

First detection of *Leishmania infantum* in dogs and wild-caught *Culicoides oxystoma* in Hainan Province, Southern China

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Abstract

Background

Leishmaniasis are neglected parasitic diseases expanding globally. In China, *Leishmania infantum*, endemic in the west, has reemerged and spread to the central part of the country, raising concerns about further potential expansion. Given limited data from southern China, this study investigated the presence of *Leishmania* spp. infection in dogs and potential vectors in Haikou, Hainan, China's southernmost Province.

Methods

Blood, conjunctival swabs, and lymph node aspirates were collected from 52 dogs at two shelters, where an entomological survey using CDC light traps was also performed. *Leishmania* DNA was amplified by kDNA and ITS1 PCR. A commercial rapid test (SNAP® Leish 4Dx®; IDEXX, USA) and immunofluorescence antibody test (IFAT) were used to detect antibodies against *L. infantum*. Insects were morphologically and molecularly identified, and tested for *Leishmania* by ITS1 PCR and sequencing. Phylogenetic relationships were inferred using Maximum likelihood and neighbour-joining trees constructed in MEGA X.

Results

Overall, 4 of 52 samples (7.7%) were serologically (n = 3) or molecularly (n = 1, conjunctival swab by kDNA PCR) positive for *Leishmania* species. Two dogs were SNAP® positive, with one showing generalized lymphadenomegaly, and one was IFAT positive (1:160). No sand flies and 1568 *Culicoides* were captured. *Leishmania* DNA sequences (10 *L. infantum*, 1 *L. tarentolae*) were detected in 11/224 non-engorged female *Culicoides oxystoma* and clustered within their corresponding phylogenetic clades.

Conclusions

This study provides the first evidence of *L. infantum* infection in dogs and biting midges from Hainan Province. Ongoing surveillance of competent vectors and dogs in this region is advocated.

Background

Leishmaniasis is a parasitic disease caused by protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) [1] and one of the most severe neglected tropical diseases, which may display various clinical forms in humans, including cutaneous, mucocutaneous, and the most severe form, visceral leishmaniasis (VL) [2]. Visceral leishmaniasis is primarily caused by *Leishmania donovani* and *Leishmania infantum* [3], the latter being zoonotic and transmitted by sand flies of the genus

Phlebotomus and *Lutzomyia* in the Old and New World, respectively [4]. Domestic dogs are major reservoir hosts for *L. infantum*, but other animals such as wild canids (e.g., red foxes, maned wolves, bush dogs), rodents, and lagomorphs have also been identified as sylvatic reservoirs [5, 6].

The major endemic regions for *L. infantum* are South America, the Mediterranean region, and parts of western and central Asia [7]. In China, *L. infantum* causes two distinct subtypes of zoonotic VL based on their geographical characteristics, sand fly vectors, and reservoir hosts, namely mountain-type (MT-ZVL) and desert-type (DT-ZVL) [8]. MT-ZVL predominates in mountainous western Provinces (Gansu, Sichuan, Shaanxi, Shanxi, Henan, and Hebei), where domestic dogs serve as primary reservoir hosts, and *Phlebotomus chinensis* as the main vector [8]. DT-ZVL predominates in northwestern desert Provinces (i.e., Xinjiang, northern Gansu, and western Inner Mongolia), where *Phlebotomus wui* and *Phlebotomus alexandri* are the main vectors. However, the animal reservoir hosts of DT-ZVL have not been identified [8].

The mean annual incidence of human VL in China was 0.0228 cases/100,000 from 2004 to 2019 [9]. Since 2020, new hotspots of MT-ZVL in central and eastern China, particularly along the Shaanxi-Shanxi border and in eastern Shanxi, have been identified through spatio-temporal cluster analysis of data from the National Notifiable Infectious Disease Reporting System, indicating the emergence of MT-ZVL in these regions [10, 11]. Investigation of canine populations in areas where recent VL emergence has been reported revealed a relatively high prevalence of *L. infantum* in dogs (14.9%, 139/929) by molecular analysis [12]. These data highlight the need to perform surveillance in canine populations alongside vector surveillance in areas where human VL has not yet been reported, as canine leishmaniasis (CanL) often precedes human cases [13].

Hainan Province, the southernmost Province in China, is an island separated from the Leizhou Peninsula in Guangdong Province by the Qiongzhou Strait. Its tropical monsoon climate is markedly different from that of most of the Chinese mainland. In addition, Hainan is China's largest free trade port and a major tourism destination, with large movements of people and animals. These factors combined increase the risk of infectious diseases, particularly vector-borne diseases such as leishmaniasis [14].

The aim of this study was to investigate the presence of *Leishmania* infection among dogs and potential vectors for leishmaniasis in Haikou, Hainan.

Results

Overall, 52 dogs, including 30 dogs in shelter 1 and 22 dogs in shelter 2, were tested for *Leishmania* spp. (n = 30, shelter 1; n = 22, shelter 2). All dogs were mongrels (39 females, 13 males), ranging from 4 months to 12 years (median 2.4 years) of age, and lived outdoors. The majority of the animals (38/52; 73%) were apparently healthy, while the remaining dogs (14/52; 27%) exhibited clinical signs consistent with possible canine leishmaniasis, including lymphadenomegaly (n = 7), mucocutaneous and cutaneous lesions (n = 5), conjunctivitis (n = 2), and weight loss (n = 1). Overall, 4/52 dogs (7.7%), all from shelter 2, were serologically (n = 3) or molecularly (n = 1) positive for *Leishmania* species.

Two dogs, a 7-month-old female and a 5-year-old male, tested positive for antibodies against *L. infantum* on the SNAP Leish 4Dx test (IDEXX Laboratories, Westbrook, ME, USA). The latter had generalized lymphadenomegaly and was co-infected with *Hepatozoon canis*. Both of these dogs tested negative on IFAT, while a third dog (4-month-old, female) tested positive by IFAT with a titre of 1:160.

Leishmania DNA was detected in one conjunctival swab sample from another 5-year-old male dog using a kinetoplast minicircle DNA (kDNA) real-time polymerase chain reaction (qPCR) assay, with a Ct value of 29. However, conventional PCRs targeting kDNA and heat shock protein 70 (hsp70) did not yield amplifiable sequences. *Leishmania* DNA was not detected in any blood samples from the 52 dogs or in any of 27 lymph node aspirates obtained.

Tests for other vector-borne pathogens (VBP) revealed the presence of *Babesia vogeli* (4/52, 7.7%), *H. canis* (17/52, 32.7%) by molecular analysis, and *Dirofilaria immitis* (1/52, 1.9%), and *Ehrlichia* spp. (1/52, 1.9%) by point-of-care (POC) assay. Half of the tested dogs (26/52; 50%) were infected with at least one VBP. A possible co-infection was detected in only one dog, which tested positive for *Leishmania* antibodies POC and *H. canis* DNA. The sequences were deposited in GenBank under accession numbers PZ343918-PZ343938.

A total of 1568 *Culicoides* specimens, but no sand flies were captured in the CDC light traps from both shelters. Four species of *Culicoides* were identified morphologically including *Culicoides oxystoma* (n = 1489; 95%), *Culicoides peregrinus* (n = 64; 4%), *Culicoides arakawae* (n = 14; 0.9%), and *Culicoides homotomus* (n = 1; 0.1%). The mitochondrial cytochrome c oxidase subunit 1 (cox1) sequences from 26 *Culicoides* DNA representing each morphologically identified species (n = 17 *C. oxystoma*; n = 5 *C. arakawae*; n = 3 *C. peregrinus*; n = 1 *C. homotomus*) were all congruent with morphological species identification results.

Overall, non-engorged females (n = 224) including *C. oxystoma* (n = 208), *C. peregrinus* (n = 11), *C. arakawae* (n = 4) and *C. homotomus* (n = 1) were identified. Of these, *C. oxystoma* from Shelter 2, tested positive for *Leishmania* DNA, including *L. infantum* in 10, and *L. tarentolae* in one. Sequences obtained from *C. oxystoma* were deposited in GenBank under accession numbers PZ350158-PZ350168.

Phylogenetic analysis of the *L. infantum* internal transcribed spacer 1 (ITS1) sequences from *C. oxystoma* showed that they clustered with *L. infantum* sequences from humans and dogs in Europe, Africa, and Asia, as well as with *L. infantum* sequences from China (Fig. 1). The single *L. tarentolae* sequence clustered with *Sauroleishmania* clade and grouped with a *L. tarentolae* sequence from a dog in Italy (Fig. 1).

Discussion

This study provides the first molecular evidence of the presence of *L. infantum* and *L. tarentolae* in Hainan, the southernmost province in the Chinese Mainland. *Leishmania* DNA was detected in a dog, which had not been imported or traveled outside of Hainan Province, suggesting that the infection is

likely autochthonous. However, the specific *Leishmania* species within the *L. donovani* complex could not be determined by sequencing, possibly due to a low parasitic burden in the infected dog. Additionally, the duplex real-time polymerase chain reaction (dq PCR) targeting the ITS1 region failed to amplify *Leishmania* DNA, probably because of its lower sensitivity in comparison with kDNA-based real-time PCR [15]. This may

be attributed to the presence of approximately 10,000 kinetoplast minicircles per parasite, which enables detection even in low parasite loads [16]. The apparent discordance between diagnostic methods should also be considered, as none of the dogs were positive by both serological and molecular methods. Seropositivity without molecular confirmation may reflect low parasite burden or previous exposure with persistence of antibodies even after parasite clearance. Serological cross-reactivity with *Trypanosoma* species is unlikely, since all dogs tested negative on PCR for *Trypanosoma evansi* [16, 17]. Additionally, seroconversion has been reported to occur between 4- and 6-months post infection by *L. infantum* in dogs under experimental conditions [18], indicating that the single molecular positive dog in this study likely reflects early phase infection (i.e., prior to detectable antibody development).

In this study, although sand flies were not captured, *Culicoides* species positive for *L. infantum* and *L. tarentolae* DNA were captured from the shelter where dogs seropositive and molecularly positive for *Leishmania* were identified. *Culicoides* species lack vector competence for *Leishmania* within the subgenus *Leishmania* [19], but are potential vectors for those within the subgenus *Mundinia* [20, 21]. For example, the growth of *L. infantum* in a controlled population of *Culicoides nubeculosus* in a laboratory setting demonstrated that *L. infantum* is unable to fully mature in the midgut of *C. nubeculosus* [19] though *Leishmania* (*Mundinia*) *enrietti* can develop to the late stage (metacyclic promastigotes) and invade the stomodeal valve of *Culicoides sonorensis* [20]. In addition, *Leishmania* (*Mundinia*) *martiniquensis* promastigotes were detected in *Culicoides peregrinus* in the absence of blood meal remnants, indicating natural infection [23]. The molecular evidence of *Leishmania* DNA in non-engorged *Culicoides* females in our study is consistent with these biting midges having taken a blood meal from *L. infantum* or *L. tarentole*-infected dogs or other wildlife reservoirs. This finding provides indirect evidence of the presence of competent *Phlebotomus* sand-fly species (Diptera: Phlebotominae) in the study area in Hainan Province.

Furthermore, *P. chinensis*, the known vector of *L. infantum* in China, has been previously identified in Hainan Province [24, 25], thus vectorial transmission could be possible in this geographic area. However, since the 1990s there have been no recent reports of *P. chinensis* in Hainan. In contrast, *P. stantoni* was detected in recent surveys in the same region [26, 27]. The role of *P. stantoni* as a potential vector of *L. infantum* has not yet been demonstrated. Nonetheless, the DNA of *L. donovani* was detected in *P. stantoni* in southern Thailand [28]. In addition, *P. stantoni* has also been reported to feed on humans [29]. Collectively, these findings suggest that *P. stantoni* may serve as a vector for species belonging to the *L. donovani* complex, warranting further investigation under experimental settings.

Other Phlebotomine sand flies, including *Sergentomyia iyengari*, *S. barraudi*, *S. kwangiensis*, *S. bailyi*, *S. fanglianensis*, and *Grassomyia indica* have also been detected in Hainan Province, predominantly in poultry sheds and livestock pens [24–27]. Sand flies of the genus *Sergentomyia* are generally considered vectors of lizard-infecting *Sauroleishmania* species such as *L. tarentolae*, but the potential role of *Sergentomyia* in transmission of mammal-infecting *Leishmania* has also raised concern [30]. Notably, flagellated parasites detected in unfed, non-gravid females of *Sergentomyia dubia* and *Sergentomyia schwetzi* were successfully cultured and molecularly identified as *L. infantum* [31]. In particular, the detection of metacyclic promastigotes in both species suggests that *L. infantum* may survive blood-meal digestion and develop in these sand flies [30]. In contrast, experimental studies using laboratory-colonized *S. schwetzi* demonstrated that *L. infantum* was unable to develop to its late infective stages [32].

The single *L. tarentolae* ITS1 sequence from our study is identical to a previously reported *L. tarentolae* isolate from a dog in Italy. *Leishmania tarentolae* has been identified in lizards from desert regions of northwestern China, but is otherwise rarely reported in the country [33]. In contrast, an undescribed *Leishmania* species belonging to the subgenus *Sauroleishmania* but phylogenetically distinct from *L. tarentolae*, based on analyses of cytochrome b, ITS1-5.8S rRNA, and hsp70 genes [34–36], has been detected in a human patient and dogs in Sichuan Province [37], as well as in lizards and snakes in the northwest desert regions in China [33, 38].

Conclusion

Overall, the results of this study show the presence of CanL in Hainan, a province that is not considered a VL-endemic area in China. This finding may reflect under-reporting or misdiagnosis of CanL, highlighting the importance of surveillance of dogs in non-endemic areas such as Hainan Province. Furthermore, the previously reported presence of *Phlebotomus chinensis* requires confirmation through new field investigations, while surveillance for *Leishmania* species in sand flies is required to investigate competent vector species and assess the risk of future transmission.

Methods

Study population and sample collection

The dogs tested in this study were recruited from two privately owned animal shelters in Haikou, Hainan, in October 2024. The distance between the two shelters was approximately 16 km. One shelter (Shelter 1) housed dogs only, and the other (Shelter 2) housed a variety of animals, including dogs, cats and chickens. Before sampling, the health status of all dogs was assessed by a veterinarian and physical examination abnormalities and signalment were recorded. Blood was sampled into EDTA and serum collection tubes from each dog, as well as exfoliated epithelial cells from the right and left conjunctivae using a sterile cotton swab. Fine needle aspirates were performed on lymph node from dogs with palpable superficial lymph nodes. Sera and EDTA blood samples were stored at – 80 °C until testing. For

insect collection, four CDC light traps with dry ice were placed in the shelters near the dogs before sunset and retrieved the following morning.

Serological analysis

Serum samples were tested for anti-*L. infantum* immunoglobulin G (IgG) antibodies by immunofluorescent assay (IFAT) as described previously [39]. The samples were considered positive at a 1:80 cut-off dilution, with clear cytoplasmic and membrane fluorescence of promastigotes. Positive sera were titrated until no fluorescence was observed. A commercially available rapid enzyme-linked immunosorbent assay (ELISA)-based POC test kit (SNAP® Leish 4Dx® test; IDEXX Laboratories, Westbrook, ME, USA) was used to detect antibodies against *L. infantum*, *Anaplasma* spp. (i.e., *A. phagocytophilum*, *A. platys*) and *Ehrlichia* spp. (i.e., *E. canis*, *E. ewingii*, *E. chaffeensis*) and antigens against *Dirofilaria immitis*.

Molecular analysis

Genomic DNA was extracted from EDTA blood using the QIAamp DNA Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), and from conjunctival swabs and lymph node aspirates using the QIAamp DNA Micro Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Conjunctival swabs, lymph node samples, and blood samples were tested for *Leishmania* spp. by real-time polymerase chain reaction (qPCR) targeting kDNA [40]. A duplex real-time PCR assay (dq PCR) was also performed to detect both *L. infantum* and *L. tarentolae* DNA, targeting ITS1 region, as previously described [41]. Samples that tested positive on qPCR samples were subject to conventional PCRs targeting a partial region of ITS1 [42] and hsp70 to obtain PCR products for Sanger sequencing [43].

Infection by other VBP (*Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Hepatozoon* spp., *Dirofilaria* spp., and *Trypanosoma evansi*) was assessed by conventional polymerase chain reaction (cPCR) on blood samples, with protocols described elsewhere [44].

Insect (*Culicoides* spp.) identification and analysis for *Leishmania* infection

Culicoides specimens were morphologically identified using taxonomic keys [45, 46], and females were classified as engorged (i.e., insects with full or partial blood meal) or non-engorged (parous and unfed midges). Genomic DNA was extracted from individual non-engorged females using an in-house protocol as previously described [47]. Extracted DNA samples were then subject to PCR targeting the ITS1 region of *Leishmania* sp. [41]. To confirm morphological identification, a subset of specimens (ranging from 1 to 17 individuals per species) from each identified *Culicoides* species was subjected to cPCR targeting a fragment of the *cox1* gene using universal invertebrate primers (LCO1490 and HCO2198) [48]. All cPCR products were analyzed by 2% agarose gel electrophoresis and purified for Sanger sequencing. Obtained sequences were edited using Geneious Prime® 2024.0.3 and the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology.

Phylogenetic analysis

The phylogenetic relationships of *Leishmania* sequences from *Culicoides* specimens were evaluated, along with sequences retrieved from GenBank (Supplementary data, Table 1). Following nucleotide sequence alignment using MUSCLE, the phylogenetic tree was inferred using the maximum Likelihood method based on the Tamura 3-parameter model [49], selected by the best-fit model analysis, and based on the lowest score obtained by Bayesian Information Criterion (BIC), using MEGA X software [50]. The evolutionary analysis was conducted with 1000 bootstrap replicates, and the tree comprised 30 nucleotide sequences with a total of 200 aligned positions, including relevant *Leishmania* species, with *Trypanosoma evansi* (Accession number: FJ416613) ITS1 sequence as the outgroup.

Abbreviations

VL

Visceral leishmaniasis

MT-ZVL

Mountain type zoonotic visceral leishmaniasis

DT-ZVL

Desert type zoonotic visceral leishmaniasis

CanL

Canine leishmaniasis

IFAT

immunofluorescent antibody test

kDNA

kinetoplast minicircle DNA

hsp70

heat shock protein 70

VBP

vector-borne pathogens

POC

point-of-care

CDC

Centers for disease control and prevention

ITS1

internal transcribed spacer 1

5.8S rRNA

5.8S ribosomal RNA

cox1

mitochondrial cytochrome c oxidase subunit 1

dq PCR

duplex real-time polymerase chain reaction

qPCR

real-time polymerase chain reaction
cPCR
conventional polymerase chain reaction
EDTA
Ethylene diamine tetraacetic acid
ELISA
enzyme-linked immunosorbent assay
BLAST
Basic Local Alignment Search Tool

Declarations

Ethics approval and consent to participate

The animal study protocol was approved by the Hainan University Institutional Animal Care and Use Committee. The study adhered to the guidelines set by the committee (approval code: HNUAUCC-00520). Samples from the dogs in this study were collected with the informed consent of the owners.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding authors upon reasonable request. In addition, the sequence data of the ITS1 and 5.8S rRNA region of *Leishmania* were deposited in GenBank under the accession numbers PZ350158 to PZ350168, and the 18S rRNA region of *Hepatozoon canis* and *Babesia vogeli* were deposited in GenBank under the accession numbers from PZ343918 to PZ343938.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Thamali Manathunga: Data curation, formal analysis, methodology, writing—original draft, writing—review and editing. Mariaelisa Carbonara: Formal analysis, methodology, writing—original draft, writing—review and editing. Marcos Antonio Bezerra-Santos: Kassim Abdullahi Jimale: Jairo Alfonso Mendoza Roldan: Data curation, methodology, writing—review and editing. Riccardo Paolo Lia: Data curation, methodology, formal analysis, writing—review and editing. Hongxiao Yu: methodology, writing—review and editing. Ping Chen: methodology, writing—review and editing. Qian Han: methodology, writing—review and editing. Domenico Otranto: conception, methodology, supervision, investigation, writing—review and editing. Vanessa R. Barrs: conception, investigation, methodology, supervision, writing—review and editing.

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Figures

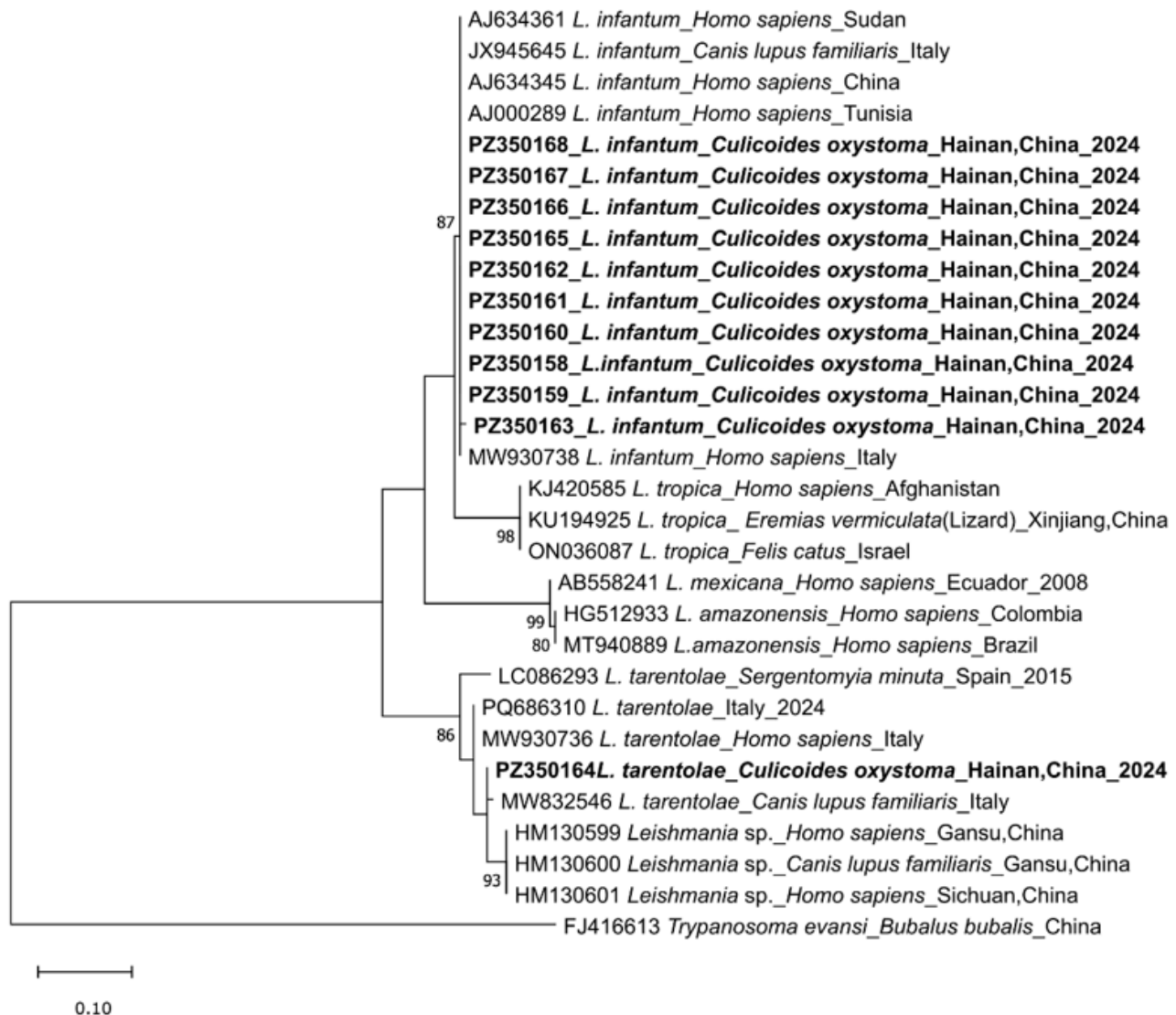


Figure 1

Maximum likelihood phylogenetic tree comparing 30 nucleotide sequences with a total of 200 aligned positions of the ribosomal ITS1 locus of *Leishmania* spp. GenBank accession numbers, *Leishmania* spp., infected host, country, and isolation date (when known) are included for each sequence. Sequences generated in this study are indicated in bold. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values (>80%) are displayed.

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