It integrates data on the geographical distribution and

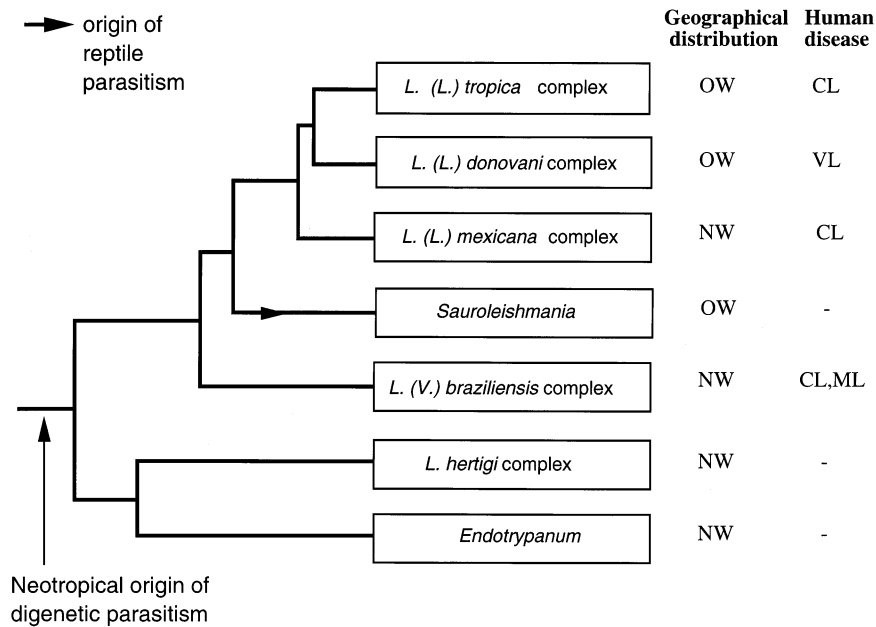


Fig. 3. Schematic tree showing the evolution of the *Leishmania/Endotrypanum* subtree of the Kinetoplastida. The origin of digenetic parasitism as shown is based on an evolutionary study of the Kinetoplastida [43]. An indication of the geographical distribution (OW, Old World; NW, New World) and typical disease pathology (CL, cutaneous; VL, visceral; MC, mucocutaneous) observed following infection by parasites from each taxon are shown on the right. Species occurring in the New World, which are members of the *L. tropica* and *L. donovani* complexes, are assumed to represent exotic species.

outcome of human infection to illustrate the evolution of these parasites. In all the analyses presented in this study, *L. (L.) tropica*, *L. (L.) donovani* and *L. (L.) mexicana* complexes were monophyletic and represent the crown of the tree. The *L. (V.) braziliensis* complex represents the earliest diverging member of the group and is found at the base of the tree. *Endotrypanum* was identified as the sister group to the *L. hertigi* complex. Furthermore mammalian and saurian *Leishmania* are monophyletic.

The number of *Leishmania* species studied, we believe, provides a sufficiently comprehensive representation of the genus so as to avoid misleading inferences resulting from species sampling [35]. At least two representatives of each species-complex were included. Furthermore, from within each species-complex divergent species are included in order to maximise representation. For example, *L. (L.) major/L. (L.) tropica*, *L. (L.) donovani/L. (L.) infantum*, *L. (L.) mexicana/L. (L.) amazonensis*, *L. (L.) hertigi/L. (L.) deanei* and *L. (V.)*

panamensis/L. (V.) braziliensis provide a considerable span of the diversity within each of their respective species-complexes, as revealed by previous studies [10,19–21].

The phylogenetic relationships among *Leishmania* inferred from POLA and POLA/RPOIILS sequences corroborate the results of most other studies, and support the taxonomy of the human pathogenic species. For example, comparison of isoenzyme profiles and other molecular sequences show the Old World *L. (Leishmania)* as being most closely related, and progressively less related, to New World *L. (Leishmania)* and *L. (Viannia)* [7,17–19,23,24]. Based on a comparison of PCR profiles, other workers suggest that the *L. (L.) tropica* and *L. (L.) mexicana* complexes are most closely related, to the exclusion of the *L. (L.) donovani* complex [21], a topology which could not be statistically rejected by the POLA/RPOIILS data. These alternative hypotheses are equally parsimonious in explaining the pathology of human disease. However, reports of some species

producing atypical disease pathologies suggest that this character is not intrinsically related to parasite evolution, thus dissolving such correlations [36,37]. Consequently, one must favour the grouping of Old World *Leishmania*, since this arrangement obtains most support from existing comparative studies.

The *Leishmania*-like parasites of reptiles have endured several taxonomic reclassifications [3,38–40], resulting in the collective assignment of these species to a separate genus, *Sauroleishmania* [3,41]. This classification emphasised the distinct host and vector groups parasitised by *Leishmania* and assumed the former to be ancestral to the latter, in coinciding with the evolution of their vertebrate hosts [3]. Recent phylogenetic hypotheses of the relationships between mammalian and saurian *Leishmania* and *Endotrypanum* require that the *Leishmania* that infect reptiles evolved secondarily from the mammalian genera [23,24,42,43]. We provide further evidence that supports this hypothesis. Consistent with this proposal, some saurian *Leishmania* develop in lizards as amastigotes [44], which is a distinguishing *Leishmania* cellular morphology in vertebrate infections. Moreover, experimental infections of humans and other mammals with *Sauroleishmania adleri* have been induced which, although ephemeral by nature, indicate that such a transition in host may be permissible in nature with few genetic alterations [45,46]. Consequently, we feel that the controversy surrounding the taxonomic identity of the *Leishmania* that infect reptiles could be resolved by including these parasites in the genus *Leishmania*. Indeed, we recommend based on the evidence provided here and in [24], that the use of the term *Sauroleishmania* to describe *Leishmania* that infect reptiles should be discontinued. Since we are unable to statistically reject a tree topology which groups the saurian *Leishmania* and Old World *Leishmania*, we refrain from further classifying these species into a new or existing subgenus. The observation that the *Leishmania* that infect reptiles may have undergone an accelerated rate of evolution requires that additional approaches be used to confirm our results. The identification of unique DNA elements [19] or comparison of the physical linkage

maps of chromosomes [47] may reveal general similarities in genomic structure between these groups of parasites which could be further used to assess their affiliation.

The enigmatic parasites of the *L. (L.) hertigi* complex possess, in comparison to other *Leishmania*, an unusual nuclear DNA base composition and extremely large promastigote forms [3]. In support of the root position inferred by DNAMLK analysis of the POLA/RPOHLS alignment, other works indicate that the *L. (L.) hertigi* complex are more closely related to *Endotrypanum* than to *Leishmania* [3,48]. In addition, a POLA amino acid sequence phylogeny of the Trypanosomatidae in which the *Leishmania-Endotrypanum* subtree was rooted by sequences of other trypanosomatid genera, also places *L. (L.) hertigi* on the *Endotrypanum* lineage (unpublished data). The proposed relationship of the *L. (L.) hertigi* complex and *Endotrypanum* is, however, problematic in that neither *L. (L.) hertigi* nor *L. (L.) deanei* possess any of the biological characteristics which typify *Endotrypanum* species. *Endotrypanum* are distinguished from *Leishmania* in that the former develop as epimastigotes or trypomastigotes in erythrocytes [49] while the latter develop as amastigotes in macrophages [3]. *L. (L.) hertigi* has been reported to develop as amastigotes in vitro; however little is known about the biology of infection in the natural host [50]. In addition, *Endotrypanum* are parasites of two and three-toed sloths exclusively (genera *Choloepus* and *Bradypus*, respectively), and neither *L. (L.) hertigi* nor *L. (L.) deanei* have been isolated from edentates. Similarly, *L. herreri* strains, although known only from sloths, have also been reported to develop as amastigotes in vitro [51], yet are clearly more closely related to *Endotrypanum* based on the results of the current and previous studies [3,23,42]. Considering the obscure background of this species, and since sloths may harbour many trypanosomatid parasites including *L. (Viannia)* spp. and *Endotrypanum* spp., one cannot rule out the possibility that characteristics of different sloth-infecting parasites were used to arrive at the description of *L. herreri* as a new species. Newly isolated strains of this parasite would be required to address whether the apparent disparity between

vertebrate development and molecular phylogeny reflects a true biological peculiarity of *L. herreri*, or is the product of an erroneous description or subsequent strain mis-labelling.

Regardless of their apparent genetic affiliation to *Endotrypanum*, it is impossible to place *L. (L.) hertigi*, *L. (L.) deanei* and *L. herreri* within this genus under the existing taxonomic concepts. If one accepts that these species do develop as amastigotes in their hosts, the implications are clear in that it would no longer be possible to discriminate between *Leishmania* and *Endotrypanum* on the basis of their morphological development in the vertebrate host. However, if these species are left in the genus *Leishmania* then our analyses indicate that this genus is paraphyletic.

The resolution of the branching order among *Endotrypanum* and the major lineages of *Leishmania* provides an opportunity to speculate on their origin and evolutionary expansion. The Neotropics accommodate the greatest diversity of *Leishmania* and *Endotrypanum* species, which some workers have considered commensurate with a Neotropical origin [3,48]. Our analyses provide compelling evidence in support of this hypothesis. The Neotropical parasites (*Endotrypanum/L. herreri*, *L. (L.) hertigi/L. (L.) deanei*, and *L. (V.) braziliensis/L. (V.) panamensis*) represent the more basal branches on the POLA and POLA/RPOIILS trees, while species at the crown of the trees predominate in the Old World. Noyes proposed a dispersal pattern from the Neotropics which included North America and, subsequently, passage into Asia via the Bering region [48]. We agree, in that a general New World to Old World evolutionary expansion, is most parsimonious in reconciling the extant parasite distribution and diversity with available molecular trees. Under this model, the identification of Old World *Leishmania* species in the New World may be attributed to the more recent exotic importation of these species, as postulated by other workers [21,52]. The incongruity between the extant distribution of the saurian *Leishmania* with their apparent New World origin, as inferred from the POLA and POLA/RPOIILS phylogenetic trees, is more difficult to appease since leishmanial infections are not found in New World lizards. The

story is further complicated by the evidence we provide for an increase in their rate of evolution.

Finally, it should be noted that interpretations made on molecular analyses alone may oversimplify the complexities of the biogeographical process. For example, the range of sloths has extended from Central America to Alaska during the Pleistocene Epoch [53]. It is probable that the geographical range of parasites such as *Leishmania* and *Endotrypanum* that were harboured by these sloths were similarly extensive before their mass extinction. Such phenomena can potentially distort the correlation between extant distribution and molecular phylogeny in proposing biogeographical models. Nevertheless, molecular phylogenetic analyses still provide a very powerful tool for the investigation of the evolutionary biology of extant organisms.

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