VECTOR-BORNE AND ZOONOTIC DISEASES
Volume XX, Number XX, 2020

DOI: 10.1089/vbz.2020.2638

# Canine Visceral Leishmaniasis in São Paulo, Brazil, the Most Populous City of South America: Isolation, Molecular Diagnosis, and Phylogenetic Inferences

Arlei Marcili,[1,2] Ryan Emiliano da Silva,[2] Vilma Pereira da Costa,[1] Fernanda A. Nieri-Bastos,[2] Roberta Carvalho de Freitas e Azevedo,[1] Jonas Moraes Filho,[1] and Renata Tonhosolo[3]



## Abstract

***Background and Objectives:*** Canine visceral leishmaniasis affects dogs, the main urban reservoirs, which favor the transmission and expansion of this zoonotic disease in areas with high anthropization process and human density. We investigated the occurence of *Leishmania infatum* based in molecular diagnosis, and phylogenetic analysis of isolates obtained from dogs in metropolitan region of São Paulo.
***Methods:*** A total of 201 dogs were tested by parasitological and molecular diagnosis. Phylogenetic analysis based sequences from SSUrDNA and gGAPDH genes were performed.
***Results:*** The parasitological diagnosis revealed 5% (10/201) of positivity, and the sequences obtained from seven isolates were clustered with L. *infantum* in phylogentic analysis based on SSUrDNA and gGAPDH genes. A total of 24.9% (50/201) of dogs were positive in molecular diagnosis based on cathepsin L-*like* marker.
***Interpretation and Conclusion:*** According to this study, it is necessary to implement a surveillance policy of visceral leishmaniasis, intensifying the actions of diagnosis, prevention, and control of this zoonosis.

**Keywords:** *Leishmania*, pathology, zoonosis, molecular diagnosis

## Introduction

Visceral leishmaniasis has a wide distribution in South America where the etiological agent of the infection is the heteroxenic protozoan *Leishmania infantum* (Marcili et al. 2014), transmitted between mammals of different orders through the blood repast of female sand flies of different species (Maroli et al. 2013). This zoonosis is one of the most important tropical diseases among the 10 infectious diseases with the highest number of cases (Hotez et al. 2004). Between 200,000 and 400,000 new human cases are estimated to occur annually, of which 90% come from India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil (Alvar et al. 2012). In a broader risk estimate, 1.7 billion people, or a quarter of the world's population, reside in areas with potential risk of leishmaniasis (Pigott et al. 2014).

In Brazil, visceral leishmaniasis affects 21 states, distributed in 1600 different municipalities with records of autochthonous human cases (Anversa et al. 2018). Transmission chains have historically been related to the northeast region; however, epidemiological data series have shown a disease expansion movement in the southeastern region (Reis et al. 2017), including the state of São Paulo (Bezerra et al. 2018).

The spatiotemporal expansion route in São Paulo state is associated with anthropic interventions and major infrastructure projects that have increased the disease frontier to the eastern portion of the state (endemic region) toward its metropolitan region, where the advancement of visceral leishmaniasis represents an alert, for both animal and human health (Cardim et al. 2016, Sevá et al. 2017, Oliveira et al. 2018).

Canids play a fundamental role in the transmission cycle (Deane and Deane 1954, 1955), mainly the domestic dog considered as the main reservoir host of the agent, especially in urban and periurban environments (Ashford et al. 1998). The main clinical manifestations in dogs involve anorexia, exfoliative, pustular, or ulcerative dermatological lesions, nonpruritic alopecia, bilateral blepharitis, depigmentation of the nasal region, onychogryphosis, popliteal lymphadenomegaly, ophthalmopathy, hepatomegaly, splenomegaly, and even chronic renal impairment (Ribeiro et al. 2018). However, there are dogs classified as asymptomatic, which, despite being infected, maintain a healthy appearance, constituting an additional

[1]Department of Medicine and Animal Welfare, Santo Amaro University, São Paulo, Brazil.
[2]Department of Veterinary Medicine and Animal Health, University of São Paulo, São Paulo, Brazil.
[3]Medical College—Santo Amaro University, São Paulo, Brazil.

challenge for epidemiological studies, making latent the need to employ sufficiently sensitive diagnostic methodologies to identify these carriers (Berrahal et al. 1996).

All studies on the spread of leishmaniasis in the state of São Paulo have pointed to the same path that the disease has been spreading west east. This dispersion follows a well-established pattern, for both canine and human cases, and for vectors, bordering mainly on highways, and that, in line with what happened in other states in the late 1990s, the metropolitan region will soon be an endemic area, example of what today is the whole region of the west of São Paulo (Matsumoto et al. 2013).

However, despite the sanitary importance of visceral leishmaniasis and epidemiological projections that indicate an increase in the prevalence values, there are few studies that evaluate the frequency and circulation of the disease in the metropolitan region of São Paulo, so this study aims to diagnose, isolate, and characterize *L. infantum chagasi* in domestic dogs of the metropolitan region of São Paulo.



## Subjects and Methods

The metropolitan region of São Paulo comprises 39 municipalities. In this study, dogs in the municipalities of Barueri, Carapicuíba, Cotia, Embu das Artes, Osasco, São Bernardo do Campo, Sâo Paulo, and Vargem Grande Paulista were sampled; dogs of both genders, of different breeds and ages, residing in the area, all belonging to São Paulo. Clinical data were recorded at the time of collection, and the respective consent terms were filled in according to the Animal Use Ethics Committee (n° 09/2016 CEUA UNISA).

From each animal, a venous blood sample was collected, placed in a sterile tube containing absolute ethanol (v/v) for molecular diagnosis. Sample of the puncture popliteal lymph node content and skin samples (biopsies) collected through a 3 mm dermatological punch for parasite isolation. In addition, the skin samples were placed in vials containing PBS1X plus gentamicin, ampicillin, and amphotericin B at the time of collection until isolation process.

### *Isolation of* Leishmania

The aspirate contents and skin biopsy were inoculated into Vacutainer tubes containing a biphasic medium consisting of 15% sheep red blood cells as the solid phase (blood agar base), overlain by liquid LIT (Liver Infusion Tryptose) medium supplemented with 20% fetal bovine serum. The culture was incubated at 28°C and grown in LIT medium for DNA preparation. The isolates were cryopreserved in liquid nitrogen in the Brazilian Trypanosomatid Collection (Coleção Brasileira de Tripanossomatídeos, CBT) in the Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine, University of São Paulo, Brazil.

### *Molecular diagnosis and phylogenetic analysis*

The DNA from *Leishmania* cultures and blood samples were extracted using Purelink Genomic DNA (Termofisher). DNA samples from *Leishmania* isolates were subjected to the conventional PCR for trypanosome barcoding (V7V8 SSUrDNA) (Maia da Silva et al. 2008) and gGAPDH gene (Hamilton et al. 2004). The Cathepsin-*like* PCR was used for *L. infantum* diagnosis (Silva et al. 2019). PCR products of the expected size were purified and sequenced in an automated sequencer (ABI Prism 310). The nucleotide sequences generated were deposited in GenBank (Table 1).

The sequences obtained from this study were aligned with sequences previously determined and available in GenBank (Table 1) using ClustalX (Thompson et al. 1997) and were adjusted manually using GeneDoc (Nicholas et al. 1997). The phylogenetic tree was constructed using maximum parsimony, as implemented in PAUP version 4.0b10 (Swofford 2002) with 500 bootstrap replicates. The Bayesian analysis was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) with 1,000,000 replicates. The first 25% of the trees represented burn-in, and the remaining trees were used to calculate the Bayesian posterior probability.

## Results

A total of 201 animals were sampled: 80 animals in Cotia, 59 in Vargem Grande Paulista, 19 in Embu das Artes, 16 in Osasco, 12 in Carapicuíba, 10 in São Paulo, and 5 in São Bernardo do Campo.

Cultures seeded with lymph node puncture material and/or skin biopsies revealed a positive frequency of 5% (10/201). Considering the municipalities sampled individually, the percentages obtained were 6.25 (5/80) for the municipality of Cotia, 10.5 (2/19) for Embu das Artes, 6.25 (1/16) for Osasco, 10 (1/10) in São Paulo, and 1.7 (1/59) in Vargem Grande Paulista, with no positive punctures found for the other territories analyzed (Fig. 1). Among these 10 positive cultures, 7 were properly isolated and cryopreserved for molecular and phylogenetic characterization (Table 1).

Molecular diagnosis based on cathepsin marker L-*like* showed a positive percentage of 24.9 (50/201), with values of 30% (24/80), 27.1% (16/59), 21% (4/19), 33.3% (4/12), 6.25% (1/16), and 10% (1/10), for the municipalities of Cotia, Vargem Grande Paulista, Embu das Artes, Carapicuíba, Osasco, and São Paulo, respectively (Fig. 1). All positive samples in the direct parasitological diagnosis were positive in the molecular reactions for cathepsin L-*like*, a coincidence that confirms the specificity of the marker and helps demonstrate its validity for conducting epidemiological surveys.

Similarity analysis (Blast) of SSUrDNA and gGAPDH gene sequences obtained from the seven isolates showed 99% similarity to *L. infantum* (XR_001203206). The phylogenetic analysis based on concatenated sequence alignments revealed the segregation of species causing visceral leishmaniasis into three distinct groups (Fig. 2). The groups formed were as follows: I. *Leishmania donovani* (100%

Table 1. Sample and Geographic Origin of *Leishmania infantum* Isolates Obtained in São Paulo Metropolitan Area

| *CBT* | *Code* | *Samples* | *Geographical origin* |
|---|---|---|---|
| 236 | A1 | Lymph node | São Paulo |
| 244 | R13 | Lymph node | Vargem Grande Paulista |
| 258 | E2 | Lymph node | Embu das Artes |
| 259 | V1 | Lymph node | Osasco |
| 274 | B47 | Skin | Cotia |
| 275 | B47 | Lymph node | Cotia |
| 276 | B9 | Lymph node | Cotia |

CBT, Coleção Brasileira de Tripanossomatídeos.

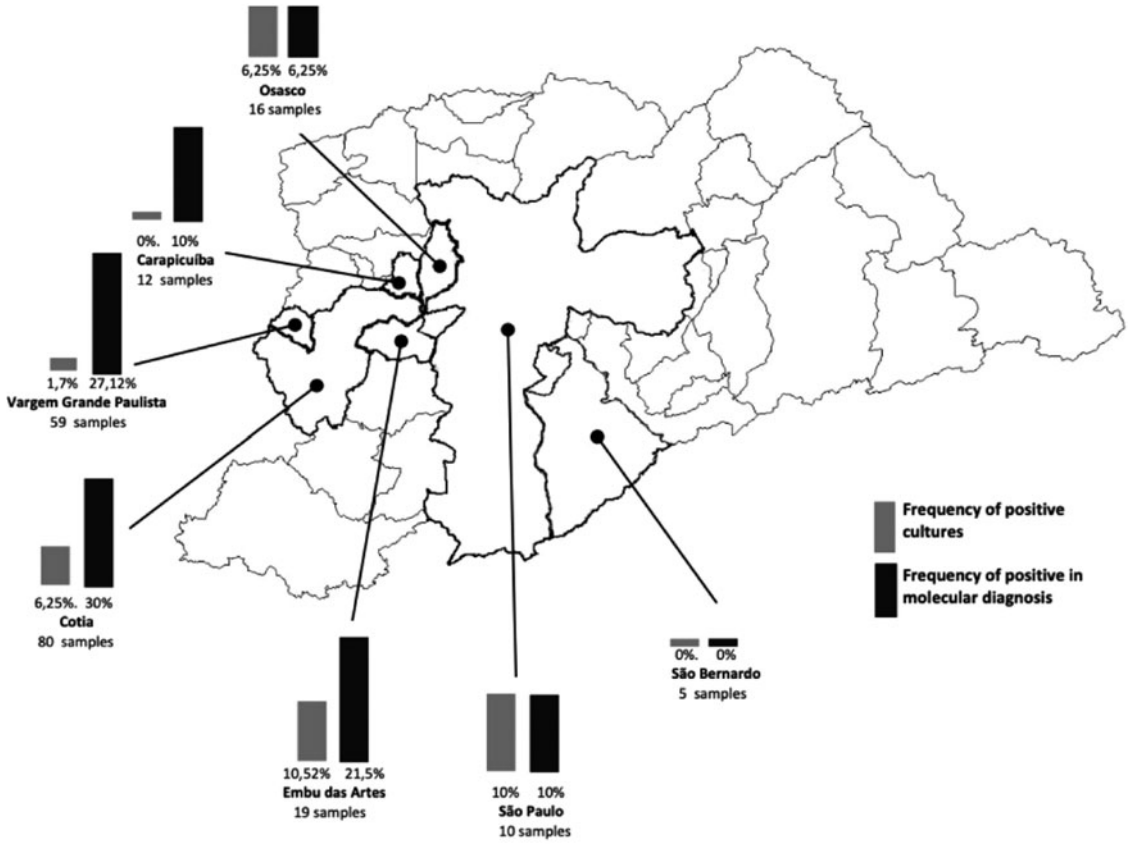


**FIG. 1.** Geographical origin of dogs caught in the São Paulo metropolitan city and frequency of parasitological and molecular diagnosis.

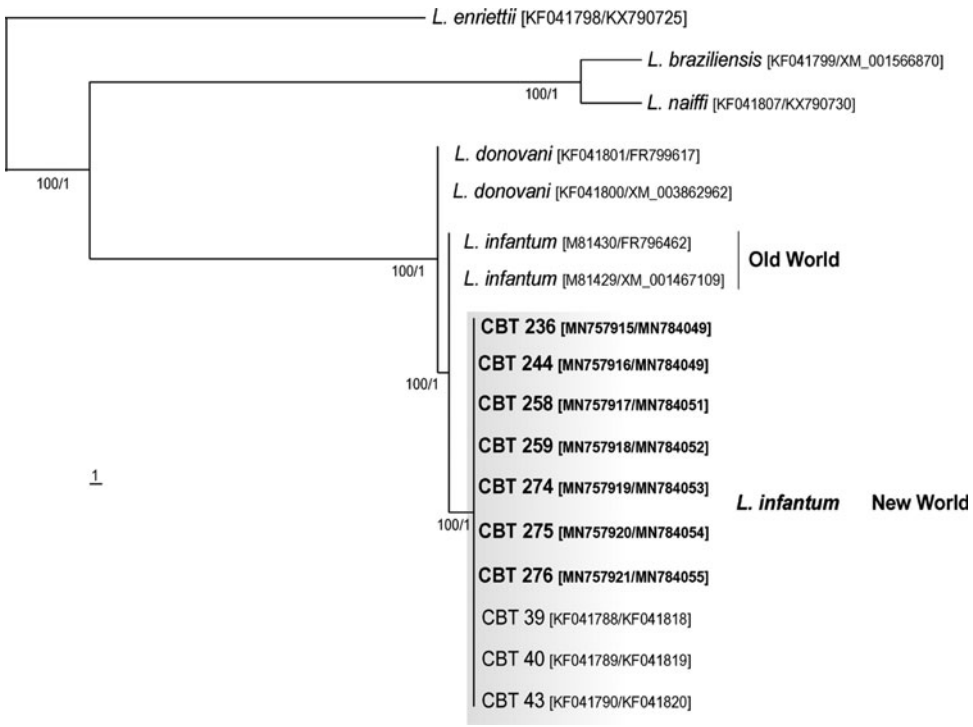


**FIG. 2.** Dendrogram based on concatenated SSU rDNA and gGAPDH sequences on 17 *Leishmania*, which was used for maximum parsimony and Bayesian inferences. Numbers at nodes are the support values for the major branches (bootstrap/posterior probability; 500 replicates). The sequences obtained from this study are indicated in *bold*. CBT, Coleção Brasileira de Tripanossomatídeos.

similarity and 100% bootstrap and 1.0 a posteriori probability), II. *L. infantum* Old World (100% similarity and 100% bootstrap and 1.0 a posteriori probability), and III. *L. infantum* New World (100% similarity and 100% bootstrap and 1.0 a posteriori probability).

The phylogenetic analysis did not show differences between the sequences of the several isolates obtained in the metropolitan region of São Paulo, nor were there any differences between these isolates and those from other geographical regions of Brazil, confirming the inexistence of intraspecific variability among the isolates of circulating *L. infantum* in the region investigated and in the country.

## Discussion

Phylogenetic inferences indicated a complete identity among the isolates. When the sequences obtained from the isolates were compared with sequences from isolates from other geographical origins, the identity profile was maintained, attesting that the isolates correspond to *L. infantum* and confirming the absence of intraspecific variations previously observed in multimarker analyses (Marcili et al. 2014, Silva et al. 2019).

The phylogenetic groups and their respective distances reflect the differences between the species causing visceral leishmaniasis and their distribution worldwide, affirming the recent segregation between *L. infantum* and *L. infantum chagasi* during the European colonization process in America and the conserved character of their sequences (Kuhls et al. 2011, Marcili et al. 2014, Espinosa et al. 2018).

Data from the parasitological and molecular diagnosis reinforce the hypothesis of the disease's tendency to expand, in which the role of human activities in ecological dynamism has allowed the number of municipalities with pathogen circulation records to grow abruptly (Camargo-Neves et al. 2003, Cardim et al. 2013, Sevá et al. 2017).

One of the objections to the hypothesis of visceral leishmaniasis transmission in the studied region was the absence of the *Lutzomya longipalpis* vector. However, findings point out that the sum of the high attractiveness by the dog, the susceptibility to protozoan infection, the life expectancy of the infected insect, and especially the high population density make *Pintomya fischeri* a potential vector of canine transmission in situations where *L. longipalpis* is absent (Galvis-Ovallos et al. 2017).

Studies demonstrated incidence rates of *P. fischeri* ranging from medium to high values in various localities of the state of São Paulo, including the municipalities of Cotia, Embu das Artes, Osasco, Vargem Grande Paulista, and São Paulo, which accumulated the positive cases diagnosed (Shimabukuro et al. 2010).

The epidemiology of the disease demonstrates that autochthonous human cases in a territory are preceded by canine notifications (Camargo-Neves et al. 2001). Thus, this work, by providing new elements that attest and corroborate the occurrence of canine visceral leishmaniasis in the metropolitan region of São Paulo, points to the need for studies that identify new ecoepidemiological elements of the disease that investigate the possibilities of human morbidity and mortality and draw attention to the need for adoption of diagnostic methodologies of high predictive value that contribute to the prevention and control of this important zoonosis.

The city of São Paulo has legislation that regulates from the possession of dogs and cats by individuals to their commercial breeding. Among the prohibitions in the city is that it is illegal to keep >10 animals in the same residence, regardless of size or species. With these legal measures, kennels (whether legal or illegal), shelters, nongovernmental organization, and animal rescuers eventually moved their property to cities in the metropolitan region where such rules do not exist and they need not worry about legal measures, such as court restraints, fines, and other penalties provided for by law.

Although city law requires the registration of all dogs and cats living in São Paulo, enforcement of this law is not effective and/or nonexistent. Sanitary control measures related to animal traffic would be more effective, given that animal traffic is mostly conditioned by guardian traffic.

Vector control measures in different municipalities and the requirement of a health certificate are effective measures to control this zoonosis, since for the initiation of the transmission cycle in new localities, the presence of both the vector and vertebrate hosts harboring the parasite is necessary. Such measures are adopted in health programs against foot-and-mouth disease, classical swine fever, and equine infectious anemia, where for animal transport a health certificate or vaccination certificate is required, and road surveillance is effective, for diseases in that the biggest damage is purely economic and not public health.

The metropolitan region of São Paulo is considered the fourth largest in the world by number of inhabitants and, consequently, high concentration of pets and the presence of dogs diagnosed as positive demonstrate that the parasite is circulating throughout the metropolitan region of São Paulo; hence urgent measures must be taken to prevent the region from becoming endemic to visceral leishmaniasis.

## Authors' Contributions

R.E.S. and A.M. conceived the study and designed the experiments; R.E.S., V.P.C., F.A.N.C., R.C.F.A., and J.M.F. assisted with sample collection, performed the experiments and analyzed the data; and R.E.S., R.T., and A.M. prepared the article. All authors read and approved the final version of the article.

## Acknowledgment

The authors thank all dog owners who accepted to collaborate in this study.

## Author Disclosure Statement

No conflicting financial interests exist.

## Funding Information

This research was financially supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq [grant number 303288/2015-9 and 302145/2018-4], Fundação de Amparo a Pesquisa do Estado de São Paulo [grant number 2015/25592-3], and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

## References


Alvar J, Vélez ID, Bern C, Herrero M, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 2012; 7:e35671.

Anversa L, Tiburcio MGS, Richini-Pereira VB, Ramirez LE. Human leishmaniasis in Brazil: A general review. Rev Assoc Med Bras 2018; 64:281–289.

Ashford DA, David JR, Freire M, David R, et al. Studies on control of visceral leishmaniasis: Impact of dog control on canine and human visceral leishmaniasis in Jacobina, Bahia, Brazil. Am J Trop Med Hyg 1998; 59:53–57.

Berrahal F, Mary C, Roze M, Berenger A, et al. Canine leishmaniasis: Identification of asymptomatic carriers by polymerase chain reaction and immunoblotting. Am J Trop Med Hyg 1996; 55:273–277.

Bezerra JMT, Araújo VEM, Barbosa DS, Martins-Melo FR, et al. Burden of leishmaniasis in Brazil and federated units, 1990–2016: Findings from Global Burden of Disease Study 2016. PLoS Negl Trop Dis 2018; 12:e0006697.

Camargo-Neves VLF, Katz G, Rodas LAC, Poletto DW, Lage LC, Spínola RMF, et al. Use of spatial analysis tools in the epidemiological surveillence of American visceral leishmaniasis, Araçatuba, São Paulo, Brazil, 1998–1999. Cad. Saúde Pública 2001; 17:1263–1267.

Camargo-Neves VLF, Spínola R, Lage L. American Visceral Leishmaniasis in São Paulo: 2001–2002. Rev Soc Bras Med Tro 2003; 36:27–29.

Cardim MF, Rodas LA, Dibo MR, Guirado MM, et al. Introduction and expansion of human American visceral leishmaniasis in the state of Sao Paulo, Brazil, 1999–2011. Rev Saude Publica 2013; 47:691–700.

Cardim MFM, Guirado MM, Dibo MR, Neto FC. Visceral leishmaniasis in the state of São Paulo, Brazil: Spatial and space-time analysis. Rev Saúde Pública 2016; 50:48.

Deane LM, Deane MP. Encontro de leishmânias nas vísceras e na pele de uma raposa, em zona endêmica de calazar, nos arredores de Sobral, Ceará. O Hospital 1954; 45:419–421.

Deane LM, Deane MP. Observações preliminares da importância comparativa do homem, do cão e da raposa (*Lycalopex vetulus*) como reservatórios de *Leishmania donovani*, em área endêmica de calazar, no Ceará. Hospital 1955; 48:61–70.

Espinosa OA, Serrano MG, Camargo EP, Teixeira MMG, et al. An appraisal of the taxonomy and nomenclature of trypanosomatids presently classified as Leishmania and *Endotrypanum*. Parasitology 2018; 145:430–442.

Galvis-Ovallos F, Silva MD, Bispo GB, Oliveira AG, et al. Canine visceral leishmaniasis in the metropolitan area of São Paulo: *Pintomyia fischeri* as potential vector of *Leishmania infantum*. Parasite 2017; 24:2.

Hamilton PB, Stevens JR, Gaunt MW, Gidley J, et al. Trypanosomes are monophyletic: Evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. Int J Parasitol 2004; 34:1393–1404.

Hotez PJ, Remme JH, Buss P, Alleyne G, et al. Combating tropical infectious diseases: Report of the Disease Control Priorities in Developing Countries Project. Clin Infect Dis 2004; 38:871–878.

Kuhls K, Alam MZ, Cupolillo E, Ferreira GE, et al. Comparative microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. PLoS Negl Trop Dis 2011; 5:e1155.

Maia da Silva F, Naiff RD, Marcili A, Gordo M, et al. Infection rates and genotypes of *Trypanosoma rangeli* and *T. cruzi* infecting free-ranging *Saguinus bicolor* (Callitrichidae), a critically endangered primate of the Amazon Rainforest. Acta Trop 2008; 107:168–173.

Marcili A, Sperança MA, Costa AP, Madereira MF, et al. Phylogenetic relationships of Leishmania species based on trypanosomatid barcode (SSU rDNA) and gGAPDH genes: Taxonomic revision of *Leishmania (L.) infantum chagasi* in South America. Infect Genet Evol 2014; 25:44–51.

Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, et al. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Med Vet Entomol 2013; 27:123–147.

Matsumoto PSS, Lima J, Casagrande B. Visceral leishmaniasis in São Paulo's state: cartographic and statistics applications Hygeia 2013; 9:195–203.

Nicholas KB, Nicholas Jr HB, Deerfield DW: GeneDoc: analysis and visualization of genetic variation. Embnew News 1997, 4:14.

Oliveira AM, López RVM, Dibo MR, Rodas LAC, et al. Dispersion of *Lutzomyia longipalpis* and expansion of visceral leishmaniasis in São Paulo State, Brazil: Identification of associated factors through survival analysis. Parasit Vectors 2018; 11:503

Pigott DM, Bhatt S, Golding N, Duda KA, et al. Global distribution maps of the leishmaniases. Elife 2014; 3:e02851.

Reis LL, Balieiro AAS, Fonseca FR, Gonçalves MJF. Changes in the epidemiology of visceral leishmaniasis in Brazil from 2001 to 2014. Rev Soc Bras Med Trop 2017; 50:638–645.

Ribeiro RR, Michalick MSM, Silva ME, Santos CCP, et al. Canine Leishmaniasis: An overview of the current status and strategies for control. Biomed Res Int 2018; 2018:3296893.

Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003; 19:1572–1574.

Sevá AP, Mao L, Galvis-Ovallos F, Lima JMT, et al. Risk analysis and prediction of visceral leishmaniasis dispersion in São Paulo State, Brazil. PLoS Negl Trop Dis 2017; 11: e0005353.

Shimabukuro PH, Silva TR, Ribeiro FO, Baton LA, et al. Geographical distribution of American cutaneous leishmaniasis and its phlebotomine vectors (Diptera: Psychodidae) in the state of São Paulo, Brazil. Parasit Vectors 2010; 3:121.

Silva RE, Sampaio BM, Tonhosolo R, Costa APR, et al. Exploring *Leishmania infantum* cathepsin as a new molecular marker for phylogenetic relationships and visceral leishmaniasis diagnosis. BMC Infect Dis 2019; 19:895.

Swofford DL. *PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), Beta Version 4.0b10*. Sunderland, MA: Sinauer and Associates, 2002.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, et al. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25:4876–4882.



Address correspondence to:
*Arlei Marcili*
*Department of Veterinary Medicine and Animal Health*
*University of São Paulo*
*Av. Prof. Eneas de Siqueira Neto, 340*
*São Paulo 04829-300*
*Brazil*

*E-mail:* amarcili@prof.unisa.br