



Phylogenetic relationships of *Leishmania* species based on trypanosomatid barcode (SSU rDNA) and gGAPDH genes: Taxonomic revision of *Leishmania* (*L.*) *infantum chagasi* in South America [☆]



Arlei Marcili ^{a,*}, Marcia Ap. Sperança ^b, Andrea P. da Costa ^a, Maria de F. Madeira ^c, Herbert S. Soares ^a, Camila de O.C.C. Sanches ^b, Igor da C.L. Acosta ^a, Aline Giroto ^a, Antonio H.H. Minervino ^a, Maurício C. Horta ^d, Jeffrey J. Shaw ^e, Solange M. Gennari ^a

^a Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária, Universidade de São Paulo, SP, Brazil

^b Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André, SP, Brazil

^c Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil

^d Colegiado de Medicina Veterinária, Universidade Federal do Vale do São Francisco, Petrolina, PE, Brazil

^e Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil

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ABSTRACT

Phylogenetic studies on trypanosomatid barcode using V7V8 SSU rRNA and gGAPDH gene sequences have provided support for redefining some trypanosomatid species and positioning new isolates. The genus *Leishmania* is a slow evolving monophyletic group and including important human pathogens. The phylogenetic relationships of this genus have been determined by the natural history of its vertebrate hosts, vector specificity, clinical manifestations, geographical distribution and molecular approaches using different markers. Thus, in an attempt to better understand the phylogenetic relationships of *Leishmania* species, we performed phylogenetic analysis on trypanosomatid barcode using V7V8 SSU rRNA and gGAPDH gene sequences among a large number of *Leishmania* species and also several Brazilian visceral *Leishmania infantum chagasi* isolates obtained from dogs and humans. Our phylogenetic analysis strongly suggested that *Leishmania hertigi* and *Leishmania equatoriensis* should be taxonomically revised so as to include them in the genus *Endotrypanum*; and supported ancient divergence of *Leishmania enriettii*. This, together with recent data in the literature, throws light on the discussion about the evolutionary southern supercontinent hypothesis for the origin of *Leishmania* ssp. and validates *L. infantum chagasi* from Brazil, thus clearly differentiating it from *L. infantum*, for the first time.

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1. Introduction

The genus *Leishmania* is an important group of parasites that is the causative agent of a complex disease called leishmaniasis, whose clinical manifestations exhibit different degrees of severity, ranging from asymptomatic, cutaneous/mucocutaneous lesions, to a more severe life-threatening visceral disease (Colmenares et al., 2002). *Leishmania* protozoans are transmitted by sandflies (Diptera; Psychodidae; Phlebotominae) (Schlein, 1993) to different species of mammals and lizards (Desjeux, 2004; Orlando et al.,

2002; Rioux et al., 1992). The mammalian *Leishmania* species are transmitted in zoonotic and anthroponotic cycles, and are divided into two subgenera according to the developmental stage of the parasite in the gut of the sandfly vector (Lainson et al., 1987a). This division is also associated with its geographical distribution: *L. (Viannia)* is composed of species restricted to the Neotropics and *L. (Leishmania)* has species distributed in both the New and the Old World (Shaw, 1994).

Visceral leishmaniasis is the most severe form of the disease with a worldwide estimate of 500,000 human cases with 59,000 deaths every year, and it is thus a serious public health problem (Postigo, 2010). The species causing visceral leishmaniasis belong to the *L* complex of the subgenus *L. (Leishmania)* including: *L. eishmania donovani* (*L.*) *donovani*, which causes anthroponotic visceral leishmaniasis in India, Bangladesh, Nepal and Pakistan; *Leishmania* (*L.*) *infantum*, responsible for zoonotic leishmaniasis in the

[☆] Note: Nucleotide sequences reported in this paper are available in the GenBank database. Accession numbers in Table 1.

* Corresponding author. Address: Av. Prof. Dr. Orlando Marques de Paiva, 87, São Paulo, SP 05508-270, Brazil. Tel.: +55 11 30911446; fax: +55 11 30917928.

E-mail address: amarcili@usp.br (A. Marcili).

Mediterranean region (Europe and Africa); and *Leishmania* (*L.*) *chagasi*, which shows high genetic similarity with *L. (L.) infantum*, and has been correlated with zoonotic leishmaniasis in different countries of the Americas (New World) (Lukes et al., 2007). Dogs are the main reservoir of all of these visceral *Leishmania* zoonotic species.

The phylogenetic relationships among *Leishmania* species have been determined through differences in the natural history of their vertebrate hosts, vector specificity, clinical manifestations, geographical distribution and, more recently, using molecular approaches with different markers (Asato et al., 2009; Boite et al., 2012; Fraga et al., 2010; Lukes et al., 2007; Mauricio et al., 2004, 2007; Orlando et al., 2002; Shaw, 1997). However, these studies have included few species and/or natural isolates and controversy still surrounds the geographical origins and spread of *Leishmania* species. Also, some species included in the genus *Leishmania* have questionable taxonomic status, such as *Leishmania hertigi*, *Leishmania equatoriensis* and *Leishmania enriettii* (Momen and Cupolillo, 2000).

In the New World, Brazil is the country that accounts for the highest number (90%) of visceral leishmaniasis cases (Romero and Boelaert, 2010). The origin of these cases is unknown, since recent studies have been unable to confirm that the human disease was in this country prior to the European invasion of South America (Momen and Cupolillo, 2000), thus suggesting that *L. (L.) chagasi* arrived in Latin America from Europe. This hypothesis is corroborated by structural and biochemical findings, including studies using various molecular markers, such as the internal transcribed spacer of the ribosomal gene (Kuhls et al., 2005; Mauricio et al., 2004), gp63 (Mauricio et al., 2001; Quispe Tintaya et al., 2004), mini-exons (Mauricio et al., 2004), cysteine proteases (Hide et al., 2007; Quispe Tintaya et al., 2004), cytochrome oxidase II (Ibrahim and Barker, 2001) and microsatellites (Kuhls et al., 2011; Lukes et al., 2007). Based on these data, the taxonomy of the species causing visceral leishmaniasis in the Americas was recently revised and it was suggested that the species name *L. (L.) infantum chagasi* should be used (Kuhls et al., 2005, 2011; Lainson and Shaw, 1988; Mauricio et al., 1999, 2001, 2004). Controversially, studies based on vertebrate hosts and the biogeography of mammals have demonstrated a scenario in which there was ancient introduction of *L. infantum chagasi* into the Americas (Lainson et al., 1987b). Independent of the initial origin of *L. infantum chagasi*, molecular markers capable of distinguishing *L. infantum* from *L. infantum chagasi* would be very useful for epidemiological investigations.

Markers based on sequences of the SSU ribosomal gene have been used to study the diversity and phylogenetic relationships of trypanosomatids (Borghesan et al., 2013; Noyes et al., 1999; Stevens et al., 1999; Teixeira et al., 2011, 1994). V7V8 SSU rDNA has been named trypanosomatid barcode and has been used in several trypanosomatid phylogenetic studies and for describing inter and intraspecific relationships (Cortez et al., 2006; Da Silva et al., 2004; Ferreira et al., 2007; Lima et al., 2012; Marcili et al., 2009a,b; Rodrigues et al., 2006; Viola et al., 2009; Teixeira et al., 2011). In addition to the ribosomal gene, the gGAPDH sequence has made it possible to position trypanosomatid species (Hamilton et al., 2004, 2007).

Thus, in an attempt to better understand the phylogenetic relationships of *Leishmania* species, we performed phylogenetic analysis on *Trypanosomatidae* barcode using V7V8 SSU rDNA and gGAPDH gene sequences among a large number of *Leishmania* species and also several Brazilian visceral *L. i. chagasi* isolates obtained from dogs and humans. The evolutionary insights from the results relating to the *L. hertigi*, *L. equatoriensis* and *L. enriettii* reference strains, and from *L. i. chagasi* isolates from humans and dogs from Brazil, strongly suggest that taxonomic revision of these species is required.

2. Material and methods

2.1. Isolation and culturing of *Leishmania* species

L. i. chagasi was isolated through culturing popliteal lymph node aspirates obtained from fine-needle puncture performed on dogs in several Brazilian regions (Table 1). The culturing was done in biphasic blood agar base and liver infusion tryptose (LIT) medium (Camargo, 1964). Promastigote forms from positive cultures were used to infect monolayers of J774 peritoneal macrophage lineage cells in a 25 cm² flask that was kept in RPMI medium containing 10% bovine calf serum at 28 °C. Infected J774 cells were monitored until cell lysis and release of promastigote forms to the RPMI medium occurred. The promastigote forms of the *Leishmania* species were transferred to and expanded in LIT medium containing 10% bovine calf serum and 2% male human urine for DNA preparation and cryopreservation. All isolates were included in the Brazilian Trypanosomatid Collection of the School of Veterinary Medicine of the University of São Paulo, Brazil. The study was approved by the Bioethical Committee in Animal Research of the Faculty of Veterinary Medicine of the University of São Paulo.

2.2. PCR amplification of 18S rDNA and glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) coding sequences

DNA from *Leishmania* isolates was extracted from culture supernatants using the phenol–chloroform method. The DNA samples were subjected to conventional polymerase chain reaction (PCR) with high fidelity Taq DNA polymerase for amplification of the barcode, which comprised a fragment of around 900 base pairs (bp) corresponding to the trypanosome V7V8 SSU rDNA (Ferreira et al., 2008; Marcili et al., 2009a,b; Viola et al., 2008), and of the approximately 850 bp fragment corresponding to the gGAPDH trypanosome coding sequence described previously (Hamilton et al., 2007). The obtained PCR products were purified using Spin-X columns (Costar) and cloned into the pCR™2.1 TA vector (Invitrogen). Sequences of three to five clones of each PCR product were determined to minimize sequence mistakes generated by Taq DNA polymerase and occurrence of different haplotypes in *Leishmania* isolates. The sequences trace files were analyzed and compiled using the Seqman program of DNASTar software. The nucleotide sequences generated were deposited in GenBank (Table 1).

2.3. Phylogenetic analysis

The sequences of trypanosomatid barcode obtained were aligned with sequences previously determined for other trypanosomatid species available in GenBank (Table 1) using ClustalX (Thompson et al., 1997) and secondary structure comparative analysis and were adjusted manually using GeneDoc (Nicholas and Nicholas, 1997). The gGAPDH sequences of the visceral leishmaniasis species obtained were aligned to evaluate intraspecific variability. The barcode and gGAPDH sequences were used to construct a phylogenetic tree using maximum parsimony, as implemented in PAUP version 4.0b10 (Wilgenbusch and Swofford, 2003) with 500 bootstrap replicates, random stepwise addition starting trees (with random addition sequences) and TBR branch swapping. Sequences of V7V8 SSU rDNA and gGAPDH genes of *L. donovani* complex were concatenated to perform intraspecific sequence variation analysis. Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) with 1,000,000 generations. Trees were sampled every 100 generations using chains, and 25% of the early sample trees were discarded as “burn-in”. The GTR+I+G substitution model was used.

Table 1
Trypanosomatid isolates, host, geographical origin and sequences of SSU rDNA and gGAPDH genes employed in the phylogenetic analyses performed in this study.

| Trypanosomatid Species | CBT ^a | Isolate code ^b | Host | Geographic origin ^c | Accession number ^d Sequences | |
|----------------------------|------------------|---------------------------|---------------------------------|--------------------------------|--|--------------------------|
| | | | | | SSUrRNA | gGAPDH |
| Phytomonas | | | | | | |
| <i>P. serpens</i> | | | | | | AF016320 |
| <i>P. sp</i> | | | | | | AF016322 |
| Herpetomonas | | | | | | |
| <i>H. megaseliae</i> | | | <i>Megaselia scalaris</i> | | | U01014 |
| <i>H. muscarum</i> | | | | | | L18872 |
| <i>H. samuelpessoai</i> | | | <i>Zelus leucogrammus</i> | | | U01016 |
| Crithidia | | | | | | |
| <i>C. fasciculata</i> | | | | | | Y00055 |
| Wallaceina | | | | | | |
| <i>W. brevicula</i> | | | <i>Nabis brevis</i> | Russia | | AF153045 |
| <i>W. inconstans</i> | | | <i>Calocoris sexguttatus</i> | Russia | | AF153044 |
| Leptomonas | | | | | | |
| <i>L. podlipaevi</i> | | | | | | DQ383649 |
| <i>L. seymouri</i> | | | <i>Dysdercus suturellus</i> | | | AF153040 |
| <i>L. costaricensis</i> | | | <i>Ricollia similima</i> | Costa Rica | | DQ383648 |
| Endotrypanum | | | | | | |
| <i>E. monterogeii</i> | | | <i>Nyssomyia trapidoi</i> | Ecuador | EC | JQ863389 |
| <i>Endotrypanum sp.</i> | | TCC 889 | <i>Psathyromyia dendrophyla</i> | Rondonia | BR | EU021240 |
| <i>Endotrypanum sp.</i> | | TCC 890 | <i>Lutzomyia gomezi</i> | Rondonia | BR | EU021238 |
| Leishmania | | | | | | |
| <i>L. adleri</i> | | | | | | M80291 |
| <i>L. tarentolae</i> | | | | | | M84225 |
| <i>L. hoogstraali</i> | | | | | | KF041810 |
| <i>L. tropica</i> | 70 | MHOM/SU/1958/STRAIN OD | <i>Homo sapiens</i> | Azerbaijão | AZ | KF041809 |
| <i>L. amazonensis</i> | | | <i>Homo sapiens</i> | Brazil | BR | GQ332354 |
| <i>L. major</i> | | | <i>Homo sapiens</i> | Turcomenistão | TM | GQ332361 |
| <i>L. major</i> | | | <i>Homo sapiens</i> | Israel | IL | AF244350 |
| <i>L. herptigi</i> | 74 | MCOE/PA/1965/C8 | <i>Coendou sp.</i> | Panamá | PA | KF041804 |
| <i>L. lainsoni</i> | 75 | MHOM/BR/1981/M6426 | <i>Homo sapiens</i> | Brazil | BR | KF041805 |
| <i>L. donovani</i> | 77 | MHOM/ET/1967/L82;HV3;LV9 | <i>Homo sapiens</i> | Ethiopia | ET | KF041801 |
| <i>L. equatoriensis</i> | 79 | MCHO/EC/1982/LSP1 | <i>Choloepus hoffmanni</i> | Ecuador | EC | KF041802 |
| <i>L. mexicana</i> | 80 | MHOM/BZ/1982/BEL21 | <i>Homo sapiens</i> | Belize | BZ | KF041806 |
| <i>L. shawi</i> | 87 | MCEB/BR/1984/M8408 | <i>Cebus apella</i> | Brazil | BR | KF041808 |
| <i>L. donovani</i> | 89 | MHOM/ET/1967/HU3 | <i>Homo sapiens</i> | Ethiopia | ET | KF041800 |
| <i>L. guyanensis</i> | 90 | MHOM/BR/1975/M4147 | <i>Homo sapiens</i> | Brazil | BR | KF041803 |
| <i>L. naiffi</i> | 91 | MDAS/BR/1979/M5533 | <i>Dasyus sp.</i> | Brazil | BR | KF041807 |
| <i>L. braziliensis</i> | | MHOM/BR/1975/M2903 | <i>Homo sapiens</i> | Brazil | BR | KF041799 |
| <i>L. enriettii</i> | 47 | | <i>Cavia porcellus</i> | Paraná | BR | KF041798 |
| <i>L. infantum</i> | | JCPM5 | | Europe | | M81430 |
| <i>L. infantum</i> | | | | Europe | | M81429 |
| <i>L. infantum</i> | 88 | MHOM/BR/2002/LPC-RPV | <i>Homo sapiens</i> | Brazil | BR | KF041793 |
| <i>L. infantum chagasi</i> | 72 | MHOM/BR/1974/PP75 | <i>Homo sapiens</i> | Brazil | BR | KF041792 |
| <i>L. infantum chagasi</i> | 13 | C808 | <i>Canis lupus familiaris</i> | Rio de Janeiro | BR | KF041776 |
| <i>L. infantum chagasi</i> | 15 | C1148 | <i>Canis lupus familiaris</i> | Rio de Janeiro | BR | KF041777 |
| <i>L. infantum chagasi</i> | 16 | C1194 | <i>Canis lupus familiaris</i> | Rio de Janeiro | BR | KF041778 |
| <i>L. infantum chagasi</i> | 22 | MCAN/BR/1984/CCC17.482 | <i>Canis lupus familiaris</i> | Ceará | BR | KF041779 |
| <i>L. infantum chagasi</i> | 24 | MCAN/BR/1995/CHULINHA | <i>Canis lupus familiaris</i> | Bahia | BR | KF041780 |
| <i>L. infantum chagasi</i> | 25 | MCAN/BR/2002/LVV-129 | <i>Canis lupus familiaris</i> | Mato Grosso do Sul | BR | KF041781 |
| <i>L. infantum chagasi</i> | 26 | MCAN/BR/2005/NMT-DOTM | <i>Canis lupus familiaris</i> | Distrito Federal | BR | KF041782 |
| <i>L. infantum chagasi</i> | 28 | MCAN/BR/2004/LIBPI-18 | <i>Canis lupus familiaris</i> | Piauí | BR | KF041783 |
| <i>L. infantum chagasi</i> | 29 | MCAN/BR/2007/LIBPI-51 | <i>Canis lupus familiaris</i> | Piauí | BR | KF041784 |
| <i>L. infantum chagasi</i> | 30 | MCAN/BR/2010/BURRINHO II | <i>Canis lupus familiaris</i> | Rio Grande do Sul | BR | KF041785 |
| <i>L. infantum chagasi</i> | 31 | MCAN/BR/2010/LAIIKA | <i>Canis lupus familiaris</i> | Rio Grande do Sul | BR | KF041786 |
| <i>L. infantum chagasi</i> | 37 | | <i>Canis lupus familiaris</i> | Pernambuco | BR | KF041787 |
| <i>L. infantum chagasi</i> | 39 | | <i>Canis lupus familiaris</i> | Pará | BR | KF041788 |
| <i>L. infantum chagasi</i> | 40 | | <i>Canis lupus familiaris</i> | Pará | BR | KF041789 |
| <i>L. infantum chagasi</i> | 43 | | <i>Canis lupus familiaris</i> | Pará | BR | KF041790 |
| <i>L. infantum chagasi</i> | 44 | | <i>Canis lupus familiaris</i> | Pará | BR | KF041791 |
| <i>L. infantum chagasi</i> | | LvA47 | <i>Homo sapiens</i> | São Paulo | BR | KF041794 |

Table 1 (continued)

| Trypanosomatid Species | CBT ^a | Isolate code ^b | Host | Geographic origin ^c | Accession number ^d | |
|----------------------------|------------------|---------------------------|-------------------------------|--------------------------------|-------------------------------|-----------------|
| | | | | | SSUrRNA | gGAPDH |
| <i>L. infantum chagasi</i> | | C1549 | <i>Canis lupus familiaris</i> | Mato Grosso | BR | <u>KF041797</u> |
| <i>L. infantum chagasi</i> | | C1149 | <i>Canis lupus familiaris</i> | Rio de Janeiro | BR | <u>KF041795</u> |
| <i>L. infantum chagasi</i> | | C1548 | <i>Canis lupus familiaris</i> | Mato Grosso | BR | <u>KF041796</u> |

^a CBT, Code number of the isolates/strains cryopreserved in the Coleção Brasileira de Tripanosomatídeos (CBT).

^b Original codes of isolates.

^c Bahia, Ceará, Distrito Federal, Mato Grosso, Mato Grosso do Sul, Pará, Pernambuco, Piauí, Rio de Janeiro, Rondonia and São Paulo are Brazilian states.

^d Sequences determined in this study and deposited in the Genbank are underlined.

3. Results

To evaluate phylogenetic relationships and polymorphisms within *Leishmania* species from different hosts and geographical origins, as well as to analyze the degree of genetic relatedness among these isolates and those of other trypanosomatid genera, we compared sequences of V7-V8 SSU rDNA genes aligned with those from reference strains from GenBank (Table 1). Most *Leishmania* species were aggregated in a monophyletic group, except for *L. equatoriensis* and *L. hertigi*, which were segregated with an isolate from the genus *Endotrypanum* (0.38% divergence of sequences and 95% bootstrap and 100% posterior probability) (Fig. 1). A single isolate of *L. enriettii* obtained from the host *Cavia porcellus* in the Brazilian state of Paraná (CBT 47) segregated at the basal branch in all phylogenetic results obtained and was placed together with *Leishmania* sp. (MAR 1), a human isolate from Martinique (100% bootstrap and 100% posterior probability). The divergence of sequences of *L. enriettii* and other *Leishmania* species ranged from 3.84% to 5.48% (Fig. 1).

The different species of the genus *Leishmania* were segregated into three groups: I. parasitic *Leishmania* species of Old World lizards including *Leishmania adleri*, *Leishmania tarentolae* and *Leishmania hoogstraali* (0.51% divergence of sequences and 92% bootstrap and 95% posterior probability); II. several human-infecting *Leishmania* species of different geographical origins that presented a broad spectrum of clinical manifestations (1.28% divergence of sequences and 83% bootstrap and 100% posterior probability); III. New and Old World species of visceral leishmaniasis (0.23% divergence of sequences and 80% bootstrap and 100% posterior probability), separated according to the geographical origin of the isolates. Phylogenetic analyses on the SSU rDNA V7V8 region grouped *L. infantum* and *L. donovani* distributed in the Old World separately from the Brazilian isolates of the New World *L. infantum chagasi*, with 100% similarity and 72/100% bootstrap and posterior probability, respectively (Fig. 1).

In all analyses using the trypanosomatid barcode region, *L. i. chagasi* isolates segregated into two branches according to their American (*L. infantum chagasi*) or Old World (*L. infantum*) origin. *L. i. chagasi* isolates from humans and dogs from South America (Brazil) were tightly clustered together (100% sequence similarities) (Fig. 1). All *L. i. chagasi* SSU rDNA V7V8 region and gGAPDH sequences were identical between them. The phylogenetic analysis based only on the gGAPDH gene separated *L. infantum* and *L. donovani* but was unable to separate the isolates of *L. infantum chagasi* from *L. infantum*. Phylogenetic analysis based on concatenated V7V8 SSU rDNA and gGAPDH genes enabled separation of the *L. donovani* complex species (Fig. 2). All *L. i. chagasi* isolates were clustered together (100% bootstrap/100% posterior probability and 100% of similarity) and were segregated from *L. infantum* and *L. donovani*, with 90% and 74% bootstrap and 100% posterior probability, respectively (Fig. 2).

4. Discussion

With the aim of gaining better understanding of the phylogenetic relationships of *Leishmania* species, we performed phylogenetic analysis on *Trypanosomatidae* barcode using V7V8 SSU rRNA and gGAPDH gene sequences among a large number of *Leishmania* species and also several Brazilian visceral *L. infantum chagasi* isolates obtained from dogs and humans.

The results from the genus *Trypanosoma* using gGAPDH and SSU rDNA have shown congruent phylogenetic relationships (Hamilton et al., 2004, 2007), while the same gene sequences used with the genus *Leishmania* revealed unrelated results (Fig. 2). The divergence values among the sequences of the *Leishmania* species ranged from 2.29% to 0.90%, thereby separating the *Sauroleishmania* species group from the *Vianna* and *Leishmania* subgenera. These values are low in comparison with the divergence rates of *Trypanosoma cruzi* intraspecific parasites (Marcili et al., 2009a,b,c), but are similar to divergence differences among *Trypanosoma rangeli* and *Trypanosoma theileri* lineages (da Silva et al., 2004; Rodrigues et al., 2006). These results are also compatible with the low evolution rates of *Leishmania* parasites, in comparison with other trypanosomatids (Stevens and Rambaut, 2001).

Our data from phylogenetic analysis on concatenated and independent V7V8 SSU rRNA and gGAPDH sequences corroborated the monophyletic origin of the genus *Leishmania* and also the data obtained using different SSU rDNA gene sequences (Briones et al., 1992; Orlando et al., 2002; Uliana et al., 1991; van Eys et al., 1992) and the gGAPDH gene (Hannaert et al., 1998), thus positioning the *Sauroleishmania* group as ancestral.

L. hertigi and *L. equatoriensis* were grouped within the genus *Endotrypanum* and were excluded from *Leishmania*. The biological cycles of *L. hertigi* and *L. equatoriensis* exhibit amastigote forms resembling *Leishmania* parasites (Lainson and Shaw, 1969), and species of the genera *Leishmania* and *Endotrypanum* are transmitted by sandflies that are genetically closely related (Cupolillo et al., 2000), which may explain the controversy regarding the taxonomic position of these organisms. However, differing from *Leishmania* species, which are able to parasitize different vertebrates such as sloths, anteaters, armadillos, opossums, rodents, canids, etc., and are distributed worldwide, the genus *Endotrypanum* is composed of parasites unique to sloth species that are found in Brazil, Colombia, Guyana and Central America (Herrer, 1971; Silva et al., 2013; Zeledon and Ponce, 1977). *L. hertigi* and *L. equatoriensis* were included in the genus *Leishmania* by molecular studies based on methodologies such as isoenzyme patterns or nucleic acid polymorphism sizes determined by enzymes restriction patterns (Cupolillo et al., 1998a,b, 2000). Such methods are useful to evaluate intraspecific polymorphisms, but have no evolutionary and/or taxonomic information. These studies, demonstrated that *L. hertigi* and *L. equatoriensis* presented polymorphisms more similar to the *Endotrypanum* than to the *Leishmania* genus species, indicating possible taxonomic prob-

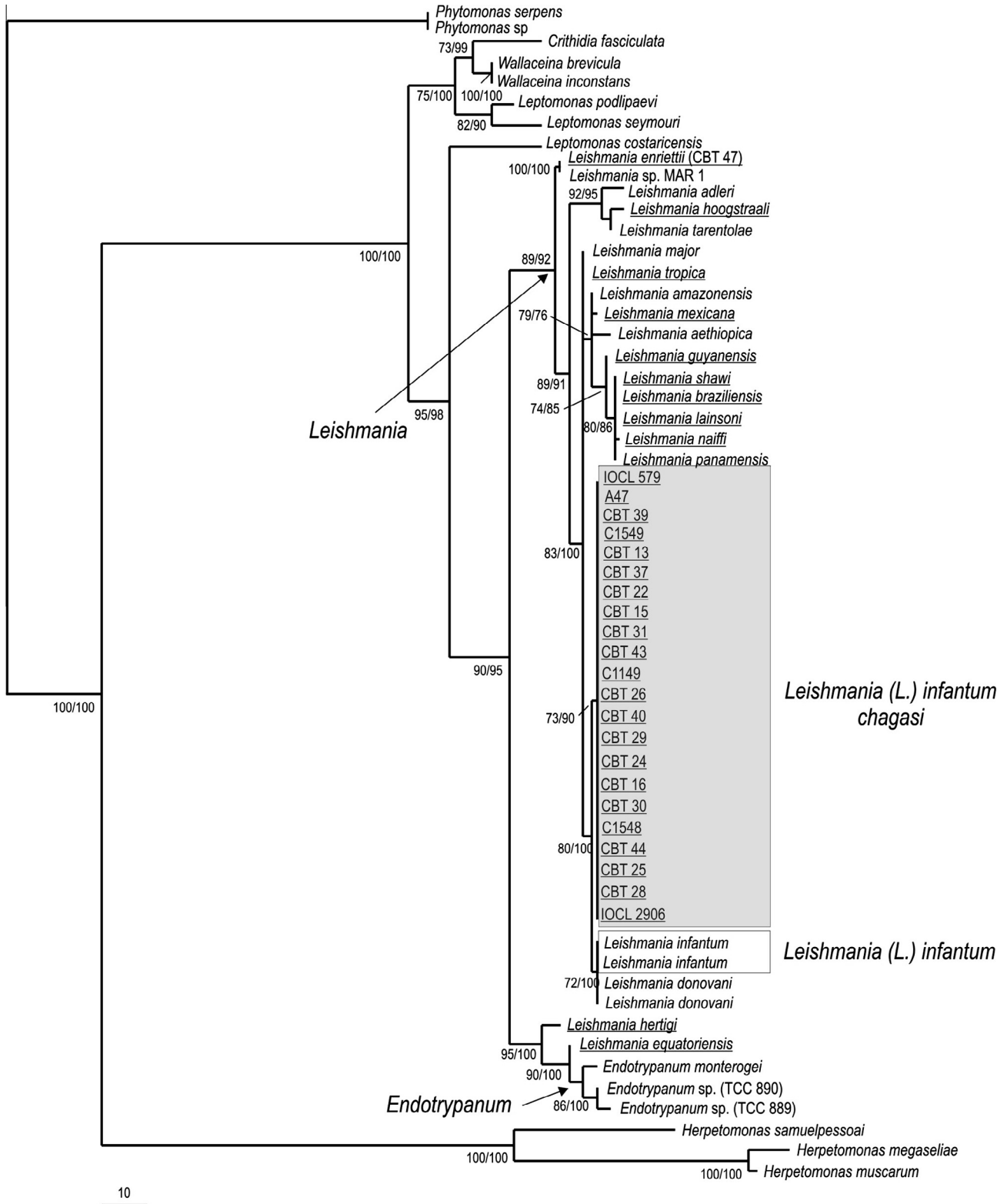


Fig. 1. Maximum parsimony and Bayesian tree inferred from SSU rDNA gene sequences on 57 trypanosomatids with *Phytomonas* as the outgroup (841 characters; 65 parsimony-informative sites). Numbers at nodes are the support values for the major branches (bootstrap/posterior probability; 500 replicates). The sequences obtained in this study are underlined.

lems. Also, the SSUrDNA gene sequences were not employed for phylogenetic positioning and taxonomic resolution of these parasites. Moreover, molecular analysis using PCR-RFLP on SSU rDNA and cytochrome b gene sequences has demonstrated that *L. hertigi* is more closely related to *Endotrypanum* than to *Leishmania* (Asato

et al., 2009; Noyes et al., 1997). Thus, we suggest that the taxonomic position of *L. hertigi* and *L. equatoriensis* should be revised so as to include these species in the genus *Endotrypanum*.

Phylogenetic analysis on the SSU rDNA gene showed that *Leishmania (L.) enriettii*, which is a parasite found in naturally

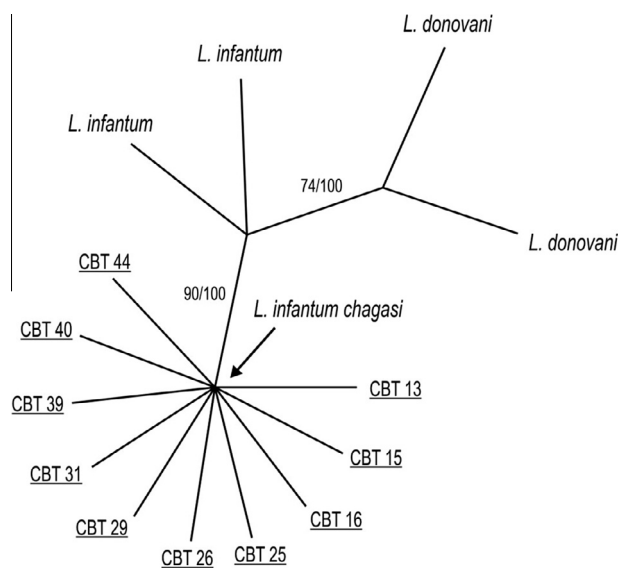


Fig. 2. Dendrogram based on concatenated SSU rDNA and gGAPDH sequences on 14 *Leishmania* species of the *L. donovani* complex, which was used for maximum parsimony and Bayesian inferences with 1469 characters. Numbers at nodes are the support values for the major branches (bootstrap/posterior probability; 500 replicates). The sequences obtained in this study are underlined.

infected domestic guinea pigs (*C. porcellus*) in the Brazilian states of Paraná and São Paulo (Machado et al., 1994; Muniz and Medina, 1948; Thomaz-Soccol et al., 1996) and probably in other sylvatic rodents (Lainson, 1997), has an ancient origin and was the first species to diverge within the genus *Leishmania*. This result is corroborated by molecular and distance analysis on the *Leishmania* cytochrome b and DNA and RNA polymerase gene sequences (Asato et al., 2009; Noyes et al., 2002). More recently, molecular characterization and distance analysis on the RNA polymerase II gene of *Leishmania* sp. isolated from skin lesions of red kangaroos in a rural area of Australia, and also from captive macropods, including several Northern wallaroos, a black wallaroo and agile wallabies, showed that these parasites were also grouped within the *L. enriettii* complex (Dougall et al., 2009; Rose et al., 2004). Considering that the phylogenetically closely related parasite *L. (L.) enriettii* is present in Australia, the early divergence and ancient origin of these species can be supported by the southern supercontinent hypothesis for the evolutionary history of other Trypanosomatidae parasites that have been isolated from bats. In this hypothesis, *T. cruzi* and related parasites evolved in isolation in the mammals of South America, Antarctica and Australia (Hamilton et al., 2012). In this scenario, to understand the evolutionary history of *Leishmania*, it would be important to characterize the biological cycle of these *L. (L.) enriettii* parasites, which is still poorly known. In the Brazilian state of Paraná, the sandfly *Lutzomyia monticola* was incriminated as a possible vector for *L. enriettii* (Luz et al., 1967), while in Australia, the potential vectors were found to be midges (Diptera: Ceratopogonidae) (Dougall et al., 2011). The evolutionary implication of different dipteran vectors in the life cycle of the *L. (L.) enriettii* group needs further investigation. In addition, it is important to note that human infection caused by parasites molecularly similar to *L. (L.) enriettii* has been described in HIV patients in Martinique, in the French West Indies (Noyes et al., 2002), suggesting that this group of ancient *Leishmania* parasites is also present in Central America.

The evolutionary history of *L. i. chagasi*, the etiologic agent of visceral leishmaniasis in South America has been widely debated. Some hypotheses suggest that *L. i. chagasi* was introduced along with the expansion of the canids in the New World about 2–3 million years ago (Lainson et al., 1987b). However, due to the failure to

distinguish *L. chagasi* from *L. infantum* through several biochemical and molecular studies performed so far (Hide et al., 2007; Ibrahim and Barker, 2001; Kuhls et al., 2005, 2011; Lukes et al., 2007; Mauricio et al., 2001, 2004; Quispe Tintaya et al., 2004), the taxonomy of the species causing visceral leishmaniasis in the Americas was recently revised and it was suggested that the name *L. (L.) infantum chagasi* should be used (Kuhls et al., 2005, 2011; Lainson and Shaw, 1988; Mauricio et al., 1999, 2001, 2004). However, our phylogenetic analysis using Trypanosomatidae barcode resulted in separation of *L. chagasi* and *L. infantum*, thus revitalizing the discussion on the origin of *L. chagasi*.

Since canids are natural reservoirs for *L. infantum* and *L. chagasi*, and the evolutionary histories of vertebrate hosts are important tools in reconstructing the evolutionary history of parasites of the family Trypanosomatidae (Hamilton, 2012; Hamilton et al., 2012), a discussion about the origin and radiation of canids could correlate with the introduction of these parasites in South America, as believed previously (Lainson et al., 1987b). The first records of the order Carnivora are from the Paleocene and Eocene, with radiation in North America and subsequently in Eurasia (Azzaroli et al., 1988; Sotnikova and Rook, 2010). The members of this order were originally absent in South America and Australia, where their niches were occupied by marsupials (Eisenberg and Redford, 1999). Procyonids were the first carnivores to migrate to South America during the Miocene, and other groups arrived only after the switching on the Isthmus of Panama during the Pliocene (Linares, 1981). Furthermore, several studies based on fossil records have shown that vast diversification of canids occurred in North America (Spassov and Rook, 2006; Sotnikova, 2006; Wang and Telford, 2007; Garrido and Arribas, 2008). Thus, if North American canids were responsible for the introduction of *L. chagasi* into South America, they should harbor *L. donovani* complex parasites. However, cases of visceral leishmaniasis in North America are rare, and are related to transplacental transmission, blood transfusion and exchange of dogs from endemic regions, without occurrences of vector transmission (Boggiatto et al., 2011; Duprey et al., 2006; Gibson-Corley et al., 2008; Giger et al., 2002; Owens et al., 2001). Furthermore, serological studies on Gray and Red Fox populations in North America have shown low positivity and possibly even cross-reactivity with *T. cruzi*, which is endemic in the region studied (Rosypal et al., 2010a,b). These pieces of evidence go against an ancient origin for *L. i. chagasi* in South America.

Therefore, the more likely origin of *L. i. chagasi* corresponds to the introduction of *L. infantum* around five hundred years ago, during the colonization of South America by Europeans, through infected domestic dogs. The low divergence found between *L. infantum* and *L. chagasi* (0.26%) based on V7V8 SSU rRNA does not explain and/or distinguish between the two hypotheses regarding the evolutionary origin of these species. However, the biogeography of canid hosts, *L. donovani* complex parasite occurrence and distribution in North America and the low divergence of *L. chagasi/L. infantum* V7V8 SSU rRNA values strongly support the notion that *L. chagasi* originated due to introduction of parasites through human actions. This hypothesis is also corroborated by the bottleneck signature of *L. chagasi* in the New World, accompanied by a thousand-fold decrease in population diversity in comparison with *L. infantum* in Europe (Kuhls et al., 2011).

The populational variability shown by microsatellites demonstrated gene flow between New and Old World *L. infantum* and corroborated the notion of recent introduction of *L. infantum* species into the Americas (Kuhls et al., 2008, 2011). However, with this approach, it was not possible to distinguish between the New and Old World parasite populations, probably due to the characteristic gene flow of microsatellites.

Nevertheless, our result validates the existence of the subspecies *L. i. chagasi* as a valid taxonomic unit, and its specific identification by

the V7V8 SSU rRNA sequence enables epidemiological studies. This is of particular importance to Brazil and other Latin American countries, where visceral leishmaniasis caused by *L. i. chagasi* is expanding, following the dispersion of its major vector, *Lutzomyia longipalpis*, which has become adapted to periurban environments (Dias et al., 2011; Nascimento et al., 2008) and has spread to new areas in Argentina and Uruguay, thereby expanding the endemic regions affected by human-threatening visceral leishmaniasis (Cordoba-Lanus et al., 2006; Gould et al., 2013; Salomon et al., 2011).

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