

# Evolution of parasitism: Kinetoplastid protozoan history reconstructed from mitochondrial rRNA gene sequences

(*Trypanosoma/Leishmania/Leptomonas/Crithidia*/kinetoplast DNA)

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**ABSTRACT** A phylogenetic tree for the evolution of five representative species from four genera of kinetoplastid protozoa was constructed from comparison of the mitochondrial 9S and 12S rRNA gene sequences and application of both parsimony and evolutionary parsimony algorithms. In the rooted version of the tree, the monogenetic species *Crithidia fasciculata* is the most deeply rooted, followed by another monogenetic species, *Leptomonas* sp. The three digenetic species *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Leishmania tarentolae* branch from the *Leptomonas* line. The substitution rates for the *T. brucei* and *T. cruzi* sequences were 3–4 times greater than that of the *L. tarentolae* sequences. This phylogenetic tree is consistent with our cladistic analysis of the biological evidence including life cycles for these five species. A tentative time scale can be assigned to the nodes of this tree by assuming that the common ancestor of the digenetic parasites predated the separation of South America and Africa and postdated the first fossil appearance of its host (inferred by parsimony analysis). This time scale predicts that the deepest node occurred at  $264 \pm 51$  million years ago, at a time commensurate with the fossil origins of the Hemiptera insect host. This implies that the ancestral kinetoplastid and its insect host appeared at approximately the same time. The molecular data suggest that these eukaryotic parasites have an evolutionary history that extends back to the origin of their insect host.

The kinetoplastids comprise a widely distributed group of parasitic protozoa that are distinguished by the possession of a single mitochondrion containing a network of catenated mitochondrial DNA minicircles and maxicircles (kinetoplast DNA) in the mitochondrial matrix adjacent to the basal body of the flagellum (1, 2). Two forms of host–protozoan relationships are known: (i) monogenetic kinetoplastids that are parasitic in a single invertebrate host and (ii) digenetic kinetoplastids that are parasitic in two hosts, usually an invertebrate and a vertebrate. These lower eukaryotic cells are the subject of both biological and medical interest.

The 123 monogenetic species that have been described so far belong to the genera *Crithidia*, *Herpetomonas*, *Blastocrithidia*, and *Leptomonas* (3). They are parasitic in arthropods (mainly in Insecta, Diptera, and Hemiptera and also in Hymenoptera, Lepidoptera, Orthoptera, and Siphonaptera). The monogenetic species are known as the “lower trypanosomatids” because the digenetic genera *Leishmania* and *Trypanosoma* are thought to have arisen from a monogenetic ancestor (4). The morphology of the insect-inhabiting stages of the pathogenic digenetic species resembles that of the monogenetic species.

In the absence of a fossil record, a phylogenetic tree must be constructed entirely from biological or, better yet, from molecular sequence data. The mitochondrial 9S and 12S

ribosomal RNAs (5–9) represent one such group of conserved sequences that lends itself to analysis. The sequences of both large and small subunit mitochondrial ribosomal RNAs are known from the lizard leishmania *Leishmania tarentolae* (6, 7), the African pathogenic trypanosome *Trypanosoma brucei* (8), and the lower trypanosomatid *Crithidia fasciculata* (9). We have also determined the sequences of both of the mitochondrial ribosomal RNAs from the monogenetic insect parasite *Leptomonas* sp. and partial sequences of the mitochondrial ribosomal RNAs from the digenetic stercorarian trypanosome *Trypanosoma cruzi*.<sup>§</sup>

## MATERIALS AND METHODS

**Cloning and Sequencing of the *Leptomonas* and *T. cruzi* 9S and 12S RNA Genes.** The *Leptomonas* genes encoding 9S and 12S RNAs were cloned on a 2.58-kilobase (kb) *Bam*HI–*Msp*I maxicircle fragment in phage M13mp10, which was detected by hybridization with the cloned 9S and 12S RNA genes from *L. tarentolae* (6, 7). Overlapping subclones were obtained by the ExoIII procedure (10). Final overlaps were obtained by use of four synthetic oligonucleotides as primers. The sequence of the 2.58-kb fragment was obtained on both strands by the dideoxynucleotide chain-termination method (11). The 5′ and 3′ ends of the 12S and 9S RNA genes were deduced from sequence homologies with the *L. tarentolae* sequences (Fig. 1A).

The *T. cruzi* 9S and 12S RNA genes were cloned on a 3.0-kb *Eco*RI maxicircle fragment, which was detected by hybridization with cloned 9S and 12S RNA genes of *L. tarentolae*. *Dra*I and *Rsa*I subfragments were cloned into the *Hinc*II site of M13mp8, and sequences were determined as above. The partial 9S and 12S RNA gene sequences were determined by sequence homology with the corresponding *L. tarentolae* sequences (Fig. 1A).

## RESULTS AND DISCUSSION

Alignments of both the 9S (small subunit) and 12S (large subunit) mitochondrial ribosomal RNA gene sequences from five kinetoplastid species (*L. tarentolae*, *T. brucei*, *Leptomonas* sp., *C. fasciculata*, and *T. cruzi*) were derived by standard methods (Fig. 1A). The *T. cruzi* sequences are partial, whereas the sequences from the other four species represent complete 9S and 12S RNA gene sequences as deduced from alignments with the *L. tarentolae* sequences for which the 5′ and 3′ ends are known. Because mitochondrial ribosomal RNAs are characterized by substitution rates

Abbreviation: Myr, million years.

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§The sequences reported in this paper are being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) [accession nos. J03814 (*Leptomonas* sp. sequence) and J03815 (*T. cruzi* sequence)].

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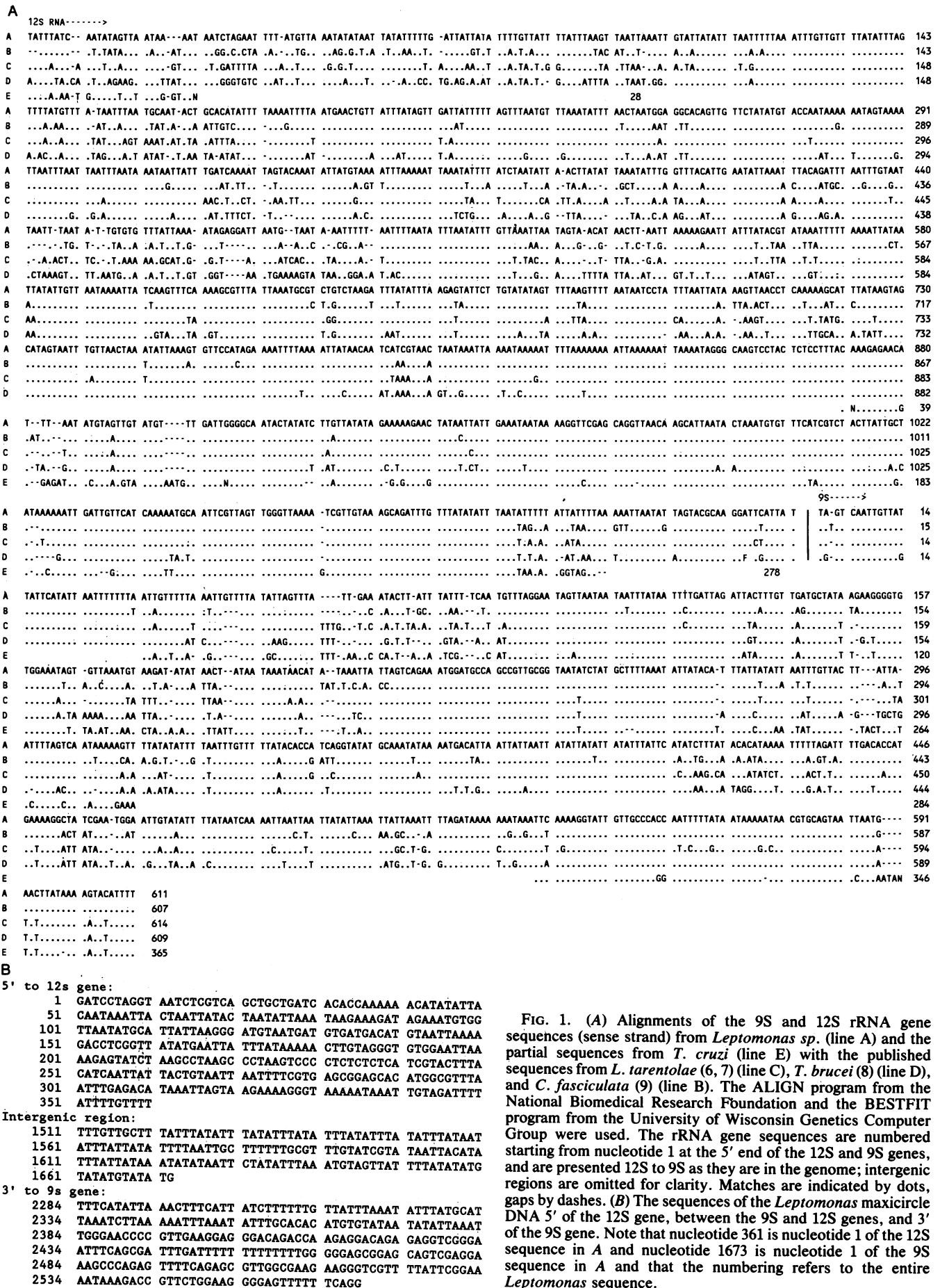


FIG. 1. (A) Alignments of the 9S and 12S rRNA gene sequences (sense strand) from *Leptomonas* sp. (line A) and the partial sequences from *T. cruzi* (line E) with the published sequences from *L. tarentolae* (6, 7) (line C), *T. brucei* (8) (line D), and *C. fasciculata* (9) (line B). The ALIGN program from the National Biomedical Research Foundation and the BESTFIT program from the University of Wisconsin Genetics Computer Group were used. The rRNA gene sequences are numbered starting from nucleotide 1 at the 5' end of the 12S and 9S genes, and are presented 12S to 9S as they are in the genome; intergenic regions are omitted for clarity. Matches are indicated by dots, gaps by dashes. (B) The sequences of the *Leptomonas* maxicircle DNA 5' of the 12S gene, between the 9S and 12S genes, and 3' of the 9S gene. Note that nucleotide 361 is nucleotide 1 of the 12S sequence in A and nucleotide 1673 is nucleotide 1 of the 9S sequence in A and that the numbering refers to the entire *Leptomonas* sequence.

that vary considerably from sequence position to position, and because rates of substitution can vary among organisms (12), we have used the algorithm of "evolutionary parsimony," which is invariant to both of these rate effects (13, 14). An unrooted dendrogram, or tree (not shown), was determined from these sequence alignments. The most parsimonious tree determined by using the PAUP package of Swofford (15) also has the same topology (data not shown). Analysis of either the small or the large subunit sequences also supports this topology. Table 1 lists the percent pairwise differences due to both transitions and transversions in the 9S and 12S RNA genes from all five species. The data indicate that transitions have nearly saturated (12), whereas the percent transversion is sensitive to change in taxa. Hence transversions were used to calculate the lengths of the branches by the method of operator metrics (13, 14).

The root of the tree is most probably in the *Crithidia* branch as shown in Fig. 2. This represents the most parsimonious rooting. Details of the rooting analysis are provided in the legend to Fig. 2. It should be noted that the indicated branching of the two *Trypanosoma* lines from the *Leishmania* line is uncertain because of the limited sequence data from the *T. cruzi* rRNA genes, and this uncertainty is indicated by a dotted ellipse in the diagram. The use of the lizard leishmania species, *L. tarentolae*, as a type specimen for the digenetic genus *Leishmania* is justified by the results of Gomez-Eichelmann *et al.* (18), which showed that lizard leishmania species (including several strains of *L. tarentolae*) are indeed more closely related to mammalian leishmania species by several criteria (kinetoplast DNA sequences, nuclear chromosomes, and membrane lipids) than to *Trypanosoma* species, as has been suggested by Wallbanks *et al.* (19).

Cladistic analysis of the biology of these organisms further supports rooting this tree in the *Crithidia* branch. All kinetoplastid protozoa have insect hosts, but *Trypanosoma* and *Leishmania* have acquired a digenetic life style and require in addition a second (vertebrate) host. This is generally presumed to be a derived adaptation (4) and, as such, supports the topology of our sequence-derived tree and places the root in either the *Leptomonas* or the *Crithidia* branch. Furthermore, *Leptomonas* shares a promastigote stage of development with *Leishmania* and with some *Trypanosoma* species (3). This then further supports the rooting in the *Crithidia* branch. In *Trypanosoma*, an epimastigote form is found in addition. Thus, our interpretation of these data support the sequence-derived rooting.

Biochemical data also support this tree. The lower trypanosomatids are characterized by ease of cultivation and less fastidious nutritional requirements than *Leishmania* and *Trypanosoma* species (20). In addition, it was demonstrated that several enzymes of ornithine-arginine metabolism are present in *Crithidia*, *Leptomonas*, and *Herpetomonas* species and not present in *Leishmania* and *Trypanosoma* (21).

Table 1. Percent pairwise differences between sequences

Species	Sequence differences				
	1	2	3	4	5
1. <i>T. cruzi</i> *	—	5.2	5.1	5.2	5.2
2. <i>T. brucei</i>	16.2	—	5.2	5.2	6.0
3. <i>L. tarentolae</i>	13.5	12.7	—	3.7	4.7
4. <i>Leptomonas sp.</i>	15.4	15.8	11.2	—	4.4
5. <i>C. fasciculata</i>	16.4	16.8	11.7	10.6	—

Percent transitions are listed above the diagonal and percent transversions are listed below the diagonal.

\*Distances have been proportionately scaled for *T. cruzi* to compensate for the partial sequences. Hence these distances are associated with a larger stochastic uncertainty.

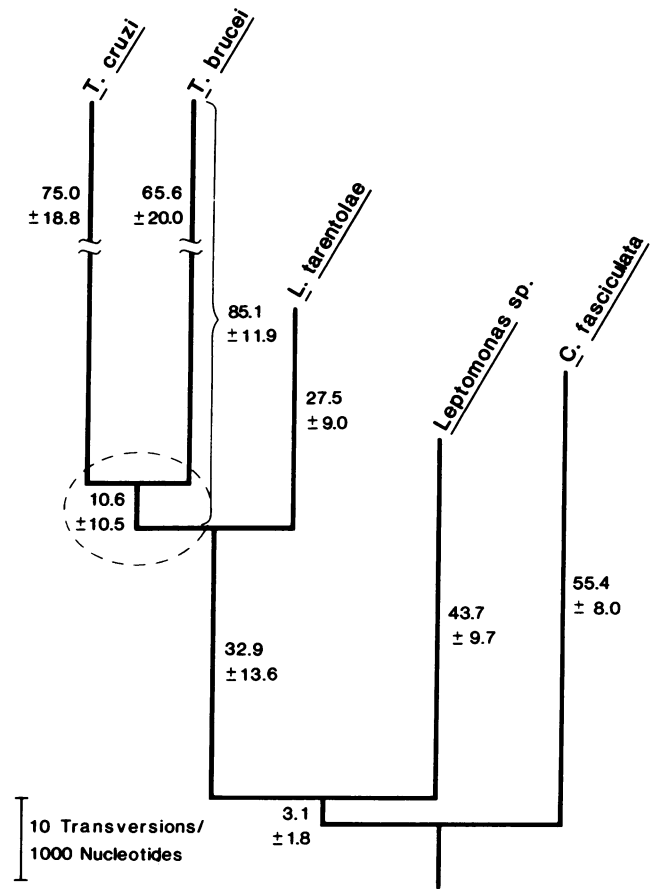


FIG. 2. Rooted tree illustrating the evolution of five taxa of kinetoplastid protozoa. The unrooted tree was calculated from an evolutionary parsimony analysis of combined (large plus small subunit) mitochondrial rRNA sequences and also checked by maximum parsimony by using the PAUP package of Swofford (15). The tree was rooted by parsimony rooting based on the simultaneous application of two criteria—namely, parsimony (16) and the relative rate test (17). The "best" tree is the one that (i) uses the minimum number of rate changes and (ii) satisfies the relative rate test. The 5% confidence limits of the "best" root are shown ( $\pm 1.5$  transversion units). The dotted ellipse indicates that the separate branching of the two *Trypanosoma* lines from the *Leishmania* line is tentative because of the high degree of statistical uncertainty associated with the limited amount of *T. cruzi* sequence data. It is equally possible that the three species share a common branch point.

The mitochondrial rRNA genes of kinetoplastid protozoa resemble those of *Drosophila* and other dipteran species in many ways and differ significantly from those of mammals. Both the dipteran and protozoan sequences are highly A+T rich, with those of the protozoa studied here being slightly higher (83% A+T) than even their dipteran counterparts (80% A+T) (22) and much higher than their mammalian counterparts (50% A+T). In the kinetoplastids, the base composition is less biased at the most conserved regions. For example, nucleotides 219–246 (Fig. 1A, 9S gene) of *Leptomonas* (corresponding to *E. coli* 16S positions 508–535) are strictly conserved among hemoflagellates and have an unbiased (50% A+T) base composition. Again this follows the pattern in flies, where the bias in base composition is most extreme at silent sites. Deficient mismatch repair has been suggested as a mechanism for creating this bias (23), and our data are consistent with the patterns observed by these workers. Furthermore, both dipteran and kinetoplastid sequences exhibit significantly unbalanced G (10.2%)/C (6.6%) ratios.

Substitution patterns in *Leishmania*, *Leptomonas*, and *Crithidia* are similar to the dipteran patterns as well. At

longer divergence times, the ratio of transitions to transversions is approximately 0.40 compared with 0.41 for the dipterans. Operator metric analysis (see Fig. 2) indicates that transversion differences accumulate in a lineage at a rate of approximately 0.023 ( $\pm 0.06$ )/million year (Myr) (excluding the trypanosomes) and at approximately 3.3 times that rate [0.080 ( $\pm 0.020$ )/Myr] for the trypanosomes. The former is very close to the 0.025%/Myr (single lineage rate) determined for fly protein-encoding mtDNAs (22).

We interpret the higher substitution rate observed in the trypanosome lineages to be statistically significant, but the true rate differences may be less than the data in Fig. 2 indicate. Evolutionary parsimony, like other treeing methods, can be strongly biased by unequal base compositions. While it is insensitive to large or small A+T/G+C ratios (provided they are derived solely from unequal transition rates), it is sensitive to some types of differences in transversion rates. For example, if the experimentally observed G  $\rightarrow$  C bias is produced by transversion rates (rather than by specific functional constraints on the rRNA structure), then the lengths of the branches may not be valid. We think such effects are not serious because the two "zero" invariants, which are expected to be most sensitive to this effect, were not significantly nonzero. In general, we emphasize the need for caution in reconstructing phylogenies from sequences with strongly biased base ratios and regard the rate differences as tentative.

Given this rooted tree, one can use parsimony analysis to reconstruct the character states of the common ancestor that led to these four taxa. The hosts for *Leptomonas* are mostly species in the arthropod taxa Diptera and Hemiptera, with a few *Leptomonas* species being parasites of Siphonaptera, Lepidoptera, and Orthoptera. Only four *Leptomonas* species have nonarthropod hosts, including one species in a marine

nematode and one in the macronucleus of *Paramecium*. The hosts for *Crithidia*, *Herpetomonas*, and *Blastocrithidia* are also mostly members of the arthropod taxa Diptera and Hemiptera, with a few species being parasites of Hymenoptera, Lepidoptera, Ixodidae, and Siphonoptera (3). Hence the most parsimonious assignment of host taxa among these groups is Hemiptera and Diptera.

The fossil and geological records, in combination with the character state analyses of the ancestors, allow one to place some practical time limits on the nodes of the tree by following the procedure of Ochman and Wilson (24) and Wilson *et al.* (25). The most useful node for this purpose is the divergence of the *Trypanosoma* and the *Leishmania* lines. This division is unlikely to have arisen earlier than approximately 140 Myr ago since this is the earliest known time of appearance in the fossil record of protoglossiniae (26), the precursor of the tsetse fly (the host of modern African trypanosomes). By a similar line of reasoning, the division is unlikely to have arisen more recently than 80 Myr ago, since the Old World species (*T. brucei*, *L. tarentolae*, *Leishmania tropica*, *Leishmania major*) and the South American species (*T. cruzi*, *Leishmania mexicana*, *Leishmania brasiliensis* complex) presumably diverged before the breakup of Gondwanaland provided for their biological isolation some 80 Myr ago (27, 28). This would then place the time of the *Leishmania/Trypanosoma* split at  $110 \pm 30$  Myr ago.

Analysis of the divergence of mitochondrial maxicircle structural genes between *L. tarentolae* and *T. brucei* (29) and analysis of nuclear DNA polymorphisms within the major *Leishmania* lineages (30) have yielded similar estimates of divergence times for the digenetic species. The estimated 13–25% nuclear sequence divergence among the major lineages of *Leishmania* implies an intragenus divergence time of 10–80 Myr ago (30). The observed 19–35% amino acid sequence

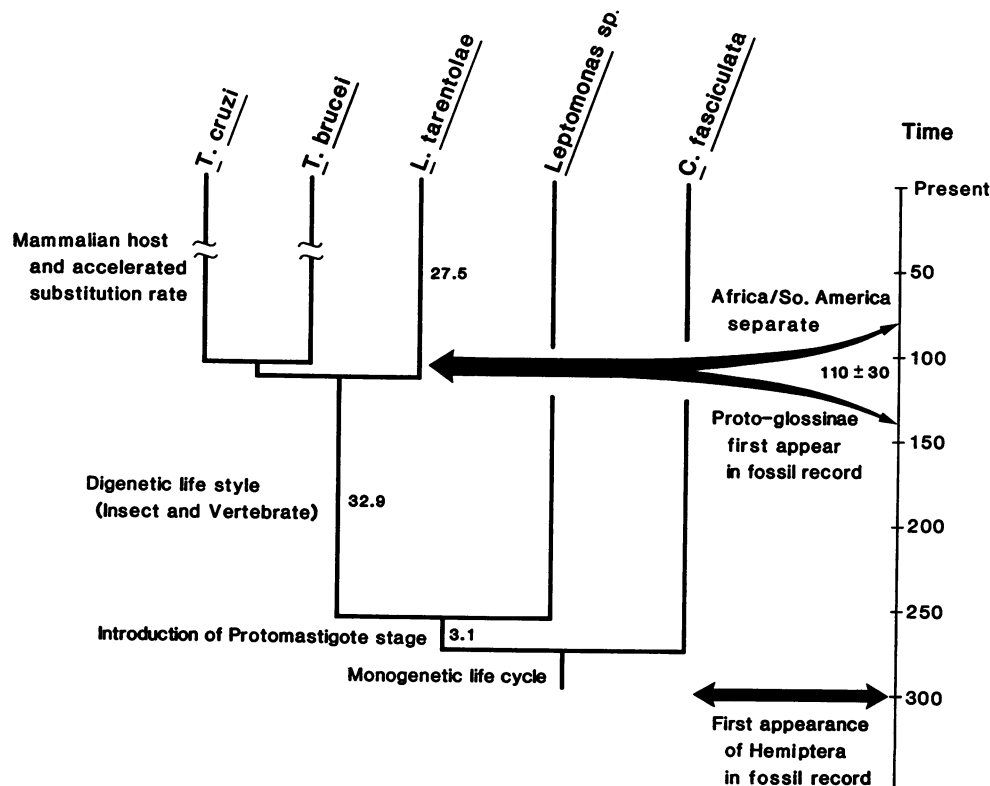


FIG. 3. Schematic illustration of the rooted tree linking kinetoplastid protozoa. Important steps in the evolution of these organisms are (i) acquisition of an insect host (monogenic life cycle), (ii) introduction of the promastigote stage of cell development, (iii) development of a digenetic life style, and (iv) acquisition of an accelerated rate of evolutionary substitutions in *Trypanosoma*. Dates of two important nodes inferred from the fossil/geological record are shown. All lengths have been standardized to the *L. tarentolae* lengths shown.

mismatch between six mitochondrial genes of *L. tarentolae* and *T. brucei* is consistent with an even older divergence time for these two genera (29).

As an independent check on the time scale, fixation of the *Leishmania/Trypanosoma* split in Fig. 3 at 110 Myr ago and assumption of a constant rate of nucleotide substitution equivalent to the *Leishmania* rate would predict that the deepest node, the root, occurred at  $264 \pm 51$  Myr ago. The most parsimonious assignment of ancestor host (for the *Leishmania* and *Crithidia* division) is Hemiptera. Fossil evidence suggests that Hemiptera arose during the Pennsylvanian period approximately 300 Myr ago (31, 32). Thus, our time scale is consistent with this fossil evidence. Furthermore, these results imply that the ancestral kinetoplastid and its insect host appeared at approximately the same time, suggesting coevolution of parasite and host.

We anticipate that inclusions of sequences of the 9S and 12S RNAs from additional kinetoplastid species should significantly reduce the uncertainties associated with the positions of these branch points. Sequences from *Herpetomonas* and *Blastocrithidia* species should prove especially informative in view of the assumption by most workers in the field that these genera represent evolutionary precursors of the more advanced digenetic genera (4).

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