Genetic Data Showing Evolutionary Links between *Leishmani*a and *Endotrypanu*m

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Striking similarities at the morphological, molecular and biological levels exist between many trypanosomatids isolated from sylvatic insects and/or vertebrate reservoir hosts that make the identification of medically important parasites demanding. Some molecular data have pointed to the relationship between some Leishmania *species and* Endotrypanum, *which has an important epidemiological significance and can be helpful to understand the evolution of those parasites. In this study, we have demonstrated a close genetic relationship between* Endotrypanum *and two new leishmanial species,* L. (V.) colombiensis *and* L. (V.) equatorensis. *We have used (a) numerical zymotaxonomy and (b) the variability of the internal transcribed spacers of the rRNA genes to examine relationships in this group. The evolutionary trees obtained revealed high genetic similarity between* L. (V.) colombiensis, L. (V.) equatorensis *and* Endotrypanum, *forming a tight cluster of parasites. Based on further results of (c) minicircle kDNA heterogeneity analysis and (d) measurement of the sialidase activity these parasites were also grouped together.*

Key words: *Leishmania colombiensis - Leishmania equatorensis - Endotrypanum* - multilocus enzyme electrophoresis - molecular characterization - numerical analysis - sialidase activity - kDNA

Parasitic protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) are biologically diverse group of microorganisms. Taxonomic studies of leishmanial isolates from the New World indicate tremendous diversity within this genus (Cupolillo et al. 1995). A number of new *Leishmania* species from sylvan areas of the Neotropics are associated with disease in humans; others appear to be restricted to lower orders of mammals, such as rodents and edentates (Grimaldi et al. 1989).

Sloths are reservoir hosts of at least five named *Leishmania* species of the subgenus *Viannia* [*L. guyanensis* Floch, 1954; *L. panamensis* Lainson & Shaw 1972; *L. shawi* Lainson et al. 1989; *L. colombiensis* Kreutzer et al. 1991 and *L. equatorensis* Grimaldi et al. 1992], responsible for human cutaneous and/or mucosal leishmaniasis (Grimaldi & Tesh 1993). Infections with other biologically distinct groups of trypanosomatid protozoa, such as *Endotrypanum* and *Trypanosoma* are also found in sloths (Deane 1961, Pipkin 1968, Travi et al. 1989, Shaw 1992).

In nature, all *Leishmania* spp. are transmitted by the bite of infected phlebotomine sand flies (Diptera:Psychodidae). However, many flagellates other than *Leishmania* commonly are found in sand flies in Neotropical forests. Arias et al. (1985) identified *E. schaudinni* and other *Endotrypanum* sp. infections in sand flies and sloths captured in the Amazon Region of Brazil. Results of kinetoplast DNA probe identifications of promastigotes present in sand flies captured near Manaus, Brazil also demonstrated *Endotrypanum* infections in *Lu. shannoni*, as well as in *Lu. umbratilis* and *Lu. anduzei* (Rogers et al. 1988). Further evidence for the development of *Endotrypanum* in phlebotomines was obtained by feeding several laboratory-reared sand fly species on infected sloths (Christensen and Herrer 1976, 1979, Shaw 1981).

Endotrypanum spp. are digenetic trypanosomatids in that they are intraerythrocytic parasites of sloths and are transmitted by phlebotomine sand flies (Shaw 1992). *Endotrypanum* shares many other charecteristics with *Leishmania*. Cul-

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tured-derived promastigotes of parasites in both genera are morphologically similar. Studies employing monoclonal antibodies for the analysis of the genus *Endotrypanum* have shown antigenic similarites between these parasites and some *Leishmania* species (Franco et al. 1997). Furthemore, molecular trees clustered the sandfly-borne digenetic parasites *Leishmania* and *Endotrypanum* together, sharing a common ancestor and representing a relatively recent lineage from the Trypanosomatidae family (Fernandes et al. 1993). Results of hybridization using kDNA probes (Pacheco et al. 1990) support the view that *Endotrypanum* and the peripylarian leishmanial parasites of the subgenus *Viannia* Lainson & Shaw 1987 are phylogenetically close (Shaw 1992). In addition, phylogenetic studies have demonstrated that the most divergent *Leishmania* species are *L. (L.) hertigi* and *L. (L.) herreri*, claimed to be closer to *Endotrypanum* than to the other *Leishmania* (Croan & Ellis 1996, Noyes et al. 1996, 1997, Croan et al. 1997).

In this study, we have shown evolutionary links between *Endotrypanum* and some leishmanial parasites based on their molecular genetics, as characterized using a broad assemblage of methodologies. The data presented here demonstrate that *E. schaudinni*, *L. (V.) colombiensis* and *L. (V.). equatorensis* form a tight phylogenetic cluster, an evolutionary linked group that should be explored to understand the origin(s) of neotropical pathogenic *Leishmania*.

MATERIALS AND METHODS

Parasites - *Leishmania* and *Endotrypanum* (Table I) were cultured in Scheneider's Drosophila medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Biolab, Rio de Janeiro, Brazil) at 24^{o}C. In the preparation of samples, the parasite (promastigotes in the late phase of growth cultures) were harvested by centrifugation (3,800x g for 15 min at 4^{o}C) and washed twice in saline pH 8.0, containing the appropriate buffer.

Biochemical/Molecular characterization - The procedures used for characterizing the parasites (multilocus enzyme electrophoresis - MLEE, measurement of the sialidase activity, PCR amplification and restriction enzyme digestion of the parasite ITSrRNA, cloning and sequencing of the conserved region of the minicircle kDNA molecules) have been described in detail in previous publications (Cupolillo et al. 1994, 1995, Medina-Acosta et al. 1994, Fernandes et al. 1996). Sialidase activity was measured using a single-cell HITACHI F-4500 spectrofluorometer (350 nm excitation and 460 nm emission wavelengths). The sequencing was performed in automatic sequencing (AbiPrisma, Applied Biosystem).

Numerical analysis - The MLEE data was analyzed by phenetic methods using the NTSYS software program (version 1.7, exeter software). Principal coordinate analysis was performed based on Euclidian distance between the samples. The similarity level between the *Leishmania* species and *Endotrypanum* was calculated using the Jaccard's coefficient. The kDNA sequences of the parasites were analyzed using the MEGA program (Kumar et al. 1993). The number of differences between the sequences were calculated and a similarity tree constructed by the Neighbor-Joining method. Bootstrap analysis was based on 500 replicates.

RESULTS AND DISCUSSION

Leishmania and *Endotrypanum* are very close protozoan parasites (Fernandes et al. 1993) commonly found in the same vertebrate and insect hosts. Recents studies have showing the relationship between *Endotrypanum* and some New World *Leishmania* species, mainly those from *L. (L.) hertigi* and *L. (L.) herreri* complex (Croan & Ellis 1996, Noyes et al. 1996, 1997, Croan et al. 1997). Moreover, DNA analysis of phylogenetically informative RNA polymerase II gene of *L. (V.) equatorensis* and *Endotrypanum* demonstrated sequence similarities among these parasites (JJ Shaw, pers. commun.). Similarly, it appears that a close antigenic links may exist between *L. (V.) colombiensis, L. (V.) equatorensis* and *Endotrypanum* (Franco et al. 1997, Grimaldi et al. 1992).

Leishmania (V.) colombiensis was found infecting humans, sloths (*Choloepus hoffmanni*), sandflies (*Lu. hartmani* and *Lu. gomezi*), and dogs in Colombia, Panama, and Venezuela (Kreutzer et al. 1991, Delgado et al. 1993, unpublished data). *L. (V.) equatorensis* is an enigmatic parasite, which was isolated from the viscera of a sloth (*C. hoffmanni*) and a squirrel (*Sciurus granatensis*), captured in humid tropical forest on the Pacific Coast of Ecuador. Data based on biological and molecular criteria, as well as numerical zymotaxonomy analysis indicated that both these parasites are clearly distinguishable from all other known species, but clustered within the *L. (V.) braziliensis* complex (Kreutzer et al. 1991, Grimaldi et al. 1992). Multilocus enzyme electrophoresis data and the restriction fragments of the internal transcribed spacers of the rRNA gene (Cupolillo et al. 1995, 1997) have indicated a close relationship between *L. (V.) equatorensis* and *L. (V.) colombiensis*, as previously demonstrated (Kreutzer et al. 1991, Grimaldi et al. 1992). In order to better understand their taxonomic position in the genus, especially in relation to the discrimi-

TABLE I

Origin and Identification of *Leishmania* and *Endotrypanum* strains used in this study

Stock number	Designation[a]	Species	Geographic origin
L565	MHOM/BR/75/M4147	*L. guyanensis*	Brazil, Pará
L566	MHOM/BR/00/M2903	*L. braziliensis*	Brazil, Pará
L575	IFLA/BR/67/PH8	*L. amazonensis*	Brazil, Pará
L579	MHOM/BR/74/PP75	*L. chagasi*	Brazil, Bahia
L888	MCHO/EC/82/Lsp1[a]	*L. equatorensis*	Ecuador, Guayas
L889	MSCI/EC/82/Lsp2	*L. equatorensis*	Ecuador, Guayas
L1023	MHOM/BR/81/M6426	*L. lainsoni*	Brazil, Pará
L1245	IGOM/PA/85/E582.34	*L. colombiensis*	Panama, Colon
L1246	IPAN/PA/85/E696.26	*L. colombiensis*	Panama, Colon
L1247	IGOM/PA/85/E582.36	*L. colombiensis*	Panama, Colon
L1545	MHOM/BR/84/M8408	*L. shawi*	Brazil, Pará
L1365	MDAS/BR/79/M5533	*L. naiffi*	Brazil, Pará
E14	MCHO/BR/80/M6159[b]	*E. schaudinni*	Brazil, Pará

a: host [M=Mammalia: CHO=*Choloepus* sp. ([a]*C. hoffmanni*, [b]*C. didactylus*), DAS=*Dasypus novemcinctus*, HOM=*Homo sapiens*, SCI=*Sciurus granatensis*; I=Insecta: FLA=*Lutzomyia flaviscultelata*, GOM= *Lu. gomezi*, PAN=*Lu. panamensis*]/country of origin/year of isolation/original code.

nation of *Leishmania* from *Endotrypanum* and evolutive studies we decided to analyze the genetic similarity among these parasites, using several biochemical and molecular methods. This information will help define the fundamental mechanisms involved in species identification and taxonomic divergence among these microorganism.

The sialidase (EC 3.2.1.18) activity alone has been shown to be a good marker to discriminate between morphologically indistinguishable flagellates isolated from human, insects and sylvatic vertebrate reservoir hosts, such as *Leishmania* and *Endotrypanum* (Medina-Acosta et al. 1994). The general concensus is that *Endotrypanum* reference stocks express clear-cut varying levels of sialidase activities whereas the *Leishmania* reference stocks do not. In this study, we measured the sialidase activity for several neotropical *Leishmania* species and for reference strain *E. schaudinni*. As expected, *Endotrypanum* exhibited high levels of sialidase activity, whilst the taxonomically unquestionable *Leishmania* stocks (*i.e., L. chagasi*) were negative for this activity. However, high levels of sialidase activity were consistently obtained from both cell lysates and culture supernatants of *L. (V.) colombiensis* and *L. (V.) equatorensis*, levels comparable with those obtained for *E. schaudinni* (this work) and those of *Trypanosoma rangeli* and *Trypanosoma leeuwenhoeki* (Medina-Acosta et al. 1994).

Further, MLEE analyses demonstrated that *L. (V.) colombiensis* and *L. (V.) equatorensis* share alleles with *Endotrypanum* for some loci, such as G6PDH and IDHNAD, that were previously admitted as monomorphic for the latter genus and as discriminative characters between *Leishmania* and *Endotrypanum* (Franco et al. 1996). Moreover, for the malic enzyme were found two distinct loci (ME1 and ME2) for *L. (V.) equatorensis* and *L. (V.) colombiensis*, as described for *Endotrypanum* but in contrast to other leishmanial parasites (Cupolillo et al. 1994, Franco et al. 1996). According to the phenetic analyses, the results showed a high level of similarity between the two *Leishmania* species, as well as a close relationship between this group and *Endotrypanum* (Fig. 1, Table II). The later parasite is genetically closest to *L. (V.) colombiensis* rather than to *L. (V.) equatorensis* (Table II). In addition, the clusters *L. braziliensis*/*L. naiffi* and *L. guyanensis*/*L. shawi* were observed, as already demonstrated (Cupolillo et al. 1994, 1997) and *L. lainsoni* made a link between *L. (V.) colombiensis*/*L. (V.) equatorensis*/*E. schaudinni* and the *Leishmania (Viannia)* species.

The Neighbor-Joining tree constructed based on kDNA sequence data using the number of differences between *Leishmania* and *Endotrypanum* shows similar clustering of MLEE for *L. (V.) equatorensis*/*L. (V.) colombiensis*/*E. schaudinni* (Fig. 2). The position of *L. lainsoni* was maintained, forming a link between the group *L. (V.) equatorensis*/*L. (V.) colombiensis*/*E. schaudinni* and other *Leishmania* species. *Leishmania (V.) lainsoni* represents a very divergent monophyletic *Viannia* species, which was clustered as an independent complex (Thomaz-Soccol et al. 1993, Cupolillo et al. 1994, Fernandes et al. 1995, Eresh et al. 1995).

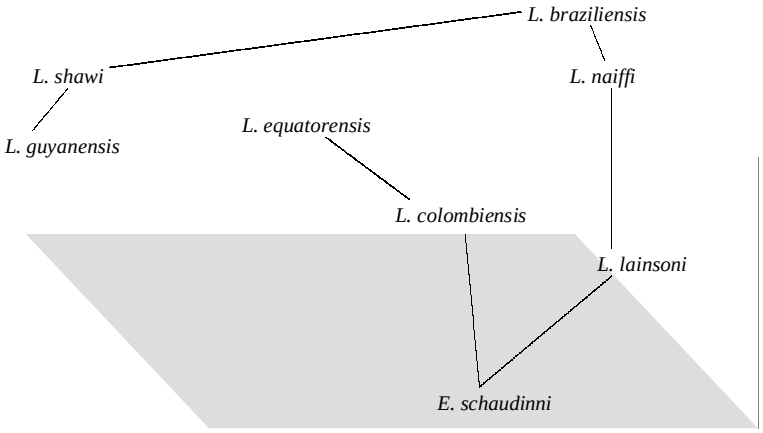

Fig. 1: principal coordinate analysis of the multilocus enzyme electrophoresis data. The three principal coordinates were claculated by Euclidian distance and plotted in 3D scale (the three principal coordinates represent 67.51% of the total variance). A minimum spanning tree was superimposed on the ordinations.

TABLE II

Similarity level among *Leishmania* and *Endotrypanum* species calculated by the Jaccard's coefficient

	1	2	3	4	5	6	7	8
1. *L. braziliensis*	-							
2. *L. guyanensis*	0.21	-						
3. *L. lainsoni*	0.22	0.16	-					
4. *L. equatorensis*	0.13	0.08	0.11	-				
5. *L. colombiensis*	0.16	0.07	0.14	0.40	-			
6. *E. schaudinni*	0.10	0.13	0.08	0.18	0.23	-		
7. *L. shawi*	0.24	0.59	0.13	0.07	0.05	0.07	-	
8. *L. naiffi*	0.47	0.15	0.26	0.12	0.18	0.12	0.20	-

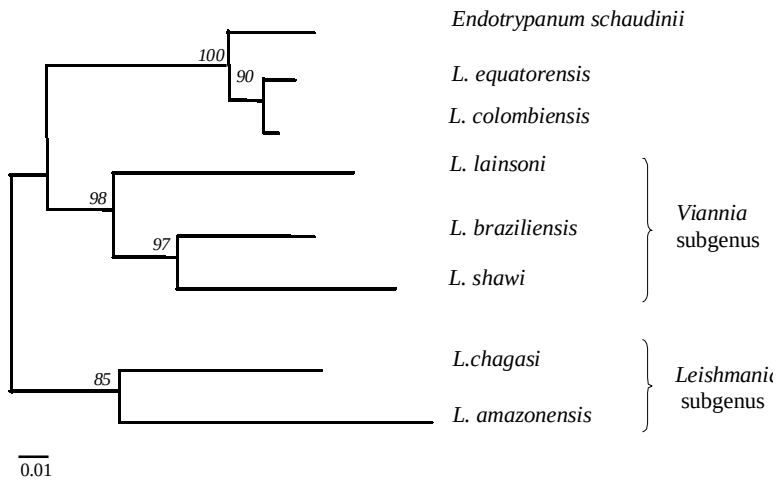

Fig. 2: phenetic analyze of sequences (83bp) of conserved region of kDNA minicircle. The similarities were evaluated by the number of differences among the sequences and the similarity tree constructed by the Neighbor-joining method. Italic numbers represent bootstrap values based on 500 replicates.

The internal transcribed spacers of the rRNA gene were amplified by PCR and the product digested with several restriction enzymes (Cupolillo et al. 1995). The RFLP profiles show a close but not identical pattern between *L. (V.) colombiensis* and *L. (V.) equatorensis*. However, through this method *Endotrypanum* can be easily discriminated from the former parasites and other *Leishmania* species by most of the restriction enzyme profiles (Fig. 3).

The genetic similarity between *Endotrypanum* and New World *Leishmania* was also demonstrated by sequencing comparisons of the small subunit of ribosomal RNA and RNA Polymerase II genes (Croan & Ellis 1996, Noyes et al. 1996, 1997, Croan et al. 1997). The results show that *L. (L.) herreri* (Zeledon et al. 1975), a sloth parasite, is closer to *Endotrypanum* than to other *Leishmania* species. *Leishmania (L.) hertigi*/*L. (L.) deanei* (Herrer 1971, Lainson & Shaw 1977), which were isolates from rodents, are also genetically closest to the *Endotrypanum*/*L. (L.) herreri* group (Croan et al. 1997, Noyes et al. 1997). Some authors suggest that *L. (L.) herreri* is a misclassified parasite and therefore probably represents *Endotrypanum* (Croan & Ellis 1996). Although *L. (L.) hertigi* and *L. (L.) deanei* are still enigmatic parasites (Lainson 1997) there are evidences supporting their classification as *Leishmania.* An interesting aspect is that these *Leishmania* species and *Endotrypanum* are biologically distinct parasites and do not share the same hosts.

In contrast to *L. (V.) colombiensis*, which has been isolated from humans (Kreutzer et al. 1991, Delgado et al. 1993), the public health importance of *L. (V.) equatorensis* remains to be determined. To date, it has only been isolated from arboreal mammals; no human infections with the parasite have been identified. Likewise, the sandfly vector (s) are unknown. However, the biological behaviour of *L. (V.) equatorensis* is indistinguishable from other members of the *L. (V.) braziliensis* complex, based on its virulence and development in laboratory animals. Inoculation of cultured promastigotes into the nose of hamster (*Mesocricetus auratus*) produced local swelling without metastasis; appearance of the lesions took 1-3 months, depending on the size of the inoculum (Grimaldi et al. 1992). Moreover, the restriction profile of the internal transcribed spacers of the rRNA gene showed a close pattern between *L. (V.) equatorensis* and *L. (V.) colombiensis*, but distinct from *Endotrypanum*, supporting the taxonomic status of the former parasite, and that the two *Leishmania* species represent a link between *Endotrypanum* and *Leishmania*.

Comparative studies will be needed to address the antiquity of this evolutionary link group and, in particular, whether or not it represents a branch point on the origin of neotropical leishmanias. It is worth noting that sloths, which have always been restricted to the American continent, are consid-

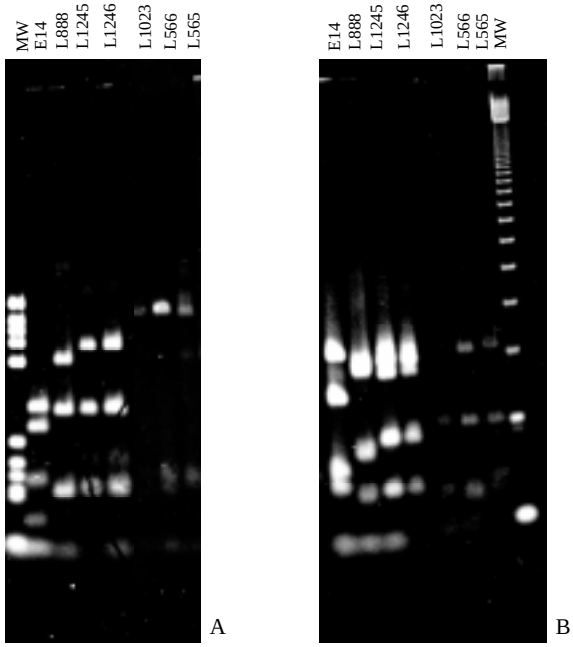

Fig. 3: restriction enzyme profile of the internal transcribed spacers of the rRNA genes for *Leishmania* species and *Endotrypanum schaudinni*. A. *Bst*UI; B. *Taq* I.

ered to have evolved from the basic Xenarthran armadillo-like stock some 60 million years ago during the Palaeocene period. These early mammals separated between the two and the three-toed groups of extant sloths later during the Miocene period. With this in mind, we feel that the neotropical leishmanias may well have evolved from a primitive endotrypanumal Miocene parasite line of South American sloths.

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