The Leishmania hertigi (Kinetoplastida; Trypanosomatidae) Complex and the Lizard Leishmania: Their Classification and Evidence for a Neotropical Origin of the Leishmania-Endotrypanum Clade

HARRY A. NOYES,†‡ BYRON A. ARANA,‡ MICHAEL L. CHANCE,* and RHAIZA MAINGON***

†‡Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK, and
***Centro de Investigaciones en Enfermedades Tropicales, Universidad del Valle, Guatemala, and

ABSTRACT. The relationships of the Leishmania hertigi complex and the lizard Leishmania species to the main groups of mammalian Leishmania and Endotrypanum parasites were examined. Restriction fragment length polymorphisms and sequences of small subunit ribosomal RNA genes and hybridization studies of kinetoplast DNA indicated that the L. hertigi complex was more closely related to the genus Endotrypanum than to the genus Leishmania. The lizard Leishmania species were found to be at the crown of the Leishmania tree. The data provides strong evidence for a Neotropical origin of the Endotrypanum/Leishmania clade since the parasites closest to the root of the tree are all found exclusively in the Neotropics. The evolution of the Leishmania/Endotrypanum clade in relation to the evolution of the known hosts of these parasites is discussed.

Supplementary key words. Biogeography, Bradypodidae, Coendu, Cricetidae, evolution, Hystricomorpha, sloth, Trypanosoma cruzi.

The genus Leishmania is an important group of protzoan parasites which is transmitted by sandflies (Diptera; Psychodidae; Phlebotominae) and is endemic amongst lizards and six orders of mammals. The majority of mammalian Leishmania species cause chronic cutaneous lesions in humans and parasites of the L. donovani complex may cause a visceral disease. There are believed to be approximately 400,000 cases of leishmaniasis each year amongst a population of about 400 million [5]. The mammalian Leishmania species are divided into two subgenera—the L. (Viannia), consisting of nine species which are restricted to the Neotropics, and the L. (Leishmania) consisting of eleven New World species and ten Old World species [29]. Two further groups of Leishmania species have an uncertain relationship to the two named subgenera, these are the Leishmania hertigi complex and the lizard Leishmania.

The L. hertigi complex consists of two species—L. hertigi and L. deanei. These parasites are restricted to Neotropical porcupines (Rodentia; Hystricomorpha; Erethizontidae; Coendu) and an unknown sandfly vector. They are not infectious to humans and only transient infections have been achieved experimentally in hamsters or mice. The L. hertigi complex species have been provisionally included in the L. (Leishmania) as both groups develop in the midgut of sandflies (supraypilarian) in the laboratory. In contrast L. (Viannia) parasites develop in the hindgut and midgut (peripylarian) and the lizard Leishmania develop in the hindgut (hypopylarian) of their respective sandfly hosts [33]. However the L. hertigi complex parasites have a nuclear DNA buoyant density which is intermediate between the ranges of the other Leishmania species and the genus Endotrypanum (Table 1).

Twelve species of Leishmania have been isolated from the blood of lizards [29]. These parasites have been placed in a separate genus—Sauroleishmania [29], principally because they infect a separate class of vertebrates and a separate genus of sandflies—Sergentomyia. In contrast mammalian Leishmania species are parasites of sandflies of the genera Phlebotomus and Lutzomyia. The haematozoic lizard Leishmania are restricted to the Old World even though some host species, such as Hemitriatulus sp. are found in both hemispheres [20], and some New World sandflies transmit Trypanosoma sp. between lizards. The genus Sauroleishmania has not been universally adopted [23, 35]. Recent phylogenies of the small subunit ribosomal RNA (SSU rRNA) gene of the Trypanosomatidae could not resolve the lizard parasite L. tarentolae and the mammalian Leishmania species [11, 38]. The Sauroleishmania is the only trypanosomatid genus for which sequence data is available that cannot be resolved by this method.

Three species of Leishmania are found mainly or exclusively in the gut of the lizard [29]. These species were all first described before 1925 when Wenyon’s definition of Leishmania was current [55]. More recent studies have shown that these parasites are probably trypanosomatids of Diptera that can survive in the lizard gut [20, 30, 53]. Therefore the lizard gut trypanosomatids will be disregarded in the following discussion of the lizard Leishmania.

Phylogenies of the SSU rRNA gene indicate that digenetic parasitism has developed independently on up to four occasions in the Trypanosomatidae [22]. One of these groups of digenetic parasites is the Endotrypanum/Leishmania clade which appears to be a sister group of the Crithidia/Leptomonas clade of monogenetic insect parasites. We have used a number of methods in order to clarify the position of the L. hertigi complex and the lizard Leishmania within the Endotrypanum/Leishmania clade. RFLP analysis of SSU rDNA has been shown to be useful for the classification of Trypanosoma cruzi strains and anuran Trypanosoma species [14, 15], we have used this method to determine the relationship of representatives of both the L. hertigi complex and the lizard Leishmania with respect to Endotrypanum sp. and the mammalian Leishmania subgenera. A partial sequence of the SSU RNA gene of L. hertigi was used to support the RFLP evidence. Hybridization studies of the variable region of kinetoplast (mitochondrial) minicircle DNA of 14 Leishmania species were also used to confirm the position of the lizard Leishmania sp.

MATERIALS AND METHODS

Parasites from the Liverpool cryobank were revived in semisolid blood agar and maintained in HOMEN [8]. DNA was prepared by standard methods [28]. Parasite strains used are shown in Table 1.

An unnamed trypanosomatid (G755) which was isolated in Guatemala was used as an outgroup. The sequence of the most variable region of the SSU rRNA gene of G755 had previously been obtained (Genbank (GB) accession number U59491) (bases 938–1,422 in the L. donovani GB:X07773 sequence). This sequence indicated that this parasite clusters between the Crithidia/Leptomonas clade and the Endotrypanum/Leishmania clade (Fig. 5). Therefore of the trypanosomatids for which se-
Table 1. Parasite strains used in the present study. All strains were from the Liverpool School of Tropical Medicine cryobank with the exception of G755 which was isolated in Guatemala by Dr Arana and MHOM/VE/XX/LBV which was isolated in Venezuela by Dr Bonfante-Garrido, Escuela de Medicina, Universidad Centro Occidental, Lara, Venezuela. Parasites are ranked primarily in descending order of nuclear DNA buoyant density and secondarily in descending order of kinetoplast DNA buoyant density. Nuclear (nDNA) and kinetoplast DNA (kDNA) buoyant densities are indicated after each strain [12, 18]. Where the buoyant densities of a given strain have not been determined the value of a closely related strain has been included in brackets where available. DNA buoyant density is related to GC content by the equation GC composition = X - 1.66/0.0098, where X = DNA buoyant density. The international code for Leishmania strains consists of four elements. The first element indicates the host class and genus in the case of vertebrate hosts and the species in the case of sandfly hosts, the second the country of isolation, the third the year of isolation and the fourth element is a local strain number [3]. In the first element M Mammal, R Reptile and I Insect; HOM strains have been described under the Liverpool School of Tropical Medicine cryobank accession numbers which begin with LV. Where this is the case the original strain number given by the isolating laboratory is followed by the Liverpool accession number in the next column.

<table>
<thead>
<tr>
<th>Species</th>
<th>Group affiliation</th>
<th>International code</th>
<th>Liverpool strain no.</th>
<th>nDNA buoyant densities</th>
<th>kDNA buoyant densities</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. (L.) tropica</td>
<td>Leishmania subgenus</td>
<td>MHOM/BR/89/ARD</td>
<td>LV357</td>
<td>(1.719)</td>
<td>(1.707)</td>
</tr>
<tr>
<td>L. (L.) tropica</td>
<td>Leishmania subgenus</td>
<td>MHOM/FR/60/LRC-L39</td>
<td>LV9</td>
<td>(1.719)</td>
<td>(1.707)</td>
</tr>
<tr>
<td>L. (L.) donovani</td>
<td>Leishmania subgenus</td>
<td>MHOM/ET/67/HU3</td>
<td>LV7</td>
<td>(1.719)</td>
<td>(1.704)</td>
</tr>
<tr>
<td>L. (L.) major</td>
<td>Leishmania subgenus</td>
<td>MHOM/SD/72/LV305</td>
<td>LV4</td>
<td>(1.718)</td>
<td>(1.703)</td>
</tr>
<tr>
<td>L. (L) major</td>
<td>Leishmania subgenus</td>
<td>MHOM/IL/67/LRC-L137</td>
<td>LV561</td>
<td>(1.718)</td>
<td>(1.703)</td>
</tr>
<tr>
<td>L. (L) mexicana</td>
<td>Leishmania subgenus</td>
<td>MNYC/BZ/62/M379</td>
<td>LV7</td>
<td>(1.718)</td>
<td>(1.700)</td>
</tr>
<tr>
<td>L. (L) amazonensis</td>
<td>Leishmania subgenus</td>
<td>MHOM/BR/73/M1845</td>
<td>LV78</td>
<td>(1.718)</td>
<td>(1.699)</td>
</tr>
<tr>
<td>L. hoagiastra</td>
<td>Lizard Leishmania</td>
<td>RHEM/SD/63/NG-26-</td>
<td>LV31</td>
<td>(1.717)</td>
<td>(1.703)</td>
</tr>
<tr>
<td>L. (V) panamensis</td>
<td>Viannia subgenus</td>
<td>MHOM/PA/71/LS94</td>
<td>LV414</td>
<td>(1.716-1.720)</td>
<td>(1.704-1.705)</td>
</tr>
<tr>
<td>L. tarentolae</td>
<td>Leishmania subgenus</td>
<td>RTAR/DZ/39/TarVI</td>
<td>LV108</td>
<td>(1.716)</td>
<td>(1.704)</td>
</tr>
<tr>
<td>L. tarentolae</td>
<td>Leishmania subgenus</td>
<td>RTAR/SE/67/G10</td>
<td>LV37</td>
<td>(1.716)</td>
<td>(1.704)</td>
</tr>
<tr>
<td>L. gymnodactyl</td>
<td>Lizard Leishmania</td>
<td>RGYM/SU/64/Ag</td>
<td>LV247</td>
<td>(1.716)</td>
<td>(1.704)</td>
</tr>
<tr>
<td>L. adleri</td>
<td>Lizard Leishmania</td>
<td>RLAT/KE/54/1433</td>
<td>LV30</td>
<td>(1.716)</td>
<td>(1.704)</td>
</tr>
<tr>
<td>L. (V) braziliensis</td>
<td>Viannia subgenus</td>
<td>MHOM/VE/XX/DBV</td>
<td>(1.716-1.717)</td>
<td>(1.691-1.692)</td>
<td></td>
</tr>
<tr>
<td>L. (V) guyanensis</td>
<td>Viannia subgenus</td>
<td>MHOM/BR/75/M147</td>
<td>(1.716-1.717)</td>
<td>(1.691-1.692)</td>
<td></td>
</tr>
<tr>
<td>L. deanei</td>
<td>L. hertigi complex</td>
<td>MCOE/BR/XX/M808</td>
<td>LV402</td>
<td>(1.716-1.717)</td>
<td>(1.691-1.692)</td>
</tr>
<tr>
<td>L. hertigi</td>
<td>L. hertigi complex</td>
<td>MCOE/PA/65/C-8</td>
<td>LV42</td>
<td>(1.714)</td>
<td>(1.700)</td>
</tr>
<tr>
<td>E. monterogenii</td>
<td>Endotrypanum sp.</td>
<td>MCHO/CR/62/A9</td>
<td>LV88</td>
<td>(1.712)</td>
<td>(1.697)</td>
</tr>
<tr>
<td>E. schaudinni</td>
<td>Endotrypanum sp.</td>
<td>MBR/A/PA/67/LV58</td>
<td>(1.712)</td>
<td>(1.697)</td>
<td></td>
</tr>
<tr>
<td>E. schaudinni</td>
<td>Endotrypanum sp.</td>
<td>MCHO/PA/68/LV59</td>
<td>(1.712)</td>
<td>(1.693)</td>
<td></td>
</tr>
<tr>
<td>Promastigote trypanosomatid</td>
<td>?</td>
<td>G755</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Quence data is available, this parasite is the most closely related to the Endotrypanum/Leishmania clade.

PCR primers were designed using the personal computer programme PRIMER (Lincoln, S. E., Daly, M. J., Lander, E. S., MIT Centre for Genome Research and Whitehead Institute for Biomedical Research, Nine Cambridge Centre, Cambridge, MA 02142, US). Primers WSSUF (GCTTGTTCAAGGACT- TAGCC) and WSSUR (GAAATATCGGTGAACTTTCGG) were designed to anneal to the ends of the coding regions of published sequences of the SSU rRNA gene of trypanosomatids and used with the following temperature profile: 30 cycles of 94°C, 30 s; 54°C, 1 min; and 72°C, 2 min.

A 561-bp sequence of the SSU rRNA gene of a number of kinetoplastids was also identified (corresponding to bases 901–1,461 of L. donovani (GB: X07773 sequence) which when analyzed using the DNAPARS program in PHYLPAR version 3.5c (J. Felsenstein, Dept. of Genetics SK-50, University of Washington, Seattle, WA 98195, USA.) generated a dendrogram of the Trypanosomatidae with a topology in general agreement with published phylogenies of the whole group [38, 39]. In an alignment of the complete SSU rRNA gene of C. fasciculata (GB: X03450), Leptomonas (GB: X53914), E. monterogenii (GB: X53911), L. donovani (GB: X07773) and L. amazonensis (GB: X53912) it was found that 58 out of 96 (60%) of the informative sites were in the region between bases 938–1,422 in the L. donovani GB: X07773 sequence. Primers SSU561F TGGGATAACAAAAAGGAGCA and SSU561R CTGAGACTGTAACCTCAAAGC were designed to amplify the sequence between bases 901 and 1,461 using 30 cycles of 94°C, 30 s; 54°C, 1 min; 72°C, 2 min. The L. hertigi (LV42) and G755 PCR products of these primers were purified on S400 spin columns (Pharmacia) and ethanol precipitated before cycle sequencing, using the same forward and reverse primers, on a model 377 automated sequencing system (Applied Biosystems).

The variable region of kinetoplast DNA minicircles was amplified using primers 13Z (ACTGGGGGTTGGTAAAA- TAG) and LiR (TGCGAGAACGCCCCCC) for 30 cycles of 94°C, 30 s and 54°C, 90 s. PCR amplified kinetoplast DNA (kDNA) variable region probes were prepared using primers 13Z and LiR and hybridized to dot blots of kDNA amplified with the same primers [9].

SSU rDNA restriction patterns were analyzed using the PHYLPAR package. The MIX parsimony programme was applied di-
Table 2. Sizes of restriction fragments found after digestion of the SSU rRNA gene. The complete gene was digested with HaeIII, HinfI, HpaII, RsaI and Sau96I of the complete SSU rDNA gene amplified with WSSUF and WSSUR and bases 901-2,142 (L. donovani GB X07773) amplified with SSUS6I and WSSUR with HhaI and bases 901-1,461 amplified with SSUS61F and SSUS61R with AlaI. HinfI generated six fragments of 230, 213, 154, 122/126 (not resolved), 63 and 901-1,461 amplified with SSU561F and SSU561R with AM.

![Fig. 1. Sau96I restriction digest of the complete 16S SSU rRNA gene of the species indicated. DNA was PCR amplified with primers WSSUF and WSSUR. Strain numbers are as indicated in Table 2. The sizes of fragments are shown in Table 2. Fragments of approximately 1,000-bp and larger in lanes 7-14 are partial digests. The mammalian Leishmania (lanes 12-14) are clearly distinguished from the lizard Leishmania (lanes 7-11) and L. deanei (lane 6) and the Endotrypanum species (lanes 3-5). This digest indicated that L. herreri (MCOE/CW 74/LV344) was an Endotrypanum species, this was confirmed by additional methods and is the subject of a separate communication [44].]

The data was also analyzed by calculating Nei's distance (equation 21 in Nei and Li [42]) for each pair of species and inserting the values into a distance matrix from which dendrograms were prepared using the FITCH programme and the UPGMA option in the NEIGHBOR programme. Bootstrap values were calculated using the SEQBOOT programme followed by MIX.

RESULTS

HaeIII, HinfI, HpaII, RsaI and Sau96I restriction digests of the complete SSU rRNA gene amplified with WSSUF and WSSUR were prepared from representatives of Leishmania, and Endotrypanum species. An AlaI digest of the partial SSU rRNA gene amplified with primers SSUS61F and SSUS61R and a HhaI digest of the products of SSUS61F and WSSUR were also used since an analysis of the alignment of the available sequences of the SSU rRNA gene indicated that these enzymes would only discriminate in these regions. The restriction fragments produced by these enzymes together with their sizes are shown in Table 2.

The enzyme that produced fingerprints most suitable for identifying mammalian Leishmania, lizard Leishmania and Endotrypanum species was Sau96I which generated unique restriction patterns for each of these groups (Fig. 1). The mammalian Leishmania species had a fragment of 452-bp absent from all other Leishmania and Endotrypanum species, although this band was present in the outgroup strain G755 this strain could be distinguished by a fragment at 500-bp which was not present in Leishmania species. The five lizard Leishmania strains had identical fingerprints with this enzyme and produced a fragment of 656-bp that was not present in the mammalian Leishmania.
Species. The *L. hertigi* complex species *L. deanei* had a unique restriction pattern with this enzyme with a fragment at approximately 1,200-bp but none at 656 or 560-bp which were found in the lizard and mammalian species. The *L. hertigi* complex the *L. tarentolae* branch and this position was supported in 90% of bootstrap replicates using the parsimony method and 95% using the distance method. The Kimura distances calculated using the DNADIST programme indicated that *L. hertigi* was closer to all the mammalian species than to the *L. deanei* species (Kimura distances 0.0361). The lizard *Leishmania* strains was proportional to loading except in the case of the homologous *L. tarentolae* strain which gave a more intense signal.

In order to confirm the position of the *L. hertigi* complex the product of PCR primers SSU561F and SSU561R from the species indicated. *Leishmania* strains which do not have a subgeneric designation are lizard parasites. The dot blot was first probed with the 13Z PCR primer which was terminal transferase labelled with 32P (Amersham) and washed in 1× SSC, 0.1% SDS at 42°C, to determine loading. The blot was then stripped and hybridized to a *L. tarentolae* variable region kDNA probe prepared by PCR using primers 132 and LiR and washed in water containing 0.1% SDS at 42°C. No hybridisation was detectable to the *L. (Viannia)* strains despite relatively high loading in the case of *L. guyanensis*. Weak hybridisation was detectable to the *L. (Leishmania)* strains despite slightly lower loading in some cases such as the *L. tropica* strains. Hybridization to the lizard *Leishmania* strains was proportional to loading except in the case of the homologous *L. tarentolae* strain which gave a more intense signal.

![Fig. 2. Dendrogram of the SSU RNA gene.](image-url)

Fig. 2. Dendrograms of the SSU RNA gene. 2. Dendrogram of the species indicated based on the presence absence data in Table 2, using the MIX parsimony programme in PHYLIP. Bootstrap percentages were based on 105 replicates. 3. Dendrogram of the species indicated prepared using DNADIST followed by FITCH from the sequence of the variable region of the SSU RNA gene between bases 938–1,422 of the *L. donovani* GB:X07773 sequence. Bootstrap percentages are based on 115 replicates. The GenBank accession numbers of the strains used are as follows: *L. major* (X53915); *L. amazonensis* (X53912); *L. tarentolae* (X53916); *L. donovani* (X07773); *L. guyanensis* (X53913); *L. hertigi* (U59492); *E. monerogei* (X53911); G755 (U59491); *Cristidna fasciculata* (X03450); *Leptomonas sp.* (X53914); *T. cruzi* (X53917); and *T. cruzi* (M31432).

Fig. 4. Dot blot of 10 ng and 1 ng of kinetoplast minicircle variable region DNA. The DNA was PCR amplified with primers 13Z and LiR from the species indicated. *Leishmania* strains which do not have a subgeneric designation are lizard parasites. The dot blot was first probed with the 13Z PCR primer which was terminal transferase labelled with 32P (Amersham) and washed in 1× SSC, 0.1% SDS at 42°C, to determine loading. The blot was then stripped and hybridized to a *L. tarentolae* (LV414) variable region kDNA probe prepared by PCR using primers 13Z and LiR and washed in water containing 0.1% SDS at 42°C. No hybridisation was detectable to the *L. (Viannia)* strains despite relatively high loading in the case of *L. guyanensis*. Weak hybridisation was detectable to the *L. (Leishmania)* strains despite slightly lower loading in some cases such as the *L. tropica* strains. Hybridization to the lizard *Leishmania* strains was proportional to loading except in the case of the homologous *L. tarentolae* strain which gave a more intense signal.
(Viannia) species (Fig. 2), a dot blot of the variable region of kinetoplast DNA minicircles was prepared to test for cross hybridisation between the lizard and mammalian Leishmania kDNA (Fig. 4). The blot was first probed with the 132 oligonucleotide primer which had been used to amplify the kDNA to test for variations in loading, some variation in loading was detected but as the L. (Viannia) species L. guyanensis appeared to have a higher loading than any of the L. (Leishmania) species this was not considered an obstacle to accurate interpretation of the data. A L. tarentolae probe hybridized strongly at high stringency (0.1% SDS, 42°C) to 10 ng of all the lizard Leishmania species tested except L. hoogstraali, weakly to 10 ng of all L. (Leishmania) species tested but there was no detectable hybridisation to L. (Viannia) species. A L. hertigi probe hybridized most strongly to L. hertigi and less strongly to L. deanei. There was a very weak signal with all other Leishmania and Endotrypanum species (not shown).

DISCUSSION

The relationship of the lizard Leishmania to the mammalian Leishmania. The SSU rDNA RFLP analysis and the pattern of cross hybridisation of the kDNA probe indicated that the lizard Leishmania are more closely related to the L. (Leishmania) than to the L. (Viannia). SSU rRNA sequence based phylogenies have been unable to resolve L. tarentolae from the mammalian Leishmania species with confidence [38] and (Fig. 3). The restriction enzymes used had been selected by a search of an alignment of L. tarentolae, L. donovani and E. monterogeii restriction enzyme sites that would discriminate between these species. The resulting restriction fragment data therefore contained relatively few uninformative sites, unlike the sequence data in which extensive regions of complete homology are found. The higher resolution of the RFLPs is therefore presumably a consequence of using mainly informative sites with an effective length of approximately 128 bp and the large number of uninformative sites in the sequence data. The cross hybridization between L. tarentolae and L. (Leishmania) species kDNA in the dot blot but not with L. (Viannia) species supports the results of the RFLP analysis. A recent RNA polymerase II phylogeny indicated that the lizard Leishmania are more closely related to the L. (Leishmania) than to the L. (Viannia) [17]. There is also some in vivo evidence of a close relationship between mammalian and lizard Leishmania species. Humans infected with lizard parasites have produced positive Montenegro skin test reactions up to two years later. Mammalian parasites have been found to be infective to lizards, and transient infections of mammals including humans have been reported with certain lizard Leishmania strains [1, 7, 37]. Only the nuclear DNA buoyant densities (Table 1) indicate that the lizard Leishmania are more closely related to the L. (Viannia) than to the L. (Leishmania).

The relationship of the L. hertigi complex to the Leishmania and Endotrypanum. The L. hertigi complex was found to be most closely related to the genus Endotrypanum when the RFLP data was analyzed with the parsimony programme MIX but most closely related to the genus Leishmania when the RFLP data was analyzed with the distance matrix programme FITCH. However the partial sequence of the 561-bp region of the SSU rRNA gene indicated that it clustered with Endotrypanum species in 95% of bootstrap replicates. Other ultrastructural, gene phylogeny and DNA buoyant density studies also suggest that the L. hertigi complex is more closely related to the genus Endotrypanum than to the genus Leishmania [17, 18, 41].

Part of the definition of the genus Leishmania is that these parasites develop as amastigotes in macrophages [54]. Although prevalence of L. hertigi in porcupines may be high (50–70%), parasites are difficult to detect except by culture and consequently the cell type parasitized is not yet known [24, 32, 58]. Until the cell type parasitized can be established it will not be clear to what extent the DNA based data may conflict with the biological characteristics of these parasites. Endotrypanum parasites develop as epimastigotes or trypomastigotes in sloth (Edentata: Bradypodidae) erythrocytes and have not been reported from any other family of mammals [48]. Consequently the L. hertigi complex would appear to have a closer affinity with Leishmania than with Endotrypanum parasites on biological grounds, since they develop as amastigotes rather than trypmastigotes or epimastigotes. Further studies of the biology of the L. hertigi complex species and of other genes will be required to determine the affiliation of these parasites with confidence. However it is clear that the L. hertigi complex appeared soon after the Endotrypanum and Leishmania genera separated, and before the lizard Leishmania species arose, an observation that has important consequences for the understanding of the evolution of the whole group.

Molecular evidence for a Neotropical origin of the Leishmania/Endotrypanum clade. In the phylogenies of the Leishmania/Endotrypanum clade (Fig. 2, 3) one of the two major branches consisted exclusively of New World parasites—L. hertigi and Endotrypanum sp., whilst the other branch consisted of Leishmania parasites from both the New World and the New World. There is only one isoenzyme based phylogeny of the mammalian Leishmania species which includes both subgenera [55]. Two major branches were identified which were distinct from the two branches of the Leishmania/Endotrypanum clade. There were some evident of a close relationship between New World parasites and the genus Leishmania with confidence [38] and in some cases both branches consisted of New World parasites. The L. guyanensis complex appeared to be most closely related to the genus Leishmania but the branch consisting of the New World L. (Viannia) subgenus and the other the New and Old World L. (Leishmania) subgenus. In the absence of an outgroup, the L. hertigi complex was found to be closely related to the L. (Leishmania) subgenus from which it branched off first. The L. mexicana complex, which is also restricted to the New World, branched off after the L. hertigi complex and the Old World complexes were placed at the crown of the tree. This phylogeny in which Old World parasites are found only at the tip of one of the sub-branches is consistent with a Neotropical origin of the Endotrypanum/Leishmania clade.

The overall GC content for a large number of mammalian and lizard Leishmania and Endotrypanum species can be calculated from their DNA buoyant densities [12, 13, 18]. The GC content of the parasites rises through the Neotropical species and then through the Old World species (Table 1). Lizard Leishmania species fall into two groups, L. adleri and L. hoogstraali and some L. tarentolae have values similar to members of the L. (Viannia) subgenus at the low end of the range whilst L. agamae and other L. tarentolae have values higher than any mammalian Leishmania species. The GC composition of the gene flanking regions of a number of trypanosomatids has been estimated [2] and follows the branching order of the trypanosomatids in the phylogeny of Fernandes et al. [22]. Consequently low GC content may be the primitive state and the genus Endotrypanum and New World species of Leishmania may be closer to the primitive state of this character than the Old World Leishmania species.

Biogeographical evidence for a Neotropical origin of the Leishmania/Endotrypanum clade. Sloths are important reservoir hosts of both Endotrypanum sp. and L. braziliensis, which are represented on both major branches of the Endotrypanum Leishmania clade. It is therefore possible that ancestors of modern sloths, which are members of the only surviving Eutherian order which was indigenous to the Neotropics throughout the Tertiary (65–6 million years ago MYA) [10, 45, 47], were early hosts of Leishmania like organisms. Marsupials were also endemic to the Neotropics throughout the Tertiary and a number
of supposedly monogenetic trypanosomatids can thrive in the anal scent glands of marsupials. It has therefore been proposed that development in these glands may have been a stage in the transition from monogenetic to digenetic parasitism in the *Leishmania* and *Trypanosoma cruzi* lines [19, 26].

The hystricomorph ancestors of the tree porcupines which are hosts of *L. hertigi* appeared in the Neotropics in the late Eocene (54–38 MYA) at the earliest, probably from the Nearctic, and the earliest Erethizontidae appeared in the fossil record in the early Oligocene (37–25 MYA) [45]. Therefore the late Eocene is probably the earliest date when the *L. hertigi* complex could have separated from the ancestors of *Endotrypanum* and *Leishmania* species. It may also be significant that the Kimura distance between the two *T. cruzi* strains in Fig. 3 (0.0760) is greater than between *Endotrypanum* and *Leishmania* (0.0167–0.0445) indicating that the *Endotrypanum* and *Leishmania* genera may have diverged after the two major groups of *T. cruzi* parasites. *T. cruzi* is a parasite of eight mammalian orders and reduvid bugs and is restricted to the New World [40]. *T. cruzi* may have evolved in South America at the end of the Cretaceous (140–65 MYA) at the time of the major mammalian radiation and the isolation of the Neotropics from the rest of the World [16, 43]. The greater distance between the two *T. cruzi* strains would be consistent with an early Tertiary separation of the *Endotrypanum* and *Leishmania* genera in edentate hosts, and a later still separation of the *L. hertigi* complex at about the time the Hystricomorpha arrived in South America in the late Eocene (54–38 MYA) [10, 45].

Representatives of all the surviving mammalian orders that were present in South America during the Oligocene and Mio- cene (37–6 MYA), except the Chiroptera, are at least occasionally infected with *Leishmania* and some are associated with highly specialized species of *Endotrypanum* or *Leishmania* that are restricted to South America and to specific hosts [25, 34, 45, 50].

**The migration of Leishmania to the Old World.** It has previously been suggested that the original radiation of the *Leishmania* species occurred from Central Asia on the grounds of the present epidemiology and the historical record [21, 36]. The radiation of the Old World *Leishmania* species from Central Asia is consistent with an earlier arrival from the Neotropics via the Isthmus of Panama and across Beringia. However it is possible that Beringia was already too cold for sandflies by the time the Neotropics became permanently reconnected to the Nearctic by the formation of the Isthmus of Panama in the early to mid Pliocene (5–3 MYA) [56, 57]. Consequently *Leishmania* parasites may have migrated to the Nearctic earlier via the Antilles or via the chain of islands that was the precursor of Central America. The presence of a distinctive strain of *L. mexicana* in the Dominican Republic which may be indigenous [27, 31], suggests that *Leishmania* parasites can be carried across open sea and become established in new habitats. The climate of the west coast of the Nearctic and Beringia reached a warm optimum in the Miocene about 13 MYA since which time temperatures at higher latitudes have declined although with several reversals [10], the passage of sandflies through this region may have been possible for much of the Miocene.

The genus *Leishmania* may therefore have arrived in Central Asia in the Miocene (24–6 MYA), possibly in cricetid rodents which are the most important hosts of *Leishmania* parasites in Central Asia and which themselves underwent a major radiation during the Miocene [4, 47]. The lizards that share burrows with cricetids may then have become infected, leading to the development of the lizard *Leishmania* in Central Asia. The lizard parasites may have developed at a time when the climate was already too cold for these species to cross back through Ber-

ingia although they could spread south into Africa. The presence of *Sergentomyia* as well as *Phlebotomus* sandflies in the rodent and lizard burrows may have permitted occasional infections of lizards with mammalian parasites to become an established cycle.

**The classification of the lizard Leishmania species.** The molecular data does not support generic status for the lizard *Leishmania* species. It has been proposed by Saf'janova that the lizard *Leishmania* species should be placed in the subgenus *L. (Sauroleishmania)* [33]. The molecular data, with the exception of the DNA buoyant densities, is consistent with the lizard parasites being a discrete group that could legitimately be accorded subgeneric status. However, if the biogeographic account of the origin of the lizard *Leishmania* is correct, these parasites are phylogenetically more closely related to the Old World *L. (Leishmania)* species than they are to the New World *L. (Leishmania)* species and the resurrection of the subgenus *L. (Sauroleishmania)* would render the subgenus *L. (Leishmania)* paraphyletic.

**Conclusions.** The DNA evidence (RFLP of SSU rRNA, kDNA dot blots, RNA polymerase II phylogeny [17] and some nDNA buoyant densities) indicates that the lizard *Leishmania* species are at the crown of the genus *Leishmania* together with the *L. (Leishmania)* species. This topology is consistent with the present distribution of these species. Consequently, although alternative hypotheses may be tenable, the hypothesis that accounts for the most of the currently available data is that the *Leishmanial/Endotrypanum* clade arose in the Neotropics in the Palaeocene (65–55 MYA) or Eocene (54–38 MYA), with a subsequent migration during the Miocene (24–6 MYA) or Pliocene (5–3 MYA) to the Nearctic and then to the Old World where the lizard *Leishmania* species evolved.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the financial support of the International Scientific Cooperation Programme of the European Union (C11-CT92-0060). We thank D. Croan (University of Technology Sydney) for discussing his unpublished data with us and Prof H. Townson and Prof M. Molyneux for critical readings of the manuscript.

**LITERATURE CITED**


