Characterization of *Endotrypanum* (Kinetoplastida: Trypanosomatidae), a Unique Parasite Infecting the Neotropical Tree Sloths (Edentata)

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This article reviews current concepts of the biology of Endotrypanum *spp. Data summarized here on parasite classification and taxonomic divergence found among these haemoflagellates come from our studies of molecular characterization of* Endotrypanum *stocks (representing an heterogenous population of reference strains and isolates from the Brazilian Amazon region) and from scientific literature. Using numerical zymotaxonomy we have demonstrated genetic diversity among these parasites. The molecular trees obtained revealed that there are, at least, three groups (distinct species?) of* Endotrypanum, *which are distributed in Central and South America. In concordance with this classification of the parasites there are further newer molecular data obtained using distinct markers. Moreover, comparative studies (based on the molecular genetics of the organisms) have shown the phylogenetic relationships between some* Endotrypanum *and related kinetoplastid lineages.*

Key words: *Endotrypanum* - Protozoa - Kinetoplastida - Trypanosomatidae - molecular taxonomy - enzyme activities - monoclonal antibodies - enzyme electrophoresis - DNA analyses - mammalian reservoirs - sandflies vectors

Biological characteristics of Endotrypanum *spp*. - Parasitic protozoa of the genus *Endotrypanum* are unique among the Kinetoplastida in that they infect erythrocytes of their mammalian host. Infection with *Endotrypanum* appears to be restricted to edentates, principally of forest-dwelling two-toed sloths of the genus *Choloepus*, but rarer infections with these flagellates seems to occur in three-toed sloths (genus *Bradypus*) (Fig. 1). As shown in Fig. 2, inside the erythocyte the *Endotrypanum* assumes an epimastigote or trypomastigote form, while in the sandfly or during *in vitro* culture the parasite assumes promastigote morphology (Shaw 1992). However, the complete life cycle of *Endotrypanum* spp. has not been reproduced in experimental studies. Other developmental stages of the parasite may occur within the mammalian host (Deane 1961).

In nature, *Endotrypanum* parasites are probably transmitted by the bite of infected phlebotomine sandflies (Diptera: Psychodidae). Arias et al. (1985) identified *E. schaudinni* and other *Endotrypanum* sp. infections in sandflies and sloths captured in the Amazon Region of Brazil. Studies using kinetoplast DNA probe for detecting parasites in sandflies also demonstrated *Endotrypanum* infections in *Lutzomyia shannoni*, *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 sandfly species on infected sloths (Shaw 1964, 1969, 1981, Christensen & Herrer 1977, 1979). In these studies, infections developing within the insect gut (Shaw 1964, 1969) were similar to those found in wild caught sandflies (Johnson et al. 1963, Arias et al. 1985). Moreover, we have reported (Franco et al. 1997b) the developmental biology of Brazilian strains of *Endotrypanum* for three sandfly species. Development of *Endotrypanum* varied for each parasite-host species association. After feeding on culture forms of *E. schaudinni*, significantly more *Lu. shannoni* (100%, 9/9) became infected than did *Lu. longipalpis* (62.3%, 33/53) or *Phlebotomus papatasi* (27.3%, 15/55). The greatest number of infections were in the midgut and hindgut from 6 to 16 days after feeding, but flagellates also were present in the Malpighian tubules. Moreover, distinct development patterns in the sandfly gut were obtained when the Callejon *Lu. longipalpis* colony was fed on cultures of other *Endotrypanum* strains.

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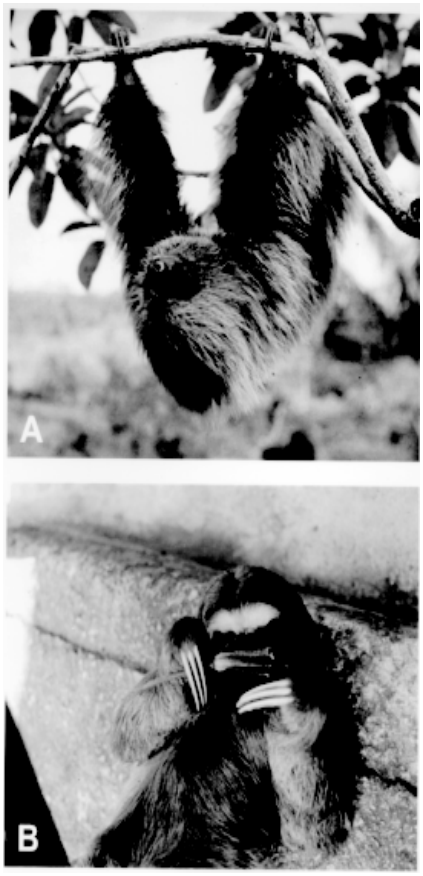

Fig.1: inhabitants of Neotropics: a two-toed sloth, *Choloepus didactylus*, the major host of *Endotrypanum* (A) and a three-toed sloth, *Bradypus variegatus* (B), reservoir-hosts of *Endotrypanum* in Central and South American Countries.

There were also individual variation in the distribution and survival of parasites within the guts of flies in each group. These data indicate that there is variation in the susceptibility to infection with *Endotrypanum* among and within sandfly species.

Laboratory diagnosis - The finding that *Endotrypanum* and *Leishmania*, as well as other distinct groups of *Trypanosoma* are commonly found in the same vertebrate and insect hosts in Neotropical forests (Table) has led to an increased interest in developing simple methods to distinguish these parasites for epidemiological purposes. Several methods based on parasite-specific markers have been proposed to discriminate these organisms. *Endotrypanum* can be distinguished from *Leishmania* species and other trypanosomatids by enzymatic profiles (Arias et al. 1985, Shaw et al. 1991, Franco et al. 1996b), monoclonal antibodies (Lopes & McMahon-Pratt 1989, Franco et al. 1997a), total DNA probes (Greig et al. 1989), hybridization probes based on kDNA (Pacheco et al.

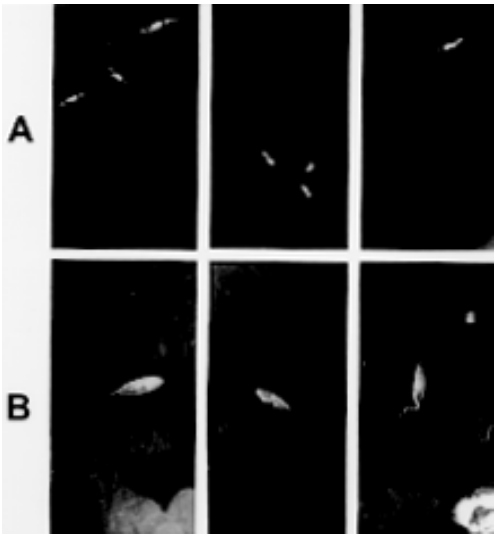

Fig. 2: promastigotes from culture of *Endotrypanum* (A) and haemoflagellates found in the blood of two-toed sloth, *Choloepus juruanus* from Rondônia State, BR (B).

1990), mini-exon gene repeats (Fernandes et al. 1993a), lectin reactivity (Lopes et al. 1987) and sialidase/trans-sialidase activities (Medina-Acosta et al. 1994). However, the classical methods used for direct demonstration of the parasite in tissues is difficult because of the paucity of organisms in infected hosts. Work is now in progress to evaluate (employing PCR and serological assays) the prevalence of *Endotrypanum* and *Leishmania* infections in sloths captured in distinct sylvan areas of the Neotropics (Pirmez et al. 1997, Mayrink et al. 1998).

Molecular characterization of the genus - The taxonomy of *Endotrypanum* spp. is still controversial. Since the original description of this parasite, by Mesnil and Brimont in 1908, only two species have been named as *E. schaudinni* Mesnil and Brimont, 1908 and *E. monterogeii*, Shaw, 1969. Croft et al. (1980) observed that the mobilities of the enzymes MDH, IDH and ME, in seven stocks of this parasite were different for the strains that had virus-like particles from those that did not. The two groups did not agree with the original identification of *E. schaudinni* and *E. monterogeii* since, ultrastructural and biochemical parameters were similar, in contrast to the description by Shaw (1969), who reported serological differences between these species. Lopes et al. (1990), also grouped both species in the same cluster by comparison of nuclear DNA restriction fragment patterns.

The main goal of our studies is to apply an integrated set of approaches towards the characterization of parasites within the genus *Endotrypanum* and to use this information in a coherent manner

TABLE

Trypanosomatid parasites commonly found infecting sloths (Edentata: Xenarthra)

Trypanosomatids/ Sloths' species[b]	Geographical distribution[a]						Reference
	BR	CO	PA	GF	EC	CR	
E. schaudinni							
C.didactylus	+			+			Mesnil & Brimont 1908, Deane 1961, Herrer & Telford 1969
C. hoffmanni			+				Shaw 1985, Herrer & Christensen 1980
B. variegatus			+			+	Shaw 1985
B.tridactylus	+						Shaw 1985
E. monterogeii							
C. hoffmanni						+	Shaw 1969
Endotrypanum sp.							
Choloepus sp.		+					ATCC 1991
Choloepus sp			+				ATCC 1991
C.hoffmanni						+	Zeledón et al. 1975c
C. juruanus	+						Franco et al. 1996b
L. panamensis							
C.didactylus			+				Shaw 1985
C.hoffmanni			+			+	Zeledón et al. 1975a
B. variegatus			+			+	Zeledón et al. 1975a
L. guyanensis							
C. didactylus	+			+			Gentile et al. 1981, Lainson et al. 1981, Shaw 1985, Dedet et al. 1989
L. herreri							
C. hoffmanni						+	Zeledón et al. 1979
B. griseus						+	Zeledón et al. 1979
L.equatorensis							
C. hoffmanni					+		Grimaldi et al, 1992
L.colombiensis							
C.hoffmanni			+				Kreutzer et al. 1991
L.shawi							
C. didactylus	+						Lainson et al. 1989
B. tridactylus	+						Lainson et al. 1989
L. braziliensis sp.							
B. infuscatus			+				Herrer & Telford 1969, Herrer et al 1973
B.griseus						+	Zeledón et al. 1975c
C. hoffmanni			+			+	Herrer & Telford 1969, Herrer et al. 1973, Zeledón et al. 1975c, Herrer & Christensen 1980, Christensen & de Vasquez 1982
T. leeuwenhoeki							
C. hoffmanni		+	+			+	Shaw 1969, Zeledón et al. 1975b, Travi et al.1989
B.variegatus			+			+	Shaw 1969, Zeledón et al. 1975b
T. mesnil-brimonti							
C.didactylus	+			+			Deane 1961, Shaw 1985
T.preguici							
C. hoffmanni			+			+	Shaw 1969, 1985
B. griseus						+	Shaw 1969
T. cruzi							
C.hoffmanni			+				Christensen & Herrer 1979, Herrer & Christensen 1980
B.infuscatus			+				Pipkin 1968
T.rangeli							
C. hoffmanni			+				Herrer & Christensen 1980
B. tridactylus	+						Miles et al. 1983
T. legeri							
B. g. griseus						+	Montero-Gei 1956, Trejos & Montero-Gei 1953

a: country of origin: BR: Brazil; CO: Colombia; PA: Panama; GF: French Guiana; EC: Ecuador; CR: Costa Rica; *b:* designations: [*E.= Endotrypanum; C.= Choloepus; B.= Bradypus; L.=Leishmania; T.=Trypanosoma*]. Original species description (ex. *B. infuscatus* e *B. griseus = B. variegatus*).

to address problems of parasite identification and taxonomy, diagnosis and developmental biology. Our results confirm previous studies reporting population diversity within this genus (Lopes et al. 1990).

Trans-sialidase and sialidase activities - By virtue of the differences observed in the rations of these enzyme activities, a large collection of trypanosomatids (comprising the major taxa of these parasites) could be separated into four expression types. *Endotrypanum* could be clustered into a group which could be easily differentiate from other trypanosomatids by their capacity of expressing comparable levels of both trans-sialidase and sialidase activities (Medina-Acosta et al. 1994). In general, the measurement of trans-sialidase and sialidase activities permits the differentiation of parasites frequently found in the same sylvatic vertebrate and invertebrate hosts that are difficult to distinguish, such as *Leishmania* and *Endotrypanum*.

Reactivities with monoclonal antibodies - Among the new approaches for identifying *Endotrypanum* is serodeme analysis using specific monoclonal antibodies, Mabs (Lopes & McMahon-Pratt 1989). In a recent study (Franco et al. 1997a), an heterogenous population of *Endotrypanum* strains have been screened against a panel of Mabs derived for selected species of *Endotrypanum* or *Leishmania*, to see whether this approach could be used to differentiate/group further among these parasites. Using different immunological assay systems, Mabs considered specific for the genus *Endotrypanum* [E-24, CXXX-3G5-F12] or strain M6159 of *E. schaudinni* [E-2, CXIV-3C7-F5] (Lopes & McMahon-Pratt 1989) reacted variably according to the test used but in the ELISA or immunofluorescence assay both reacted with all the strains tested. Analyses using these Mabs showed antigenic diversity occurring among the *Endotrypanum* strains, but no qualitative or quantitative reactivity pattern could be consistently related to parasite origin (i.e., host species involved) or geographic area of isolation. Western blot analyses of the parasites showed that these Mabs recognized multiple components and differences existed either in the epitope density or molecular forms associated with the antigenic determinants, which allowed the assignment of the strains to specific antigenic groups.

Multilocus enzyme electrophoresis (MLEE) - As a result of using numerical zymotaxonomy for classifying these organisms (Franco et al. 1996b), the taxonomic horizon of *Endotrypanum* spp. has been widened. We have analyzed enzyme polymorphism among a group of *Endotrypanum* parasites (17 stocks isolated from sloths in the Amazon Region in Brazil and 6 reference strains). The 16 enzymic loci were analyzed, and the strains were classified into zymodemes, each representing parasites with unique enzyme profiles. Using numerical analyses the genus was shown to be monophyletic and the 12 zymodemes characterized could be divided into three groups (A, B, C) (Fig. 3). The data indicate that *E. schaudinni* is a species complex. The heterogeneous population of parasites showed, however, no correlation with the origin of *Endotrypanum* stocks. Eight isolates from the State of Rondônia (Brazil) and a parasite strain from Panama were clustered together into a zymodeme (IOC/EZ01), which was phenetically closely related to the *E. monterogeii* from Costa Rica. All these data reinforce the discussion about the existence of the later parasite species.

Minicircle kDNA heterogeneity and molecular karyotypes - Comparisons of kDNA restriction enzyme fragments profiles from strains representing selected *Endotrypanum* zymodemes were carried out by polyacrylamide gradient gel electrophoresis. As the degree of heterogeneity within minicircles varied among species or strains of *Endotrypanum*, the stocks could be clustered into distinct groups of parasites, according to the major sequence classes (minicircle fragments or digests) released by the restriction endonucleases tested (Franco et al. 1996a). In parallel study using PFGE and appropriate gel running conditions, chromosomes were polymorphic in number (10-25) and size (290-2,200 kb) of bands among parasites of this genus. Distinct karyotypes were constructed among *E. schaudinni*, *E. monterogeii* and *Endotrypanum* sp. strains. All of the isolates (5) of *Endotrypanum* sp. from the same species of sloth (*C. juruanus*) and geographical origin (Rondônia, Brazil) had the same karyotype (Franco et al. 1992). Interesting, these parasites were grouped in the same zymodeme (EZ01) with two other stocks originating from Central America (Franco et al. 1996b). However, RAPD analysis using selected primer was able to differentiate the EZ01 parasites, but the strains did not group on the basis of their geographic origin (Franco et al. 1998a).

Evolutionary links among parasites and taxonomic problems - The difficult taxonomy of *Endotrypanum* spp. is mainly because the parasite life cycle is unknown. Although this genus is unique among the Trypanosomatidae in that the parasites are found inside the erythrocyte of the mammalian host, both forms (epimastigotes of *E. schaudinni* and trypomastigotes of *E. monterogeii*) are found rarely in infected sloths. *Endotrypanum* spp. have other distinct form in their lyfe cycle: a motile flagellated promastigote stage that lives extracellularly within the alimentary tract of the

vector, at least in experimentally infected phlebotomine sandfly (Franco et al. 1997b).

The *Endotrypanum* shares many characters with other trypanosomatids, particularly those of the genus *Leishmania* (Shaw 1992). Phylogenetic reconstruction studies of the various kinetoplastid lineages (Fernandes et al. 1993b, Maslov & Simpson 1995) have shown that *Leishmania* and *Endotrypanum* are more closely related organisms to each other than either is to *T. cruzi*. The genetic similarity between *Endotrypanum* and leishmanial parasites, such as *L.* (*L.*) *herreri, L.* (*L*). *hertigi*/ *L.* (*L*). *deanei, L.* (*V.*) *colombiensis* or *L.* (*V.*) *equatorensis* was demonstrated by (*i*) 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), (*ii*) numerical zymotaxonomy, (*iii*) intergenic region typing of the internal transcribed spacers of the rRNA genes, (*iv*) minicircle kDNA heterogeneity analysis, and (*v*) measurement of the sialidase activites of the parasites (Cupolillo et al. 1998). The data show that *L.* (*L.*) *hertigi*/*L.* (*L.*) *deanei* (Herrer 1971, Lainson & Shaw 1977) are genetically closest to the *Endotrypanum*/*L.* (*L.*) *herreri* group. Interesting, *L.* (*L.*) *herreri* was genetically closer to *Endotrypanum* than to the pathogenic *Leishmania* species (Croan et al. 1997, Noyes et al. 1997). The former parasite was originally isolated from two (*C. hoffmanni*) and three-toed (*B. griseus*) sloths, and sandflies (*Lu. trapidoi, Lu. ylephiletor* and *Lu. shannoni*) in Costa Rica (Zeledón et al. 1979). Studies employing monoclonal antibodies have also shown antigenic similarities among these parasites (Grimaldi et al. 1992, Shaw 1992, Franco et al. 1997a).

Moreover, studies by Touch-down PCR with primers from kinetoplastid housekeeping genes (ribosomal P proteins and repetitive sequences, SIRE) examine the relationships among *Endotrypanum* (N= 22 strains, comprising the major taxa of this genus) and *L.* (*L.*) *herreri* (ISHA/ CR/74/LV341 and IYLE/CR/74/LV342 strains, both isolated from sandflies, and MCHO/CR/74/ LV344 strain, isolated from sloth). The data reveal differences among the leishmanial strains, but showing closed genetic similarity between LV342 and *Endotrypanum* strains clustered within the same group (group A) by zymodeme analysis (Franco et al. 1996b). However, both strains LV341 and LV344 were distinct from all *Endotrypanum* stocks analyzed (Franco et al. 1998b). Comparative phylogenetic reconstruction studies are in progress to address the evolutionary links among these related kinetoplastid lineages.

The known vertebrate hosts of *Endotrypanum* spp., two and three-toed sloths, are considered as unique examples of South American fauna and they evolved some 60-70 million years ago during the Palaeocene period. The separation between these two genera of sloths occurred later during the Miocene period and their presence in Central America, is thought to be a result of migration from South America (Webb 1985, Sarich 1985). In *Choloepus* the body temperature ranges from 24º to 33º C. This variation results in a geographical barrier to these mammals (Nowak 1991) and consequently to the parasites. Lopes et al. (1990) based on results of restriction fragment hybridization conclude that *Endotrypanum* originated in South America. Considering these observations, zymodeme EZ01 (Fig. 3) has migrated towards Central America and the parasites isolated in this area are representative of the most recent group (group A). Moreover, the results suggest that the dispersal of the genus *Endotrypanum* has arisen from group C, which is probably the most ancient organism (Franco et al. 1996b).

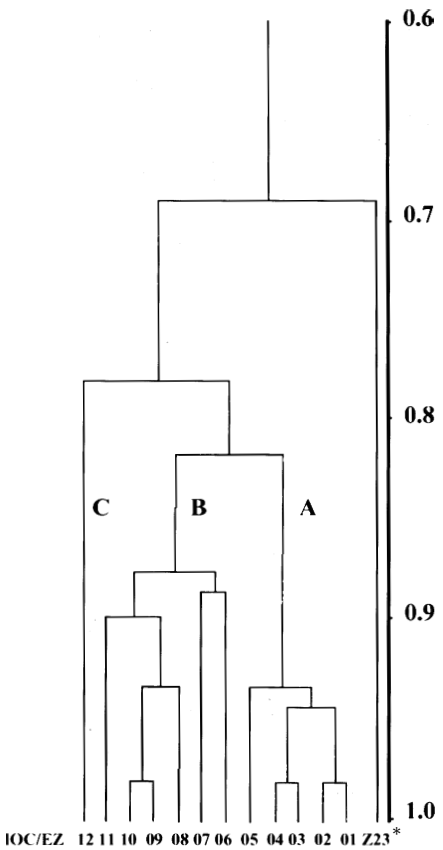

Fig. 3: phenetic analysis of enzyme data from *Endotrypanum* stoks. The Simple Matching Coefficient of Similarity was calculated between all combinations of pairs of strains and the similarity matrix transformed into a dendogram by the UPGMA algorithm.
(* Corresponds to *Leishmania* zymodeme - IOC/Z23).

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