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Evolution of the genus *Leishmania* as revealed by comparisons of nuclear DNA restriction fragment patterns

(protozoan parasites/evolutionary trees/visceral leishmaniasis/species identification/divergence times)

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ABSTRACT **Restriction endonuclease DNA fragment patterns have been used to examine the relationships among 28 isolates of *Leishmania* as well as *Crithidia, Endotrypanum*, and *Trypanosoma cruzi*. Fragments of nuclear DNA were generated with six restriction enzymes, and blots were hybridized with probes from three loci. Among the major lineages the fragment patterns are essentially completely different, while within the major lineages various degrees of divergence are found. Molecular evolutionary trees were constructed using the method of Nei and Li to estimate the percent nucleotide sequence divergence among strains from the fraction of fragments shared. Defined groups, such as species or subspecies within the major lineages, are also grouped by nuclear DNA comparisons. Within the *donovani* complex, we find *Leishmania donovani chagasi* and *Leishmania donovani infantum* to be as similar as strains within *Leishmania donovani donovani*, consistent with the proposal by other workers that New World visceral leishmaniasis originated quite recently.**

Protozoans belonging to the genus *Leishmania* are frequently parasites in humans, causing a spectrum of diseases whose severity ranges from mild through severely disfiguring to lethal (1). A variety of clinical, biological, and geographical criteria were initially used to classify species (1). With further study it became evident that geographical and pathological criteria were often inadequate to discriminate among different isolates, and biochemical and molecular approaches were employed, including comparisons of isoenzymes (2–5), kinetoplast DNA (6–9), proteins (10), and antigens (11–14). Currently the genus *Leishmania* includes the following several species complexes: the *tropica* complex, consisting of the species *L. tropica, L. major*, and *L. aethiopica*, and the *mexicana, donovani*, and *braziliensis* complexes, consisting of the subspecies *L. mexicana, L. donovani*, and *L. braziliensis*, respectively (1). Some workers have proposed that the subspecies within the species complexes should be elevated to species status (1, 15).

We have examined the relationships of species within *Leishmania* with the aim of developing quantitative molecular evolutionary trees. Comparisons of suitable macromolecules allow the construction of molecular evolutionary trees whose topology reflects that of species (orthologous molecular comparisons; ref. 16) and whose molecular distances reflect the approximate time of evolutionary divergence. Molecular evolutionary trees thus provide a quantitative temporal framework with which to view the evolution of species (17).

Comparisons of nuclear DNA have been successfully employed in other organisms for the estimation of temporal and cladistic relationships of species (18–20). We used Southern blot hybridization with various defined nuclear DNA probes to obtain data that was analyzed by the method of Nei and Li (21) to estimate first the fraction of DNA fragments shared among species and then the percent of nucleotide sequence divergence. This method yields results that generally agree with comparisons of DNA sequences or of other parameters (refs. 20, 22, and 23; K. Helm-Bychowski and A. C. Wilson, personal communication). However, the calculation of percent divergence assumes that all fragment differences are attributable to point mutations. This assumption may not always be valid for nuclear DNA, where the presence of detectable length mutations would lower the fraction of fragments shared between two species and thus inflate the estimate of sequence divergence (24). To detect any anomalies caused by length mutations, we examined three independent regions of the nuclear genome and analyzed each data set separately as well as in combination. Our analysis indicates that the fragment comparison method is indeed suitable for comparisons within the major lineages of *Leishmania*.

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MATERIALS AND METHODS

Strains. Thirty-one strains and species of kinetoplastid parasites were examined (Table 1). The World Health Organization recommended nomenclature for the taxonomic names of *Leishmania* isolates has been followed (1).

DNA Isolation and Blot Hybridization. Total cellular DNAs were isolated as described for *L. major* (25, 26). Blot hybridization was performed as described (27), except that the hybridization and washing temperature was 60°C.

Hybridization Probes. The hybridization probes were as follows: pLT-1, corresponding to the β-tubulin repeat unit of *L. major* strain WR309 (28); pLTS-108-S48 and pLTS-2-S50, 4.8-kilobase (kb) and 5.0-kb *Sal* I fragments from *L. major* strain LT252 that are amplified within the H and R regions, respectively, in methotrexate-resistant lines (29). The H and R region probes identify stable RNAs in RNA gel blots (G. Kapler, T. Ellenberger, and S.M.B., unpublished data).

The parasite DNAs were digested with six different enzymes (*Sal* I, *Kpn* I, *Sac* I, *Hin*dIII, *Pst* I, and *Sph* I). These six enzymes were selected because they digest *Leishmania* DNA at a reasonable frequency, and their recognition sequences include every dinucleotide pair at least once, thereby minimizing any potential dinucleotide bias (30). The total number of DNA fragments visualized was 1788.

Calculation of Molecular Divergence and Evolutionary Tree Construction. With data from blot hybridization fragment patterns, the equations of Nei and Li (21) were used to calculate the fraction of fragments shared (F) and the percent nucleotide sequence difference (Δ). All fragments were weighted equally, regardless of the intensity of hybridization, including the unit repeat of the β-tubulin locus evident in many species (ref. 28; this is appropriate because these genes are probably evolving in a concerted manner). Δ values were used to construct evolutionary trees using the method of

Table 1. *Leishmania* strains used in this work

Isolate no.*	Species	Designation	Source†
1	*L. braziliensis guyanensis*	MHOM/BR/83/BOS-5	a
2	*L. braziliensis guyanensis*	MHOM/BR/75/M4147	a
3	*L. braziliensis braziliensis*	MHOM/BR/76/LTB0014	b
4	*L. braziliensis braziliensis*	MHOM/BR/75/M2903	a
5	*L. braziliensis panamensis*	MHOM/PA/74/WR120	c
8	*L. hertigi deanei*	MCOE/BR/00/M5088	a
9	*L. hertigi hertigi*	MCOE/PA/65/C8	a
10	*Leishmania* sp.	MHOM/BR/81/Xabi	d
12	*L. mexicana* ssp.	MHOM/DR/00/Isabel	d
14	*L. mexicana mexicana*	MHOM/BZ/82/Bel 18C	e
15	*L. mexicana amazonensis*	MHOM/BR/77/LTB0016	b
17	*L. enrietti*	MCAV/BR/45/L88	f
19	*L. tarentolae*	LTC-1	g
21	*L. aethiopica*	MHOM/ET/72/L100	h
22	*L. aethiopica*	MHOM/ET/00/TWG	h
23	*L. tropica*	MHOM/AF/00/LRC-L8	h
24	*L. tropica*	MHOM/SU/60/LRC-L39	h
25	*L. tropica*	MHOM/IR/00/LRC-L18	h
26	*L. major*	MHOM/IL/79/LRC-L251	c
27	*L. major*	RHO/IR/75/ER	i
28	*L. major*	MHOM/IR/83/LT252	j
29	*L. major*	MHOM/SN/00/DK-106	k
30	*L. major*	CAN/EG/84/D-1	k
31	*L. donovani donovani*	MHOM/ET/67/HV-3	l
32	*L. donovani donovani*	MHOM/SD/80/WR378	c
33	*L.donovani donovani*	MHOM/SD/62/1S	k
34	*L. donovani chagasi*	MHOM/HN/1974/1151-III	l
35	*L. donovani infantum*	MHOM/FR/00/SXI	l
37	*Crithidia fasciculata*	CFC-1	g
7	*Endotrypanum shaudini*	M6159	a
40	*Trypanosoma cruzi*	Peru	m

*Isolate number is in our data set.
†Sources of strains were as follows: a, J. J. Shaw and R. Lainson; b, P. Marsden; c, L. Hendricks; d, F. Neva; e, D. Evans, f, M. Hommel; g, L. Simpson; h, L. Schnur; i, F. Modabber; j, S. Meshnick; k, S. Giannini; l, P. McGreevey and P. Jackson; m, C. C. Wang.

Fitch and Margoliash (31), which were evaluated as described (32).

RESULTS

Fragment Hybridization Data. The nuclear DNA fragment patterns of many *Leishmania* species are very different, usually as different from one another as they are from the patterns of other genera such as *Crithidia* (Fig. 1). We define species (or groups of species) whose fragment patterns are essentially completely different as major lineages. By this criterion the major lineages consist of the *Leishmania tropica, mexicana, donovani*, and *braziliensis* species complexes, *L. hertigi, L. enrietti, L. tarentolae*, and the three genera *Endotrypanum, Crithidia*, and *Trypanosoma*. Interestingly, the DNA of an unusual isolate from patients, which was tentatively classified as a leishmania ("Xabi"), is not detectably related to any other species, as reported by others (33).

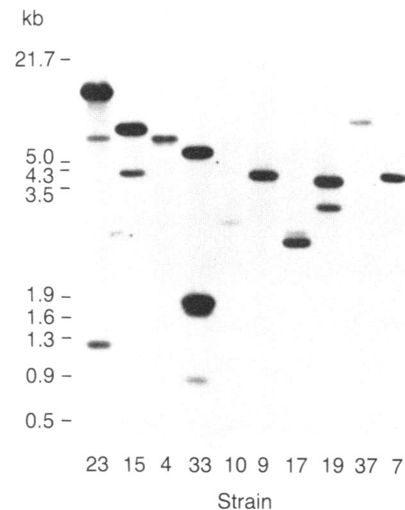

FIG. 1. DNA fragment patterns among species complexes of *Leishmania* and other genera of the order Kinetoplastida. DNAs from the indicated isolates (Table 1) were digested with *Sac* I, separated on 0.8% agarose gels, transferred to GeneScreenPlus, and hybridized with the probe pLTS-S50. Molecular size markers (kilobase pairs, kb) are indicated on the side.

As the fragment patterns among the major lineages are very different, exhibiting what are apparently only fortuitous identities in a small proportion of fragments, it is impossible to accurately quantify the differences among these groups using the fragment comparison method (21). This is represented in the evolutionary tree of Fig. 2 as a thick ancestral "divergence" event, and reflects the limitations of the fragment method when applied to distantly related species rather than a specific statement of the relative relationships of these groups. In our data set the divergences among the major lineages correspond to an apparent Δ of about 13%, the true Δ values being indeterminately greater. This value agrees well with estimates for the limits for the usage of Δ by other workers (M. Nei, personal communication; refs. 34 and 35). Because the DNA probes hybridized with all species DNA and to roughly the same number of fragments (Tables 2–5), we estimate that the average percent sequence divergence among the nuclear DNA of the major lineages is <25%.

Comparisons Within Species and Species Complexes. Within the major lineages the fragment patterns are related to various degrees, allowing quantitation by the fragment comparison method. This is shown in Fig. 3 for isolates belonging to the *tropica* complex of Old World cutaneous *Leishmania*, consisting of the species *L. major, L. tropica*, and *L. aethiopica*. Members within each species are quite similar to each other, while intermediate degrees of DNA fragment pattern relatedness are observed among the three species.

The nuclear DNA of isolates within a taxonomic group are more closely related than they are to different groups. Δ is 0.44 between two isolates of *L. braziliensis guyanensis* and 0.49 between two isolates of *L. b. braziliensis*, whereas the mean intersubspecies Δ for the *braziliensis* complex is 1.5 (Table 4). Within *L. donovani donovani* the average Δ is 0.4 compared to 1.7 for the intersubspecies comparisons within the *donovani* complex (Table 3). Within the *tropica* complex the average values of Δ within species (0.1, 0.8, and 1.0) are all less than the interspecific comparisons (3.1, 5.5, and 5.8;

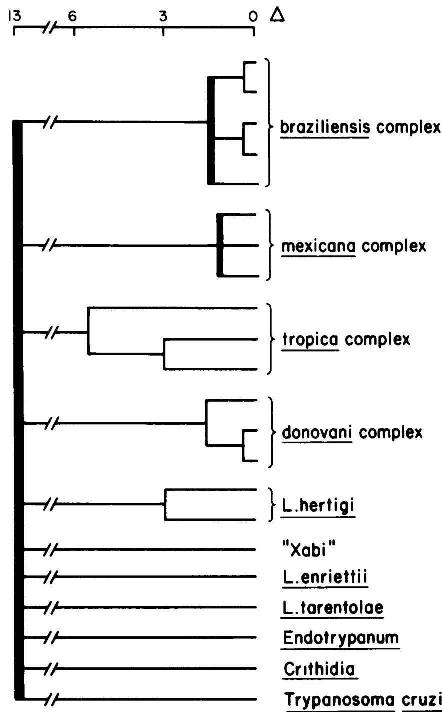

FIG. 2. Molecular tree depicting DNA fragment relationships within the order Kinetoplastida. Relationships of nuclear DNA fragment patterns are presented in the form of a molecular evolutionary tree. The horizontal scale is that corresponding to Δ, the estimated percent of nucleotide sequence difference. The thick vertical bar located at Δ = 13 corresponds to the approximate point at which the fragment patterns are completely different, which is a minimal estimate of the true value of Δ among the major lineages, and does not imply that all descendant lineages are equally related or unrelated. The relationships along the lineages descendant from the thick black bar do indicate molecular relationship. The branching orders of these evolutionary trees were calculated using the method of Fitch and Margoliash (30), using the goodness-of-fit parameter "F" (31) to choose the trees most closely in agreement with the data. A thicker vertical line is used to indicate where the exact relationships are ambiguous by the aforementioned criterion. The position of each branchpoint is plotted as the average of all comparisons passing through the branchpoint (33). The three lineages within the *braziliensis* complex correspond to the subspecies.

Table 2). The subspecies of *L. hertigi* are also closely related (F = 0.6; Δ = 3.0; 41–48 fragments). The relationships of nuclear DNA may be visualized in the form of evolutionary trees (Figs. 2 and 4).

Table 3. DNA fragment pattern relationships within *L. donovani*

Species	Isolate no.	Isolate no. 31	32	33	34	35
L. d. donovani	31	**71**	0.29	0.44	1.8	1.6
L. d. donovani	32	0.95	**68**	0.41	1.6	1.6
L. d. donovani	33	0.92	0.93	**74**	1.9	1.6
L. d. chagasi	34	0.74	0.75	0.72	**65**	0.4
L. d. infantum	35	0.76	0.76	0.76	0.93	**66**

For a description of the isolates see Table 1 and of the data see Table 2.

Factors Affecting the Accuracy of Molecular Trees. Δ values obtained using each probe separately for each pairwise comparison are correlated (r = 0.86–0.87). Interestingly, the Δ values for the H and R region probes are 1.9-fold higher than those obtained for the β-tubulin probe, possibly because of conservation of the tubulin protein during evolution (36). The relationships presented in Figs. 2 and 4 are unchanged by subdivision of the data by enzyme or by probe (results not shown).

The steadiness of the rate of evolution of Δ within the *tropica* complex was examined using the method of Nei (37) and relative rate tests (17). Δ appears to evolve as steadily as other molecular indices such as amino acid or nucleotide sequence difference (data not shown). This finding, as well as the invariance of the molecular evolutionary trees for the subdivided data, suggests that DNA rearrangements are not responsible for a major fraction of the fragment pattern variability within the species complexes, since extensive DNA rearrangement would be visualized as radical alterations in fragment pattern and relationships among the different lineages.

DISCUSSION

Evolutionary Relationship and Nuclear DNA Relatedness. Our data indicate that relationships of *Leishmania* and other members of the Trypanosomatidae, as revealed by quantitative comparisons of nuclear DNA fragment patterns, correspond to those predicted from previous taxonomic and molecular studies (1). The following two levels of divergence are apparent in the nuclear DNA: that among the major lineages, in which the fragment patterns are very different, and that within the major lineages, in which various degrees of relatedness are observed. The major lineages determined by nuclear DNA correspond to recognized organismal divisions, including the species complexes of human *Leishmania tropica, mexicana, donovani, braziliensis*), *L. hertigi, L.*

Table 2. Nuclear DNA fragment pattern relationships within the *tropica* complex of *Leishmania*

Species	Isolate no.	Isolate no. 21	22	23	24	25	26	27	28	29	30
L. aethiopica	21	**60**	1.0	3.2	3.2	3.2	5.6	5.9	5.9	5.2	5.9
L. aethiopica	22	0.84	**60**	3.0	3.1	3.0	5.9	6.2	5.9	5.4	6.2
L. tropica	23	0.58	0.60	**64**	0.1	0.2	5.5	6.1	5.8	5.2	6.8
L. tropica	24	0.57	0.59	0.98	**66**	0.10	5.2	5.6	5.4	4.8	6.2
L. tropica	25	0.58	0.60	0.97	0.98	**64**	5.0	5.5	5.3	4.7	6.1
L. major	26	0.39	0.37	0.39	0.42	0.43	**58**	0.61	0.83	0.6	1.4
L. major	27	0.37	0.36	0.36	0.39	0.39	0.90	**58**	0.5	0.71	0.87
L. major	28	0.37	0.37	0.38	0.40	0.41	0.86	0.91	**58**	0.71	0.74
L. major	29	0.42	0.40	0.42	0.44	0.45	0.90	0.88	0.88	**60**	1.1
L. major	30	0.37	0.36	0.32	0.35	0.36	0.78	0.86	0.88	0.82	**47**

This table summarizes comparisons among *Leishmania* strains whose numbers are listed at the head of each column and row. See Table 1 for a description of each isolate. Values (bold numbers) on the diagonal are the number of fragments visualized for each species; values below the diagonal are F (the fraction of fragments shared between two species), and values above the diagonal are Δ [the percent of nucleotide sequence divergence, calculated according to Nei and Li (21)].

Table 4. DNA fragment pattern relationships within *L. braziliensis*

Species	Isolate no.	Isolate no. 1	2	3	4	5
L. b. guyanensis	1	**52**	0.44	2.0	1.6	1.1
L. b. guyanensis	2	0.92	**54**	1.5	1.3	1.2
L. b. braziliensis	3	0.71	0.77	**55**	0.49	1.7
L. b. braziliensis	4	0.75	0.79	0.92	**52**	1.4
L. b. panamensis	5	0.82	0.81	0.74	0.78	**55**

For description of entries see Table 2 and of isolates see Table 1.

enrietti, *L. tarentolae*, and the genera *Endotrypanum*, *Crithidia*, and *Trypanosoma cruzi*. Due to the limitations of the fragment method, when applied to distantly related species, we cannot currently determine the relative relationships among the major lineages. This task will require the analysis of a more slowly evolving molecule, such as rRNA (38, 39) or the coding region of intracellular proteins (17). Nonetheless, the major lineages of nuclear DNA accurately reflect divisions established by organismal and molecular criteria of these species. Similarly, the nuclear DNA relationships of isolates within the major lineages are in agreement with current views (ref. 1; see below). Our findings suggest that comparisons of nuclear DNA fragment patterns can be used to establish molecular evolutionary relationships within the major lineages of *Leishmania*.

The estimated nuclear DNA sequence divergence among the major lineages of *Leishmania* is 13–25%, which is comparable to that observed between animal species that diverged 10–80 million years ago (19). This suggests that divergence times within the *Leishmania* may be surprisingly ancient. Comparisons of the kinetoplast DNA maxicircle also indicate an old divergence among major lineages of the Trypanosomatidae (40).

The divergence of the *tropica* complex members *L. tropica* and *L. aethiopica* from *L. major* is the most distant intracomplex divergence, with a Δ of ≈ 6 compared with Δs of <2 among other subspecies within the other complexes. The relative molecular divergence of the intracomplex species and subspecies could be viewed as supporting the current classification (1), as opposed to proposals to elevate the subspecies of *L. donovani* and other *Leishmania* to species (1, 15). It is premature, however, to use quantitative molecular studies solely to decide this point, as molecular divergence in many taxa is frequently "uncoupled" from morphological and taxonomic divergence [refs. 17, 41, and 42; for example, humans (family Hominidae) and chimpanzees (family Pongidae) are as molecularly related as sibling species of *Drosophila*]. For this reason the elevation of the subspecies of *Leishmania* to species must rest upon the evaluation of other criteria.

Comparison of Nuclear DNA and Isoenzyme Divergence. Several workers have employed comparisons of isoenzymes to quantify genetic divergence and construct molecular evolutionary trees for *Leishmania* (43–46). The Δ values are correlated with the isoenzymatic estimates of genetic distance, D ($r = 0.87$, $n = 18$; unpublished data). Furthermore, the relationships within the complexes depicted in Figs. 2 and 4 are consistent with those predicted from isoenzyme comparisons. Surprisingly, we find more nuclear DNA divergence within *L. major* than within *L. tropica*, whereas three studies of isoenzymes (3, 43, 44, 47) find the opposite result. The discrepancy may be due to the limited number of DNAs examined from strains within *L. tropica*. Nonetheless, overall the agreement between the isoenzymatic and nuclear DNA data is good.

Table 5. DNA fragment pattern relationships within *L. mexicana*

Species	Isolate no.	Isolate no. 12	14	15
L. mexicana sp.	12	**58**	1.0	1.2
L. m. mexicana	14	0.83	**53**	1.5
L. m. amazonensis	15	0.81	0.77	**50**

For description of entries see Table 2 and of isolates see Table 1.

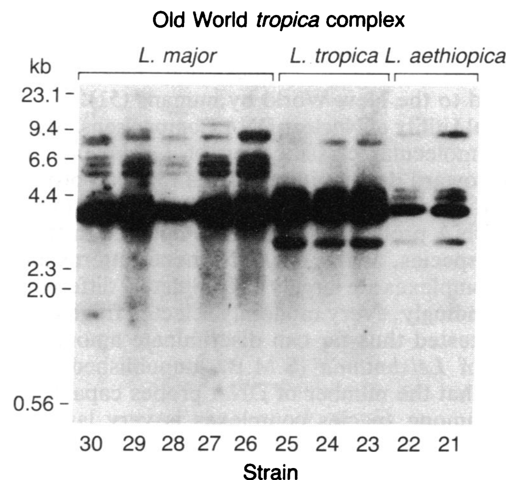

FIG. 3. DNA fragment patterns within the *tropica* species complex of *Leishmania*. DNAs from the indicated isolates (Table 1) were analyzed as described in the legend to Fig. 1, except that the probe was pLT-1 (β-tubulin), and the DNAs were digested with *Sph* I.

Origin of New World Visceral Leishmaniasis. Our data indicate that the New World *Leishmania donovani chagasi* is very closely related to the Old World *Leishmania donovani infantum* ($\Delta = 0.5$) and *L. d. donovani* ($\Delta = 1.7$). The divergence of the subspecies is comparable to that observed within animal populations, which ranges from 0.3 to 4% (20, 48, 49); from our data we calculate the average intrasubspecific divergence of nuclear DNA in *Leishmania* to be 0.5%. *L. d. infantum* and *L. d. chagasi* may, therefore, be as closely related to each other as two random individuals from the same population. Thus, comparisons of nuclear DNA as

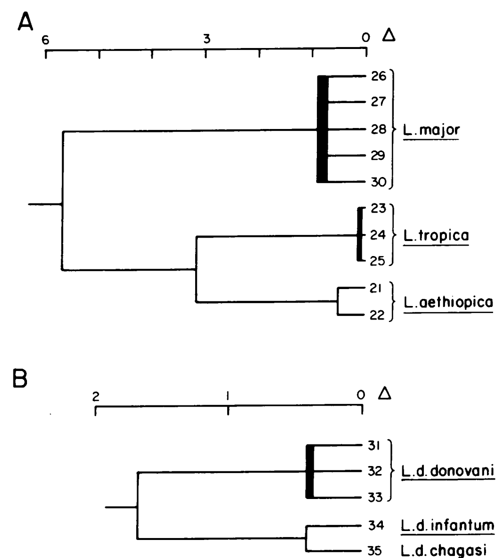

FIG. 4. Molecular trees depicting DNA fragment relationships with the *tropica* and *donovani* complexes of *Leishmania*. (*A*) The *tropica* complex. (*B*) The *donovani* complex. For methods of tree construction and display see the legend to Fig. 2. The small numbers correspond to the species and isolates listed in Table 1.

well as isoenzymes (46, 50) clearly indicate a recent separation of *L. d. chagasi* and *L. d. infantum* and lend credence to the proposal that *L. d. chagasi* may have in fact been introduced to the New World by humans (51).

Potential Utility of Nuclear DNA Comparisons. Much of the work on molecular classification of *Leishmania* has been oriented toward developing methods that can provide rapid identification of species (2, 7, 9, 11). Our data indicate that nuclear DNA probes may also be employed in the identification of species, as the DNA fragment patterns among the species complexes are largely or completely different (Fig. 1). Correspondingly, every randomly selected fragment of DNA we have tested thus far can discriminate among the major lineages of *Leishmania* (S.M.B., unpublished data). This suggests that the number of DNA probes capable of distinguishing among species complexes is very large, possibly equal to the number of restriction DNA fragments (about 10,000 for the genome of *Leishmania*). Similar results have been obtained for comparisons within *Trypanosoma* (52, 54) and *Plasmodium* (53).

While suitable for use in identifying *Leishmania* species in the laboratory, nuclear DNA probes are probably not well suited for clinical diagnostic purposes, as the sensitivity is low and the analysis requires considerable time. The major value of nuclear DNA probes is that they allow one to develop quantitative molecular evolutionary trees depicting the relationships among species. Additionally, as nuclear DNA and many other molecular metrics appear to evolve in a relatively steady, time-dependent manner within many lineages (17–19), these quantitative methods approximately estimate the relative timing of evolutionary divergence events among species. For protozoans such as *Leishmania*, where there is essentially no fossil record, molecular methods currently offer the only means for accomplishing this goal. A knowledge of the evolutionary history of this important genus will allow the examination of a number of interesting questions, including the coevolution of host, of parasite, and of insect vectors, and the molecular and morphological adaptations of the *Leishmania* parasite during evolution.

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