RESEARCH ARTICLE

Multi-locus characterization and phylogenetic inference of *Leishmania* spp. in snakes from Northwest China

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Abstract

Background

Leishmaniasis caused by protozoan parasite *Leishmania* is a neglected disease which is endemic in the northwest of China. Reptiles were considered to be the potential reservoir hosts for mammalian Leishmaniasis, and *Leishmania* had been detected in lizards from the epidemic area in the northwest of China. To date, few studies are focused on the natural infection of snakes with *Leishmania*.

Methods

In this study, 15 snakes captured from 10 endemic foci in the northwest of China were detected *Leishmania* spp. on the base of mitochondrial *cytochrome b*, *heat shock protein 70* gene and *ribosomal internal transcribed spacer 1* regions, and identified with phylogenetic and network analyses.

Result

In total, *Leishmania* gene was found in 7 snakes. The phylogenetic inference trees and network analysis suggests that the species identification was confirmed as *Leishmania dono- vani*, *L. turanica* and *L. (Sauroleishmania) sp.*

Conclusion

Our work is the first time to investigate the natural *Leishmania* spp. infection of snakes in the northwest of China. Mammalian *Leishmania* (*L. donovani* and *L. turanica*) was discovered in snakes and the reptilian *Leishmania* (*Sauroleishmania* sp.) was closely related to the clinical strains both prompt the importance of snakes in the disease cycle. To indicate the epidemio- logical involvement of snakes, a wide sample size in epidemic area and the pathogenic fea- tures of reptilian *Leishmania* promastigotes are recommended in the future research.

Introduction

Leishmaniasis is a neglected disease caused by infection with flagellate protozoan parasite *Leishmania* of the family Trypanosomatidae. There are three main forms of leishmaniasis: visceral or kala-azar (VL), cutaneous (CL) and mucocutaneous (MCL). About 20 *Leishmania* species known to be infective to humans are transmitted by the bite of infected sand flies of which about 166 species were proven or probable vectors worldwide [1]. An estimated 2 million new cases and 50 000 deaths occur in over 98 countries annually [2]. In China, which is one of 14 high-burden countries of VL [3], there exists three epidemiological types: anthroponotic VL (AVL), mountain-type zoonotic VL (MT-ZVL), and desert-type ZVL (DT-ZVL) [4]. Information on the epidemiology including the vector and animal reservoir hosts of the disease is important to understand the disease and its control.

In view of the topography in the northwest of China, DT-ZVL is found largely in oases and deserts including the southern and eastern of Xinjiang Uygur Autonomous Region, the western part of the Inner Mongolia Autonomous Region, and northern Gansu province [5]. Surveillance of the disease reflects that DT-ZVL is persistent with sporadic outbreaks and still not under control now [5]. According to the data from Disease Reporting Information System of China CDC, there were two outbreaks of VL occurred in Xinjiang Uygur autonomous region, of which the highest incidence rate was in Kashgar Prefecture [6,7]. Sand flies (Phlebotominae) were recognized as the transmission vector in the traditional sense, while ticks (Ixodoidea) had been reported to be the vector in the transmission of canine VL [8–11]. In addition to humans, there are various kinds of reservoir hosts, mainly mammals such as canids, hyraxes and rodents, which could be the source of infection. Because transmission occurs in a complex biological system involving the human host, parasite, vectors and in some cases an animal reservoir host, the control of Leishmaniasis is relatively complex. As for the desert in the northwest of China, there is no consistent agreement regarding dissemination of the actors playing the key roles in leishmaniasis.

Moreover, reptiles, mainly lizards, were found harboring *Leishmania* parasites with controversies concerning their role in spreading the disease [12]. Blood cells of lizards containing amastigotes were first found by Chatton and Blanc in *Tarentola mauritanica* from southern Tunisia [13] and later several cases were reported from the same lizard species [14]. In 1920, Wenyon provisionally named the leptomonad flagellates in a gecko as *Leishmania tarentolae* [15]. Killick-Kendrick recognized *Sauroleishmania* as a separate genus for the leishmanial parasites of reptiles in 1986 [16], later DNA sequence-based phylogenies had clearly placed *Sauroleishmania* within the *Leishmania* genus as a secondarily derived development from the mammalian species [17–19]. Most studies were focused on the infection of *Leishmania* parasites in lizards, but Belova and Bogdanov were able to culture promastigotes from the blood of five species of snakes in the Turkmen S.S.R. in 1969 [16,20].

In reality, the detection of reptilian *Leishmania* is rare in China. Zhang et al. was the first team to detect *Leishmania* via molecular methods from lizards captured in the northwest of China [21]. Interestingly, besides reptilian *Leishmania*, mammalian *Leishmania* species (*L. donovani* and *L. tropica*) were also isolated in their study. There are few studies reporting that mammalian *Leishmania* can parasitize in reptilians. In addition, *Sauroleishmania* was once considered not to infect humans or other mammals [1,22], however, *L. adleri* was found to infect rodents [23] and *L. tarentolae* could invade human macrophages in later studies [24].

Similarly, we employed the highly sensitive polymerase chain reaction (PCR) to detect the presence of *Leishmania* spp. in snakes. The sequences of various genetic markers have been used successfully to infer the phylogenetic relationships within *Leishmania* including the sequences of DNA polymerase α [17], RNA polymerase II [17], 7SL RNA [25–27], ribosomal

internal transcribed spacer [28,29], mitochondrial *cytochrome b* gene [30,31], *heat shock protein 70* gene [32,33] and the *N-acetylglucosamine-1-phosphate transferase* gene [34]. Therefore, this study was based on three genetic loci, i.e., *cytochrome b* (*cyt b*), *heat shock protein 70* (*Hsp70*), and *internal transcribed spacer 1* (*ITS1*) region to genetically characterize *Leishmania* spp. detected in 15 snakes, and conduct tree-based species delimitation to infer the phylogenetic positions by comparison with some representative *Leishmania* sequences retrieved from GenBank. It was the first time to survey infected snakes of *Leishmania* in the northwest of China and to conclude on the role of snakes in the transmission of human leishmaniasis.

Materials and methods

Study area and sampling

15 snakes identified as 3 species by morphological characters were captured alive by hand and snake hooks at 10 sites across the endemic foci of VL in the northwest of China (Table 1). These sites are located in arid desert areas with the altitude ranging from 537 to 1222 m above sea level. Franchini [35,36] had observed rare amastigotes in the liver of lizards, and Shortt & Swaminath [37] cultured a strain *Leishmania* on NNN (Novy-MacNeal-Nicolle) medium from the liver of lizards. Consequently, a part of the liver was taken from the abdominal cavity of each snake to detect the presence of *Leishmania* via PCR. In total, 15 liver specimens were collected in Eppendorf tube and stored at -20˚.

All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The protocol was approved by the ethics committee of Sichuan University (Protocol Number: K2018056).

DNA extraction, amplification, cloning and sequencing protocols

Total genomic DNA was extracted from liver tissues using the commercial kit, TIANamp Genomic DNA Kit (TIANGEN Bio, Beijing, China) following the protocols of the manufacturer. PCR primers specific for the *Leishmania* synthesized by Tsingke Biological Technology Co., Ltd (Chengdu, China) were used to amplify *cyt b* [38], *Hsp70* [39] and *ITS1* [40] gene fragments by PrimeSTAR Max DNA polymerase (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instruction. We picked the Chinese *L.* (*Sauroleishmania*) sp. strain MHOM/CN/90/SC10H2 as the positive control while preparing the mixture, and the negative control was treated without template DNA. The PCR products were purified by excision of the band from agarose gel using the Universal DNA Purification Kit (TIANGEN Bio, Beijing, China),

Table 1. List of sampling localities, snake species and sample size in this study.

Site Label	Locality	Species	Sample size
P1	Shawan county, Tarbagatay Prefecture, Xinjiang	*Psammophis lineolatus*	1
P2	Tekes County, Ili Kazak Autonomous Prefecture, Xinjiang	*Gloydius halys*	1
P3	Tekes County, Ili Kazak Autonomous Prefecture, Xinjiang	*Natrix tessellate*	1
P4	Yiwu County, Hami, Xinjiang	*Psammophis lineolatus*	1
P5	Yizhou Area, Hami, Xinjiang	*Psammophis lineolatus*	5
P6	Dunhuang, Jiuquan City, Gansu Province	*Psammophis lineolatus*	1
P7	Heshuo, Bayingol Mongolian Autonomous Prefecture, Xinjiang	*Psammophis lineolatus*	2
P8	Makit County, Kashgar Prefecture, Xinjiang	*Psammophis lineolatus*	2
P9	Yopurga County, Kashgar Prefecture, Xinjiang	*Psammophis lineolatus*	1
P10	Yengisar County, Kashgar Prefecture, Xinjiang	*Psammophis lineolatus*	2
total			15

and ligated into a pGEM-T vector (Promega Co., Madison, USA) with added the poly (A) tails. After transformation of *Escherichia coli* DH5α competent cells with the ligation product and X-gal blue-white screening, 8 positive colonies were selected by PCR using the plasmid primers T7 and SP6 and grown in LB/ampicillin medium. DNA was extracted and sequenced at Tsingke Biological Technology Co., Ltd (Chengdu, China). The chromatograms were validated and assembled in BioEdit v7.2.6 [41].

Sequence alignment and phylogenetic analyses

GenBank searches were performed to initially identify the species of the original DNA sequences using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). And all the nucleotide sequences generated in this study have been submitted to the GenBank database. The sequences were multiple-aligned with a set of *Leishmania* strains of each locus examined in this study retrieved from the GenBank (see S1 Table in Supporting information) using ClustalW of MEGA (Molecular Evolutionary Genetic Analysis v7.0.26 [42]) with its default option and refined manually. The alignments were further trimmed to exclude regions with missing data and then distinct haplotypes were defined by DAMBE v7.0.1 [43,44]. The evolutionary history was inferred by phylogenetic tree construction using Bayesian inference (BI). Gaps were treated as missing data and each haplotype was treated as a taxon in the analyses. The program PartitionFinder v2.1.1 [45] was used to select the most appropriated substitution model of all phylogenetic analyses. Bayesian analyses were carried out using the program MrBayes v3.2 [46] which the trees were started randomly. 2 parallel sets of 4 simultaneous Monte Carlo Markov chains (3 hot and 1 cold) were run for 10,000,000 generations until convergence was reached (stopval = 0.01) and the trees were sampled for every 1000 generations. Temperature heating parameter was set to 0.2 (temp = 0.2) and burn-in was set to 25% (burninfrac = 0.25). The results of Bayesian analyses were visualized in FigTree v1.4.3 (available at http://tree.bio.ed.ac.uk/software/figtree/).

Network reconstructions

Due to the limit of bifurcating tree to represent intraspecific gene evolution, haplotype networks may more effectively portray the relationships among haplotypes within species. The phylogenetic networks were constructed by using the Median Joining (MJ) network reconstruction method in NETWORK v5.0.0.3 (available at http://www.fluxus-engineering.com/sharenet.htm) [47,48].

Results

Leishmania infection of the captured snakes in the study area

Through preliminary determination of identity using BLASTn, the total prevalence of *Leishmania* DNA detected via PCR was 46.7% (7/15). 5 (33.3%) snakes were detected as positive for *cyt b*, 4 (26.7%) snakes for *Hsp70* and 2 (13.3%) for *ITS1*. Considering the sensitivity of different loci, two samples (Guo4707 and Guo4708) from P1 and P2 were amplified successfully in all three loci, while other samples were detected by only one locus in this study. As shown in Fig 1, the snakes from P1, 2, 3, 4, 5, 6 and 9 with colored spots were *Leishmania*-positive.

Sequence characteristics

After the haplotypes were defined, 10 *cyt b* sequences, 7 *Hsp70* sequences and 10 *ITS1* sequences were obtained in this study and deposited in the GenBank database under Accession No. MK330198-MK330207, MK330208-MK330214 and MK300708-MK300717, respectively
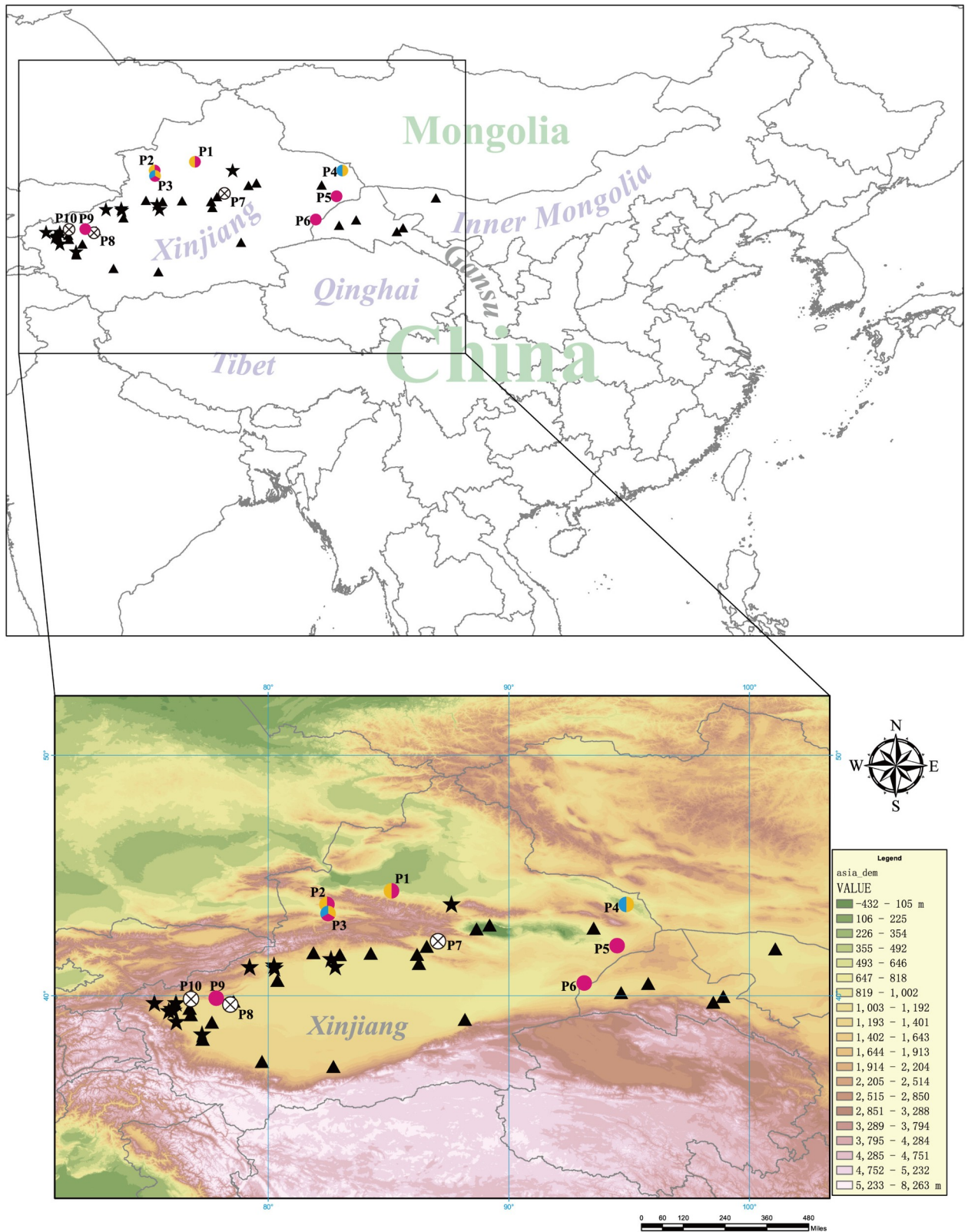
Fig 1. Sampling sites of snakes in Northwest China, along with the current foci of endemicity of anthroponotic visceral leishmaniasis (AVL) and desert-type zoonotic visceral leishmaniasis (DT-ZVL) in China. The map image of countries with boundary lines and the topographic map layer of areas were downloaded from ArcGIS online via ArcMap program (http://www.esri.com). The site numbers P1-P10 correspond to those in

(Table 2). PCR amplification of each target locus resulted in amplicons of the expected sizes as follows: *cyt b* (543 bp), *Hsp70* (738–767 bp), and *ITS1* (328–334 bp). The GC contents were 23.4% for *cyt b*, 64.9% for *Hsp70* and 41.8% for *ITS1*, respectively. Thus, both their lengths and their GC contents are within the range of corresponding sequences of other *Leishmania* species.

Phylogenetic relationship

Prior to the phylogenetic analyses, the most adequate models of nucleotide substitution were selected by PartitionFinder v2.1.1: GTR +G and GTR+I+G for *cyt b*, HKY+I and GTR for *Hsp70*, K80+G for *ITS1*. Using Bayesian inference method, the trees showed similar phylogenetic topology for all three loci supported by posterior probability (PP) values.

Regarding the BI tree inferred from each locus, the phylogenetic analyses inferred from the aligned matrix of *cyt b* (Fig 2) separated two distance clades, the *Viannia* isolates apparently formed an independent clade (PP = 1.0) outside the clusters of those other species (PP = 1.0), while the subgenera *Leishmania* and *Sauroleishmania* were still separated into different branches (PP = 1.0 and 0.53, respectively). The isolates C1, C2 and C3 clustered with *L. donovani* and *L. infantum* (PP = 1.0). The isolate C4 shared an identical *cyt b* sequence to a *L. turanica* isolate and they were grouped with *L. arabica*, *L. major*, *L. tropica*, *L. aethiopica* and *L. killicki* in a subclade (PP = 0.99) separated with *L. donovani* complex. The isolate C6 shared identical sequences with 5 Chinese *Leishmania* sp. isolates from Yang et al. [29] clustered in a separated clade (PP = 0.96) together with C5 and other *L.* (*Sauroleishmania*) sp.

The *Hsp70* analysis (Fig 3) showed a similar structure to the phylogenetic tree of *cyt b*: *Viannia* (PP = 1.0), *Sauroleishmania* (PP = 1.0) and *Leishmania* (PP = 0.84). The isolates H1 and H2 searching by BLASTn (Megablast) revealed over 98% identity with *L. donovani* and *L. infantum* respectively, both of them were nested within *L. donovani* complex. In this subclade, the isolates of other species of *Leishmania* subgenus (except *L. mexicana* complex clustered by the PP 1.0) were not in reciprocally monophyletic groups. The isolates H3, H4 and H5 clustered in a separated clade (PP = 1.0) with *L.* (*Sauroleishmania*) sp. from China.

For the *ITS1*-5.8S region, the phylogenetic tree as Fig 4 shown was constructed by three clades with high support (PP = 1.0, 0.96 and 1.0, respectively), corresponding to the three subgenera *Viannia*, *Leishmania*, and *Sauroleishmania*. All the 10 fragments from snakes formed a strongly supported cluster with *L.* (*Sauroleishmania*) sp. isolated from lizards [21] (PP = 1.0), and revealed a close relationship to isolates from *Sergentomyia minuta* in Europe.

Median joining network

To get additional insight into the relationships among the strains, we analyzed our data sets using the Median Joining algorithm network approach. The MJ analysis of three regions was congruent with the topology described in the BI trees, and no haplotype was shared among the species groups.

The network of *cyt b* in Fig 2 showed that the isolate C6 seemed to be the central haplotype. C1 (MK330198 isolated from two snakes captured at P1 and MK330200 from P2), together with C2 (MK330201 from P3) and C3 (MK330205 from P4) were most closely related to the central HQ908267, within 2 mutational steps. C4 (from P3 and P4) shared the same haplotype with one strain of *L. turanica* from Russia, and only 2 and 3 steps away from the Chinese

Table 2. List of the snake samples, origin, isolated *Leishmania* spp. and GenBank accession numbers.

Snake species	Voucher number	Origin	Isolated *Leishmania* spp.	Haplotype number	GenBank accession	Sequence length (bp)
(A) *cyt b*						
Psammophis lineolatus	Guo4707	P1	*L. donovani*	C1	MK330198	543
			L. (Sauroleishmania) sp.	C6	MK330199	543
Gloydius halys	Guo4708	P2	*L. donovani*	C1	MK330200	543
Natrix tessellata	Guo4709	P3	*L. donovani*	C2	MK330201	543
			L. turanica	C4	MK330202	543
			L. (Sauroleishmania) sp.	C5	MK330203	543
			L. (Sauroleishmania) sp.	C6	MK330204	543
Psammophis lineolatus	Guo4710	P4	*L. donovani*	C3	MK330205	543
			L. turanica	C4	MK330206	543
Psammophis lineolatus	Guo6995	P9	*L. (Sauroleishmania)* sp.	C6	MK330207	543
(B) *Hsp70*						
Psammophis lineolatus	Guo4707	P1	*L. donovani*	H1	MK330208	738
			L. infantum	H2	MK330209	738
Gloydius halys	Guo4708	P2	*L. infantum*	H2	MK330210	738
Psammophis lineolatus	Guo4023	P6	*L. (Sauroleishmania)* sp.	H3	MK330211	767
			L. (Sauroleishmania) sp.	H4	MK330212	766
			L. (Sauroleishmania) sp.	H5	MK330213	767
Psammophis lineolatus	Guo4026	P5	*L. (Sauroleishmania)* sp.	H5	MK330214	767
(C) *ITS1*						
Psammophis lineolatus	Guo4707	P1	*L. (Sauroleishmania)* sp.	I1	MK300708	334
			L. (Sauroleishmania) sp.	I2	MK300709	328
			L. (Sauroleishmania) sp.	I3	MK300710	332
			L. (Sauroleishmania) sp.	I4	MK300711	330
			L. (Sauroleishmania) sp.	I5	MK300712	330
			L. (Sauroleishmania) sp.	I6	MK300713	330
			L. (Sauroleishmania) sp.	I7	MK300714	330
Gloydius halys	Guo4708	P2	*L. (Sauroleishmania)* sp.	I8	MK300715	330
			L. (Sauroleishmania) sp.	I9	MK300716	332
			L. (Sauroleishmania) sp.	I10	MK300717	328

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strains *L. turanica* (from Xinjiang). The *L. (Sauroleishmania)* sp. isolate C6 (from P1 and P9) as the central haplotype shared the same sequences to *L. (Sauroleishmania)* sp. from China with a wide geographical distribution, including Shandong (HQ908255), Sichuan (HQ908260, HQ908264), Gansu (HQ908263, HQ908271).

For the *Hsp70* region, the haplotype network of Fig 3 could intuitively reflect the genetically small distances between the obtained sequences H1 (MK330208 isolated from P1), H2 (MK330209 and MK330210 from P1 and P2, respectively) and the reference *L. donovani* and *L. infantum* strains from India, Sudan and the northwest of China. The haplotype H5 shared by the isolates from P5 (MK330213) and P6 (MK330214) was the interior haplotype of *L. (Sauroleishmania)* sp. and may be older than other top haplotypes H3 (MK330211) or H4 (MK330212) which were both from P6). It reflected a close distance between JX021439 (Xinjiang, China) and the interior haplotype as three mutational steps.

As shown in the haplotype network of *ITS1*-5.8S in Fig 4, the *L. (Sauroleishmania)* sp. group was centered around haplotype KU194967 (Dunhuang, China). All haplotypes found in this group of *Sauroleishmania* isolated from lizards of the northwest of China were very closely related to each other. One strain from Tuokexun (KU194973) shared the same haplotype with
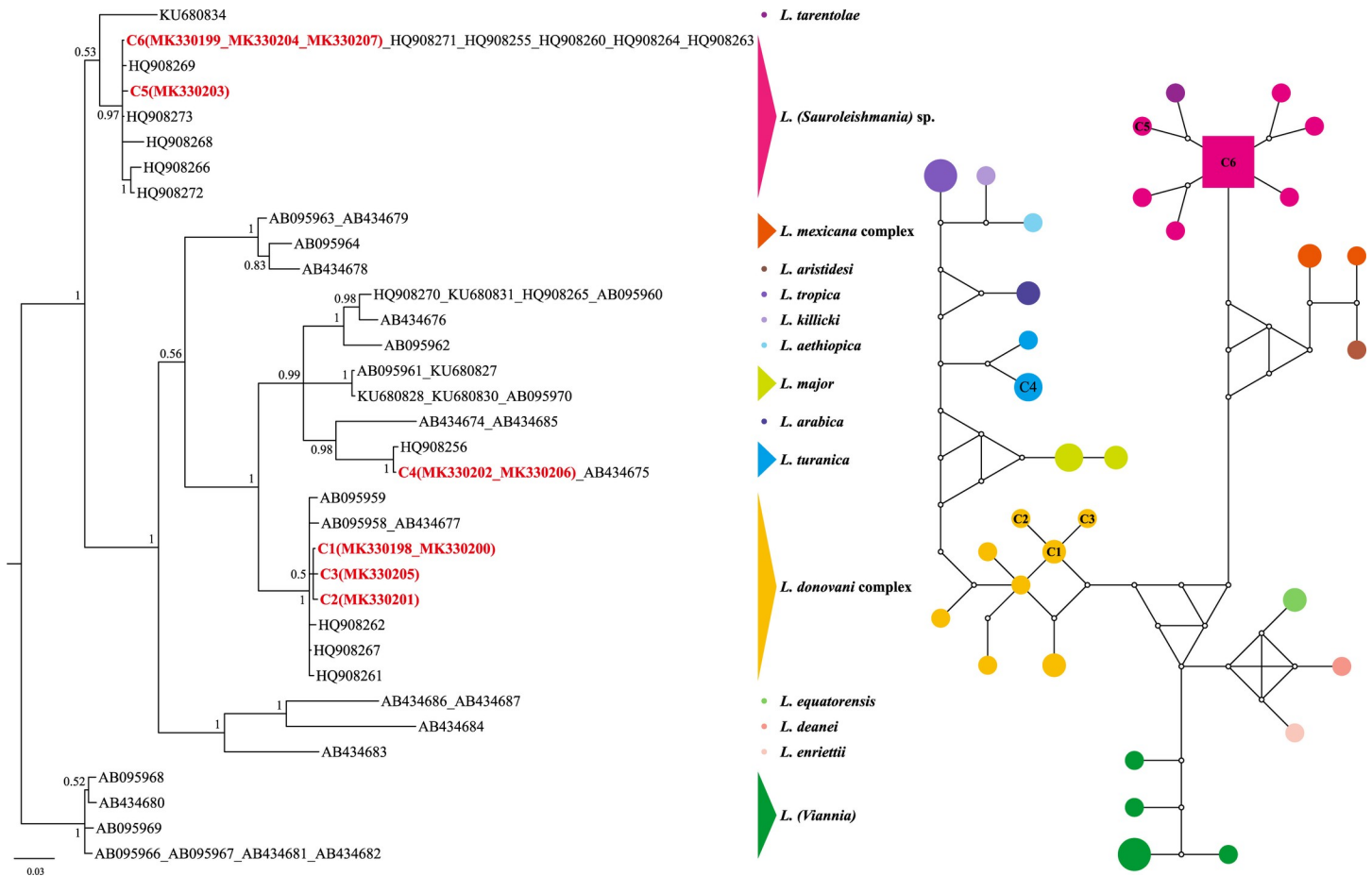
Fig 2. The majority-rule consensus tree (midpoint rooted) of *Cyt b* inferred from Bayesian inference by using MrBayes v.3.2, and the corresponding median-joining network implemented by NETWORK v5.0.0.3. Different colors represent the corresponding *Leishmania* species obtained in this study; numbers at the nodes of the trees are the posterior probability (PP) values; circles of the networks indicate the haplotypes and squares for the central haplotypes, the small hollow circles indicate median vectors; the information of reference sequences see S1A Table.

I4 (MK300711 from P1). Similarly, two strains from Dunhuang (KU194969, KU194971) and another one from Lukchun (KU194939) shared the same haplotype with I10 (MK300709 from P2). In addition, three strains isolated from *Sergentomyia minuta* of Europe shared a close distance with the *Sauroleishmania* group as less than 3 steps.

Discussion

This study was the first time to detect the infection of *Leishmania* in snakes by DNA sequencing. Our result showed that the snakes from 7 loci of the 10 study areas were detected positive for *Leishmania*. Yopurga county, Hami and Dunhuang are the DT-ZVL epidemic foci [5], while for Shawan and Texes county current data are lacking.

Three *Leishmania* species (including *L. donovani*, *L. turanica* and *L. (Sauroleishmania)* sp.) were detected from one snake (Guo4709, *Natrix tessellate*) which was captured from Texes, whereas only 1–2 species were detected in the other *Leishmania*-positive snakes. *L. (Sauroleishmania)* sp. was the most widely distributed species. Owing to the limit of the sample size, the other two *Leishmania* species (*L. donovani* and *L. turanica*) were lack of significant geographic specificity in this study.
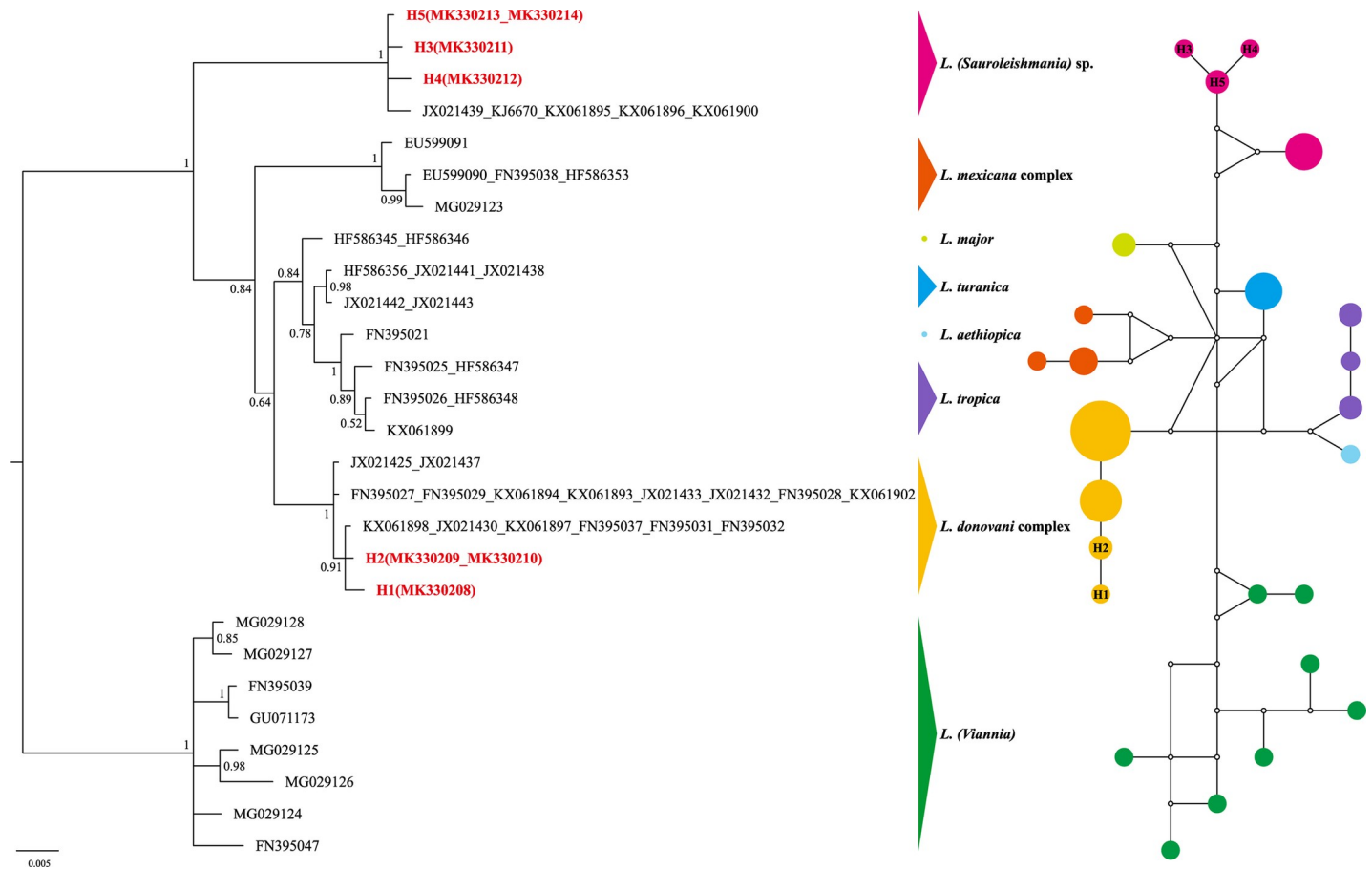
Fig 3. The majority-rule consensus tree (midpoint rooted) and the median-joining network based on *Hsp70*. Different colors represent the corresponding *Leishmania* species obtained in this study; numbers at the nodes of the trees are the posterior probability (PP) values; circles of the networks indicate the haplotypes and squares for the central haplotypes, the small hollow circles indicate median vectors; the information of reference sequences see S1B Table.

The effect of many factors (e.g., the condition of specimen, the DNA extraction methodology, the length of amplified gene sequences and the PCR protocols) would cause different rates in PCR positivity. Amplification of multiple gene sequences could significantly increase the comprehensiveness of the *Leishmania* infections to be investigated in snakes. Various genetic loci had been developed to detect *Leishmania*, such as kinetoplast DNA (kDNA) genes, nuclear DNA (nDNA) genes and ribosomal RNA (rRNA) genes [28,49,50]. *Cyt b*, one of the cytochromes encoded on the kinetoplast DNA maxicircle, is considered one of the most useful markers that had delimitated most tested *Leishmania* species for phylogenetic work. *Hsp70* gene, an antigen gene on the chromosome, is the most optimal marker for the species discrimination and phylogenetic inference. *ITS1*, one of the highly variable regions of rRNA gene, have been successfully used to resolve taxonomical questions and to determine phylogenetic affinities among closely related *Leishmania* species. Therefore, three genetic loci were amplified in this study and the positivity rates were different indeed. For two samples from Shawan and Tekes, *L.* (*Sauroleishmania*) sp. and *L. donovani* were identified by all the three genetic loci while for other samples only one of the three genetic loci could be amplified. It was suggested that different *Leishmania* primers might have different selection preferences for the gene amplification. At the same time, the use of different primers to amplify the corresponding gene fragments did significantly improve the detection rate of infected snake samples.
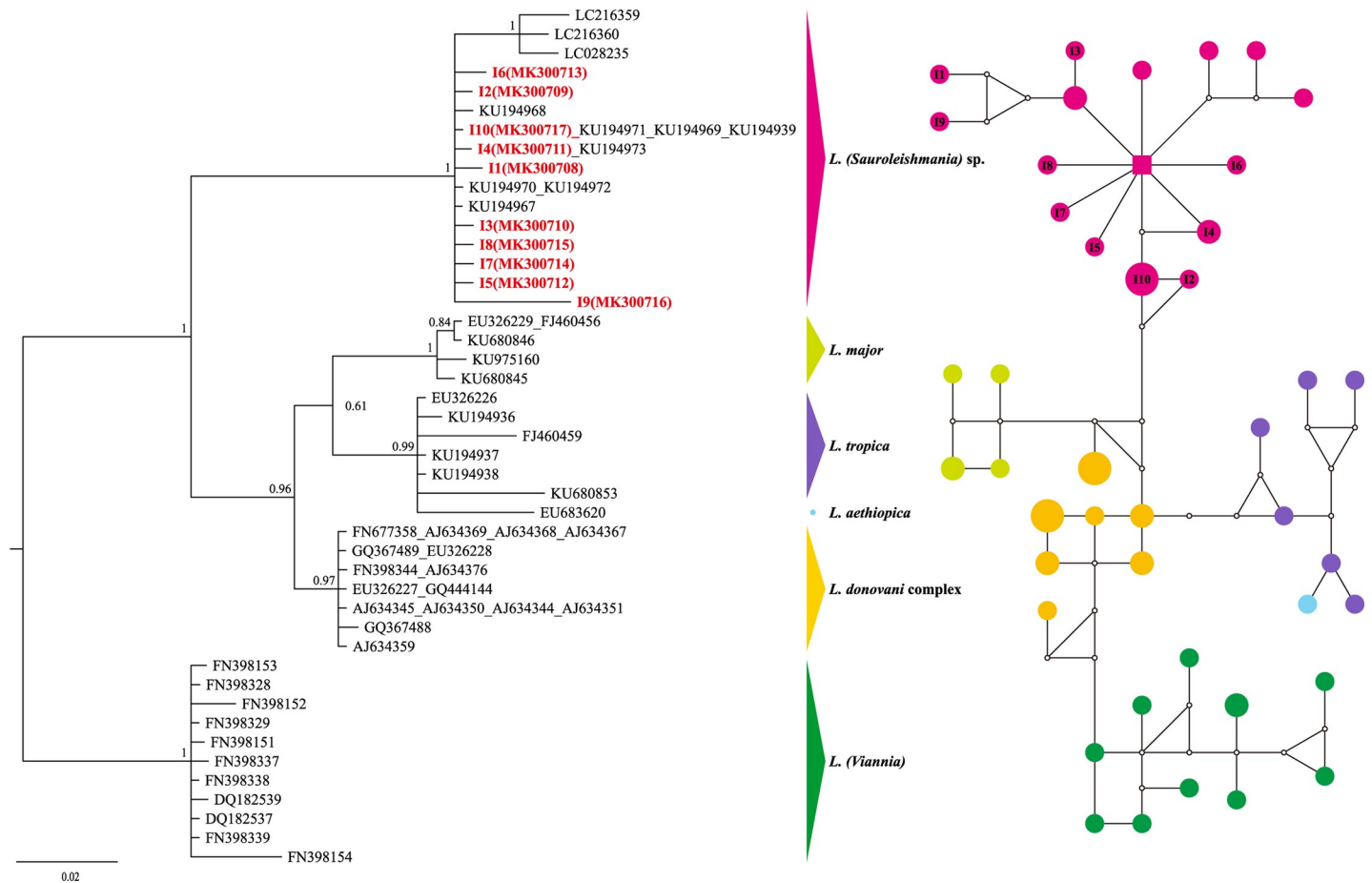
Fig 4. The majority-rule consensus tree (midpoint rooted) and the median-joining network based on ITS1. Different colors represent the corresponding *Leishmania* species obtained in this study; numbers at the nodes of the trees are the posterior probability (PP) values; circles of the networks indicate the haplotypes and squares for the central haplotypes, the small hollow circles indicate median vectors; the information of reference sequences see S1C Table.

The spreading mechanism of *Leishmania* in reptiles is still debated. Wilson & Southgate assumed that transmission was achieved by the reptile host eating an infected sand fly [51,52]. Nevertheless, there are species of *phlebotomine* that are known to feed on reptiles [53,54]. The same as lizards, it was reasonable that snakes could be infected naturally. In view of the small sample size per location and species, the calculation of infection rates does not make much sense. *Leishmania* DNA was detected in all three species of snakes captured in endemic foci of VL, even though the sample size of both *Gloydius halys* and *Natrix tessellate* was 1. Therefore, a bigger sample size would be necessary for further studies to enrich our understanding on natural infections of *Leishmania* parasites in snakes.

One of the most noteworthy findings in the present study was that two mammalian *Leishmania* taxa, i.e., *L. donovani* complex and *L. turanica* appeared to occur also in snakes. Previously, Zhang et al. had a similar discovery in lizards [21]. They believe that lizards may be putative reservoirs for human leishmaniasis because of the common ancestry of the haplotypes obtained for *L. tropica* and clinical samples. At present, apart from the above study, there are few studies of mammalian parasites natural infections in reptiles. Belova [12] carried out intensive research on reptilian and mammalian *Leishmania* and found that the mammalian parasites (such as *L. donovani*, *L. tropica*, and some other promastigotes recovered from sandflies)

were infective to lizards. To determine whether reptiles might be reservoir hosts for human leishmaniasis, it is necessary to sample of *Leishmania* strains from VL patients and from reptiles in the epidemic foci and to compare them by phylogenetic analyses.

On the other hand, the *L.* (*Sauroleishmania*) sp. isolated from snakes in this study shared the same haplotypes or were located in close proximity to strains from VL patients or canines in China as shown by the networks. Indeed, the Chinese *L.* (*Sauroleishmania*) sp. isolates were speculated to be closely related to the lizard-infected strains, *L. tarentolae*, on the basis of COII [55] and *cyt b* [31] genes. Analogously, *L. adleri* was found to cause transient CL in humans by Manson-Bahr & Heisch [56], and could infect rodents from which the strains were isolated by Adler [23] and Coughlan et al. [57]. *L. tarentolae* was chosen as a candidate vaccine against VL in virtue of the character that the parasites could invade in human macrophages and exist as amastigotes in mammals with slower replication [24]. These studies seem to be contradictory with the former which indicated that *Sauroleishmania* was not pathogenic to human [12]. Since the Chinese strains from patients or canines were isolated in the 70s to 90s of the last century, medical records were no longer available, and we could not confirm whether the patients got leishmaniasis due to *Sauroleishmania* or co-infection with other mammalian *Leishmania*. Thus, to further explore the pathogenicity of *Sauroleishmania*, it is recommended to isolate and culture the promastigotes from liver or blood of reptiles and study the virulence to mammals.

In conclusion, the present study is the first which investigates natural *Leishmania* spp. infections of snakes in the northwest of China. The species identification was primarily achieved by comparisons of the obtained sequences with the GenBank database and determined the belongs with phylogenetic and network analyses, including *L.* (*Sauroleishmania*) sp., *L. donovani*, *L. turanica*. For the first time mammalian *Leishmania* were discovered in snakes, after the similar finding in lizards. The *Sauroleishmania* strains in this study were closely related to clinical isolates, which suggests us to be cautious about the widely accepted view that *Sauroleishmania* is nonpathogenic to human. These findings invite us to ponder the importance of snakes in the disease cycle. However, more snake samples in epidemic foci and the pathogenic features of *Sauroleishmania* promastigotes are required for indicating their epidemiological involvement.

Supporting information

S1 Table. Accession numbers for *Leishmania* sequences downloaded from GenBank and used for the phylogenetic analyses presented in Fig 2.
(DOCX)

Acknowledgments

Author Contributions

Conceptualization: Xianguang Guo, Dali Chen, Jianping Chen.

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References

1. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies. PLoS Negl Trop Dis. 2016; 10: e0004349. https://doi.org/10.1371/journal.pntd.0004349 PMID: 26937644

2. WHO Expert Committee on the Control of the Leishmaniases, World Health Organization, editors. Control of the leishmaniases: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22–26 March 2010. Geneva: World Health Organization; 2010.

3. WHO | Leishmaniasis in high-burden countries: an epidemiological update based on data reported in 2014. In: WHO [Internet]. [cited 16 Nov 2018]. Available: http://www.who.int/leishmaniasis/resources/who_wer9122/en/

4. Lun Z-R, Wu M-S, Chen Y-F, Wang J-Y, Zhou X-N, Liao L-F, et al. Visceral Leishmaniasis in China: an Endemic Disease under Control. Clin Microbiol Rev. 2015; 28: 987–1004. https://doi.org/10.1128/CMR.00080-14 PMID: 26354822

5. Wang J-Y, Cui G, Chen H-T, Zhou X-N, Gao C-H, Yang Y-T. Current epidemiological profile and features of visceral leishmaniasis in people's republic of China. Parasit Vectors. 2012; 5: 31. https://doi.org/10.1186/1756-3305-5-31 PMID: 22316234

6. Zheng CJ, Xue CZ, Wu WP, Zhou XN. Epidemiological characteristics of Kala-azar disease in China, during 2005–2015. Zhonghua Liu Xing Bing Xue Za Zhi Zhonghua Liuxingbingxue Zazhi. 2017; 38: 431–434. https://doi.org/10.3760/cma.j.issn.0254-6450.2017.04.004 PMID: 28468057

7. Maimaitiyiming Y, Hai-Ting Z, Song Z, Zhumahong R, Abuduwayiti S. Epidemic analysis of kala-azar in Xinjiang Uygur Autonomous Region from 2004 to 2014. Bull Dis Control Prev China. 2015;

8. Coutinho MTZ, Bueno LL, Sterzik A, Fujiwara RT, Botelho JR, De Maria M, et al. Participation of Rhipicephalus sanguineus (Acari: Ixodidae) in the epidemiology of canine visceral leishmaniasis. Vet Parasitol. 2005; 128: 149–155. https://doi.org/10.1016/j.vetpar.2004.11.011 PMID: 15725545

9. Paz GF, Ribeiro MFB, Michalsky ÉM, da Rocha Lima ACVM, França-Silva JC, Barata RA, et al. Evaluation of the vectorial capacity of Rhipicephalus sanguineus (Acari: Ixodidae) in the transmission of canine visceral leishmaniasis. Parasitol Res. 2009; 106: 523. https://doi.org/10.1007/s00436-009-1697-1 PMID: 20012095

10. Dantas-Torres F, Lorusso V, Testini G, de Paiva-Cavalcanti M, Figueredo LA, Stanneck D, et al. Detection of Leishmania infantum in Rhipicephalus sanguineus ticks from Brazil and Italy. Parasitol Res. 2010; 106: 857–860. https://doi.org/10.1007/s00436-010-1722-4 PMID: 20127362

11. Solano-Gallego L, Rossi L, Scroccaro AM, Montarsi F, Caldin M, Furlanello T, et al. Detection of Leishmania infantum DNA mainly in Rhipicephalus sanguineus male ticks removed from dogs living in endemic areas of canine leishmaniosis. Parasit Vectors. 2012; 5: 98. https://doi.org/10.1186/1756-3305-5-98 PMID: 22613502

12. Belova EM. Reptiles and their importance in the epidemiology of leishmaniasis. Bull World Health Organ. 1971; 44: 553. PMID: 5316256

13. Chatton E, Blanc G. Existence de corps leishmaniformes dans les hématoblastes d'un gecko barbaresque Tarentola mauritanica L Gunth. CR Soc Biol. 1914; 77: 430–433.

14. Sam R. Telford J. Hemoparasites of the Reptilia: Color Atlas and Text [Internet]. CRC Press; 2016. https://doi.org/10.1201/9781420080414

15. Wenyon CM. Observations on the Intestinal Protozoa of Three Egyptian Lizards, with a Note on a Cell-invading Fungus. Parasitology. 1920; 12: 350–365. https://doi.org/10.1017/S0031182000014347

16. Killick-Kendrick R, Lainson R, Rioux J-A, Sar'janova VM. The taxonomy of Leishmania-like parasites of reptiles. 1986;

17. Croan DG, Morrison DA, Ellis JT. Evolution of the genus Leishmania revealed by comparison of DNA and RNA polymerase gene sequences. Mol Biochem Parasitol. 1997; 89: 149–159. https://doi.org/10.1016/S0166-6851(97)00111-4 PMID: 9364962

18. Noyes HA, Arana BA, Chance ML, Maingon R. The Leishmania hertigi (Kinetoplastida; Trypanosomatidae) Complex and the Lizard Leishmania: Their Classification and Evidence for a Neotropical Origin of the Leishmania-Endotrypanum Clade. J Eukaryot Microbiol. 1997; 44: 511–517. https://doi.org/10.1111/j.1550-7408.1997.tb05732.x PMID: 9304821

19. Orlando TC, Rubio MAT, Sturm NR, Campbell DA, Floeter-Winter LM. Intergenic and external transcribed spacers of ribosomal RNA genes in lizard-infecting Leishmania: molecular structure and phylogenetic relationship to mammal-infecting Leishmania in the subgenus Leishmania (Leishmania). Mem Inst Oswaldo Cruz. 2002; 97: 695–701. https://doi.org/10.1590/S0074-02762002000500020 PMID: 12219138

20. Belova EM, Bogdanov OP. [Incidence of leptomonad infection in snakes of the Turkmen SSR]. Med Parazitol (Mosk). 1969; 38: 304–306.

21. Zhang J-R, Guo X-G, Liu J-L, Zhou T-H, Gong X, Chen D-L, et al. Molecular detection, identification and phylogenetic inference of Leishmania spp. in some desert lizards from Northwest China by using internal transcribed spacer 1 (ITS1) sequences. Acta Trop. 2016; 162: 83–94. https://doi.org/10.1016/j.actatropica.2016.06.023 PMID: 27338182

22. Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J. Taxonomy of Leishmania. Use of isoenzymes. Suggestions for a new classification. Ann Parasitol Hum Comparée. 1990; 65: 111–125. https://doi.org/10.1051/parasite/1990653111 PMID: 2080829

23. Adler S. The Behaviour of a Lizard Leishmania in Hamsters and Baby Mice. Rev Inst Med Trop Sao Paulo. 1962; 4: 61–4. PMID: 13859453

24. Breton M, Tremblay MJ, Ouellette M, Papadopoulou B. Live Nonpathogenic Parasitic Vector as a Candidate Vaccine against Visceral Leishmaniasis. Infect Immun. 2005; 73: 6372–6382. https://doi.org/10.1128/IAI.73.10.6372-6382.2005 PMID: 16177308

25. Zelazny AM, Fedorko DP, Li L, Neva FA, Fischer SH. EVALUATION OF 7SL RNA GENE SEQUENCES FOR THE IDENTIFICATION OF LEISHMANIA SPP. Am J Trop Med Hyg. 2005; 72: 415–420. https://doi.org/10.4269/ajtmh.2005.72.415 PMID: 15827278

26. Guan W, Cao D-P, sun K, Xu J, Zhang J, Chen D-L, et al. Phylogenic analysis of Chinese Leishmania isolates based on small subunit ribosomal RNA (SSU rRNA) and 7 spliced leader RNA (7SL RNA). Acta Parasitol. 2012; 57: 101–113. https://doi.org/10.2478/s11686-012-0022-9 PMID: 22807046

27. Sun K, Guan W, Zhang J-G, Wang Y-J, Tian Y, Liao L, et al. Prevalence of canine leishmaniasis in Beichuan County, Sichuan, China and phylogenetic evidence for an undescribed Leishmania sp. in China based on 7SL RNA. Parasit Vectors. 2012; 5: 75. https://doi.org/10.1186/1756-3305-5-75 PMID: 22515334

28. Dávila AMR, Momen H. Internal-transcribed-spacer (ITS) sequences used to explore phylogenetic relationships within Leishmania. Ann Trop Med Parasitol. 2000; 94: 651–654. https://doi.org/10.1080/00034983.2000.11813588 PMID: 11064767

29. Yang B-B, Guo X-G, Hu X-S, Zhang J-G, Liao L, Chen D-L, et al. Species discrimination and phylogenetic inference of 17 Chinese Leishmania isolates based on internal transcribed spacer 1 (ITS1) sequences. Parasitol Res. 2010; 107: 1049–1065. https://doi.org/10.1007/s00436-010-1969-9 PMID: 20617444

30. Asato Y, Oshiro M, Myint CK, Yamamoto Y, Kato H, Marco JD, et al. Phylogenic analysis of the genus Leishmania by cytochrome b gene sequencing. Exp Parasitol. 2009; 121: 352–361. https://doi.org/10.1016/j.exppara.2008.12.013 PMID: 19159626

31. Yang B-B, Chen D-L, Chen J-P, Liao L, Hu X-S, Xu J-N. Analysis of kinetoplast cytochrome b gene of 16 Leishmania isolates from different foci of China: different species of Leishmania in China and their phylogenetic inference. Parasit Vectors. 2013; 6: 32. https://doi.org/10.1186/1756-3305-6-32 PMID: 23383990

32. Fraga J, Montalvo AM, De Doncker S, Dujardin J-C, Van der Auwera G. Phylogeny of Leishmania species based on the heat-shock protein 70 gene. Infect Genet Evol. 2010; 10: 238–245. https://doi.org/10.1016/j.meegid.2009.11.007 PMID: 19913110

33. Yuan D, Qin H, Zhang J, Liao L, Chen Q, Chen D, et al. Phylogenetic analysis of HSP70 and cyt b gene sequences for Chinese Leishmania isolates and ultrastructural characteristics of Chinese Leishmania sp. Parasitol Res. 2017; 116: 693–702. https://doi.org/10.1007/s00436-016-5335-4 PMID: 27942942

34. Waki K, Dutta S, Ray D, Kolli BK, Akman L, Kawazu S-I, et al. Transmembrane Molecules for Phylogenetic Analyses of Pathogenic Protists: Leishmania-Specific Informative Sites in Hydrophilic Loops of Trans- Endoplasmic Reticulum N-Acetylglucosamine-1-Phosphate Transferase. Eukaryot Cell. 2007; 6: 198–210. https://doi.org/10.1128/EC.00282-06 PMID: 17142569

35. Franchini G. Sur les flagelles intestinaux du type Herpetomonas du Chamaeleon vulgaris et leur culture, et sur les flagelles du type Herpetomonas de Chalcides (Gongylus) ocellatus et Tarentola mauritanica. Bull Soc Path Exot. 1921; 14: 641.

36. Telford SR. Evolutionary implications of Leishmania amastigotes in circulating blood cells of lizards. Parasitology. 1979; 79: 317–324. https://doi.org/10.1017/S0031182000053725 PMID: 542323

37. Shortt HE, Swaminath CS. Preliminary Note on three Species of Trypanosomidae. Indian J Med Res. 1928; 16. Available: https://www.cabdirect.org/cabdirect/abstract/19281000711

38. Luyo-Acero GE, Uezato H, Oshiro M, Takei K, Kariya K, Katakura K, et al. Sequence variation of the Cytochrome b gene of various human infecting members of the genus Leishmania and their phylogeny. Parasitology. 2004; 128: 483–491. https://doi.org/10.1017/S0031182004004792 PMID: 15180316

39. Garcia L, Kindt A, Bermudez H, Llanos-Cuentas A, Doncker SD, Arevalo J, et al. Culture-Independent Species Typing of Neotropical Leishmania for Clinical Validation of a PCR-Based Assay Targeting Heat Shock Protein 70 Genes. J Clin Microbiol. 2004; 42: 2294–2297. https://doi.org/10.1128/JCM.42.5.2294-2297.2004 PMID: 15131217

40. El Tai NO, Osman OF, El Fari M, Presber W, Schönian G. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans R Soc Trop Med Hyg. 2000; 94: 575–579. https://doi.org/10.1016/S0035-9203(00)90093-2 PMID: 11132393

41. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series. [London]: Information Retrieval Ltd., c1979-c2000.; 1999. pp. 95–98.

42. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016; 33: 1870–1874. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

43. Xia X. DAMBE7: New and Improved Tools for Data Analysis in Molecular Biology and Evolution. Mol Biol Evol. 2018; 35: 1550–1552. https://doi.org/10.1093/molbev/msy073 PMID: 29669107

44. Xia X. DAMBE6: New Tools for Microbial Genomics, Phylogenetics, and Molecular Evolution. J Hered. 2017; 108: 431–437. https://doi.org/10.1093/jhered/esx033 PMID: 28379490

45. Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B. PartitionFinder 2: New Methods for Selecting Partitioned Models of Evolution for Molecular and Morphological Phylogenetic Analyses. Mol Biol Evol. 2017; 34: 772–773. https://doi.org/10.1093/molbev/msw260 PMID: 28013191

46. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Syst Biol. 2012; 61: 539–542. https://doi.org/10.1093/sysbio/sys029 PMID: 22357727

47. Bandelt HJ, Forster P, Röhl A. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol. 1999; 16: 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036 PMID: 10331250

48. Polzin T, Daneshmand SV. On Steiner trees and minimum spanning trees in hypergraphs. Oper Res Lett. 2003; 31: 12–20. https://doi.org/10.1016/S0167-6377(02)00185-2

49. Feridoun Mahboudi MA Mohsen Abolhassani, Mohammad Yaran, Hamid Mobtaker. Identification and Differentiation of Iranian Leishmania Species by PCR Amplification of kDNA. Scand J Infect Dis. 2001; 33: 596–598. https://doi.org/10.1080/00365540110026746 PMID: 11525354

50. Victoir K, Bañuls AL, Arevalo J, Llanos-Cuentas A, Hamers R, Noël S, et al. The gp63 gene locus, a target for genetic characterization of Leishmania belonging to subgenus Viannia. Parasitology. 1998; 117: 1–13. PMID: 9695095

51. Wilson V, Southgate BA. Lizard leishmania. Biol Kinetoplastida. 1979; 2: 241–268.

52. Bates PA. Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol. 2007; 37: 1097–1106. https://doi.org/10.1016/j.ijpara.2007.04.003 PMID: 17517415

53. Stuckenberg BR. New fossil species of Phlebotomus and Haematopota in Baltic Amber (Diptera: Psychodidae, Tabanidae). Ann Natal Mus. 1975; 22: 455–464.

54. Morayma Solórzano Kraemer M. Systematic, palaeoecology, and palaeobiogeography of the insect fauna from Mexican amber. Palaeontogr Abt A. 2007; 1–133. https://doi.org/10.1127/pala/282/2007/1

55. Cao D-P, Guo X-G, Chen D-L, Chen J-P. Species delimitation and phylogenetic relationships of Chinese Leishmania isolates reexamined using kinetoplast cytochrome oxidase II gene sequences. Parasitol Res. 2011; 109: 163–173. https://doi.org/10.1007/s00436-010-2239-6 PMID: 21221640

56. Manson-Bahr PEC, Heisch RB. Transient Infection of Man with a Leishmania (L. adleri) of Lizards. Ann Trop Med Parasitol. 1961; 55: 381–2. PMID: 14469433

57. Coughlan S, Mulhair P, Sanders M, Schonian G, Cotton JA, Downing T. The genome of *Leishmania adleri* from a mammalian host highlights chromosome fission in *Sauroleishmania*. Sci Rep. 2017; 7: 43747. https://doi.org/10.1038/srep43747 PMID: 28256610