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# Phylogenic analysis of Chinese *Leishmania* isolates based on small subunit ribosomal RNA (SSU rRNA) and 7 spliced leader RNA (7SL RNA)

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## Abstract

The leishmaniases are zoonotic diseases caused by protozoan parasites of the genus *Leishmania*. Leishmaniases are still endemic in China, especially in the west and northwest froniter regions. To revalue the preliminary phylogenetic results of Chinese *Leishmania* isolates, we amplified partial fragment of small subunit ribosomal RNA (SSU rRNA) and 7 spliced leader RNA (7SL RNA), then tested the phylogenetic relationships among Chinese *Leishmania* isolates and their relatives by analyzing SSU rRNA gene sequences and 7SL RNA gene sequences. 19 SSU RNA sequences and 9 7SL RNA sequences were obtained in our study, then analyzed with 42 SSU RNA sequences and 32 7SL RNA sequences retrieved from Genbank, respectively. In the Bayesian analysis of the SSU RNA gene, the isolate MHOM/CN/93/GS7 and the isolate IPHL/CN/77/XJ771 are members of *Leishmania donovani* complex, while the isolate MHOM/CN/84/JS1 clustered with *Leishmania tropica*. The other 11 Chinese *Leishmania* isolates (MHOM/CN/90/WC, MCAN/CN/90/SC11, MHOM/CN/80/XJ801, MHOM/CN/85/GS4, MHOM/CN/84/SD1, MCAN/CN/86/SC7, MHOM/CN/54/#3, MHOM/CN/83/GS2, MHOM/CN/90/SC10H2, MHOM/CN/89/GS6 and MHOM/CN/89/GS5) form an unclassified group, defined as *Leishmania* sp., and the most relative species to this group is *L. tarentolae*. In the Bayesian analysis of the 7SL RNA gene, 9 Chinese *Leishmania* isolates also formed an unclassified group with *L. tarentolae*, including canine isolate 10, MHOM/CN/85/GS4, MHOM/CN/84/SD1, MCAN/CN/86/SC7, MHOM/CN/54/#3, MHOM/CN/83/GS2, MHOM/CN/90/SC10H2, MHOM/CN/89/GS6 and MHOM/CN/89/GS5. We concluded that: (1) Chinese *Leishmania* isolates are non-monophyly group; (2) an unclassified group may exist in China, and the most relative species to this group is *L. tarentolae*; (3) MHOM/CN/84/JS1, which was previously assigned as *L. donovani*, was most genetically related to *L. tropica* strain MHOM/SU/74/K27.

## Keywords

*Leishmania*, phylogeny, SSU RNA, 7SL RNA, China

## Introduction

The leishmaniases are zoonotic diseases caused by protozoan parasites of the genus *Leishmania*, which are geographically widespread severe parasitic diseases with an incidence of two million cases per year and 350 million people at risk (Desjeux 2004). Leishmaniases are various manifestations in clinic, eg., cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis, mucocutaneous and visceral leishmaniasis (VL) diseases, mainly caused by species of the *Leishmania donovani* complex, and an intermediary form known as post-Kala-Azar dermal leishmaniasis (PKDL). In the current context of worldwide (re)emergence and spreading of leishmaniasis, the relevance of species identification gains further importance.

As with many parasites, the taxonomy of the genus *Leishmania* is very complex because species boundaries are difficult to define (Schönian *et al*. 2010). The simplest and

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most widely used classifications are the clinical features and the geographic distribution. Morphological classifications are not always reliable because of closely related species and intraspecies variation. Currently, multilocus enzyme electrophoresis (MLEE, also known as zymedome typing) is the reference method for identification of *Leishmania* species and strains. However, it has several drawbacks: strains with the same enzyme phenotype may in fact have distinct amino acid sequences; it requires large volume *Leishmania* cultures, thus it is costly and time consuming (Kuhls *et al*. 2005; Bañuls *et al*. 2007); sufficiently discriminatory markers are not available. A simpler and more discriminatory method is required. Genetic markers have been explored for the development of reliable tools for species identification as well as an understanding of intraspecies genetic diversity and population structure (Aransay *et al*. 1999; Barroso *et al*. 2007; Barón *et al*. 2008).

Meanwhile, DNA-based methods have replaced traditional methods to analyze the phylogenetic relationship among species with various targets such as kDNA genes, introns and nuclear genes (Piarroux *et al*. 1995; Noyes *et al*. 1997; El Tai *et al*. 2001; Ibrahim and Barker 2001; Fraga *et al*. 2010), as they trend to be more specific and stable. These studies suggest that the taxonomy of the *L. donovani* complex needs to be revised (Lukeš *et al*. 2007). They used a combination of widely used DNA analysis techniques, further demonstrated that geographic origin of a strain is a more important predictor of genetic relatedness than clinical features (visceral versus cutaneous leishmaniasis). Unfortunately, few Chinese *Leishmania* isolates were included in these studies.

Leishmaniasis is still endemic in China, especially in the west and northwest frontier regions of China. According to different geographical origin, infective agent, and clinical manifestations, epidemic foci of VL in China were classified into three types, i.e., plain foci, hill foci, and desert foci (Lu *et al*. 1994). Different epidemic foci and types of leishmaniasis in China have brought forth the difficulty of identifying the strains of *Leishmania*, as the causative agents cannot be distinguished easily on the basis of morphological characteristics. Up to the present, efforts have been directed towards the identification of Chinese *Leishmania* isolates from three foci in China by analyzing kDNA or nDNA (Lu and Hu 1988; Hu *et al*. 1992; Zheng *et al*. 1999; Bu *et al*. 2000; Lu *et al*. 2001; Tian and Chen 2005). Their results suggested that the differences at genetic level did exist in *Leishmania* isolates from different foci in China.

In order to shed further light on the phylogenetic position of Chinese *Leishmania* isolates in the genus *Leishmania*, Yang *et al*. (2010) and Cao *et al*. (2011) used ITS1 (internal transcribed spacer 1) and COII (cytochrome oxidase C subunit II) as genetic markers to analyze the phylogeny of Chinese *Leishmania* isolates, respectively. Their results on the phylogeny of Chinese *Leishmania* isolates have challenged some aspects of the traditional taxonomy and cladistic hypotheses. Therefore, more data and analyses are needed for disagreements with respect to relationships within Chinese *Leishmania* isolates. SSU rRNA gene sequence comparisons have been used successfully to infer phylogenetic relationships within other genera of the Kinetoplastida (Uliana *et al*. 1991; Landweber and Gilbert 1994; Marche *et al*. 1995; Maslov *et al*. 1996). The mitochondrial genome is particularly valuable for understanding evolutionary relationships among individuals, populations and species of different organisms (Ibrahim and Barker 2001; Luyo-Acero *et al*. 2004; Asato *et al*. 2009). 7 spliced leader RNA, a component of the signal recognition particle which mediates the protein translocation across the endoplasmic reticulum in eukaryotes, has also been used as a genetic marker for phylogenetic analysis in the genus *Leishmania* (Zelazny *et al*. 2005). Although the 7SL RNA used as marker contains only 137–139 nts, the phylogeny was robust and powerful based on it. As many researches have verified, more isolates and more phylogenetic information will change the phylogenetic results (Botilde *et al*. 2006; Asato *et al*. 2009; Kuwahara *et al*. 2009). Thus, further studies on classification and phylogeny of Chinese *Leishmania* isolates are still necessary.

In this study, we amplified partial fragment of small subunit ribosomal RNA (SSU rRNA) and 7 spliced leader RNA (7SL RNA), then tested the phylogenetic relationships among Chinese *Leishmania* isolates and their relatives by analyzing SSU rRNA gene sequences and 7SL RNA gene sequences. 19 SSU RNA sequences and 9 7SL RNA sequences were obtained in our study, and then analyzed with 42 SSU RNA sequences and 32 7SL RNA sequences retrieved from Genbank, respectively. The aims of our study are as follows: (1) to report a set of original SSU RNA gene sequences for 16 *Leishmania* isolates and a set of original 7SL RNA sequences for 9 *Leishmania* isolates from different foci in China; (2) to revalue the phylogeny of Chinese *Leishmania* isolates; (3) to determine the most relative species to *Leishmania* sp.; (4) to compare the differences among the different results based on different molecular markers.

## Materials and methods

*DNA extraction*

Promastigotes (including 16 Chinese *Leishmania* isolates, and 3 *Leishmania* representatives of other *Leishmania* complex isolated from other countries) stored in liquid nitrogen, and kept in the department of parasitology, Western China school of preclinical and forensic medicine, Sichuan university. All isolates used in this study were listed in Table I. Promastigotes were cultivated in medium 199 supplemented with 15% heat-inactivated fetal bovine serum at 25°C. Approximately 1–5 × $10^9$ promastigotes were collected at room temperature by centrifugation at 4,000 rpm for 10 min and washed with distilled water. Total nDNA was extracted from the promastigotes using a standard sodium dodecyl sulfate-proteinase K proce-

**Table I.** List of *Leishmania* strains, origin, and database accession numbers

| Species | GenBank accession number | WHO code | Sequence length (bp) | Origin | Reference |
|---|---|---|---|---|---|
| *Leishmania* sp. | HQ895954 | IPHL/CN/77/XJ771 | 394 | Xinjiang, China | this study |
| *Leishmania* sp. | HQ895849 | MHOM/CN/93/GS7 | 393 | Gansu, China | this study |
| *Leishmania* sp. | HQ895856 | MHOM/CN/90/WC | 397 | Sichuan, China | this study |
| *Leishmania* sp. | HQ895857 | MCAN/CN/90/SC11 | 396 | Sichuan, China | this study |
| *Leishmania* sp. | HQ895846 | MHOM/CN/80/XJ801 | 383 | Xinjiang, China | this study |
| *Leishmania* sp. | HQ895847 | MHOM/CN/85/GS4 | 355 | Gansu, China | this study |
| *Leishmania* sp. | HQ895853 | MHOM/CN/84/SD1 | 393 | Shandong, China | this study |
| *Leishmania* sp. | HQ895852 | MCAN/CN/86/SC7 | 393 | Sichuan, China | this study |
| *Leishmania* sp. | HQ895845 | MHOM/CN/54/#3 | 390 | Peking, China | this study |
| *Leishmania* sp. | HQ895855 | MHOM/CN/83/GS2 | 343 | Gansu, China | this study |
| *Leishmania* sp. | HQ895858 | MHOM/CN/90/SC10H2 | 353 | Sichuan, China | this study |
| *Leishmania* sp. | HQ895848 | MHOM/CN/89/GS6 | 391 | Gansu, China | this study |
| *Leishmania* sp. | HQ895850 | MRHO/CN/88/KXG-2 | 396 | Xinjiang, China | this study |
| *Leishmania* sp. | HQ895860 | MGER/CN/60/GS-GER20 | 391 | Gansu, China | this study |
| *Leishmania* sp. | HQ895851 | MHOM/CN/89/GS5 | 392 | Gansu, China | this study |
| *Leishmania* sp. | HQ895959 | MHOM/CN/84/JS1 | 392 | Jiangsu, China | this study |
| *L. tropica* | HQ895861 | MHOM/SU/74/K27 | 393 | Soviet Union | this study |
| *L. mexicana* | HQ895863 | A90 | 393 | – | this study |
| *L. amazonensis* | HQ895862 | A90 | 393 | – | this study |
| *L. colombiensis* | AF133836 | IGOM/PA/85/e582 | 394 | Panama | Cupolillo *et al*. 2000 |
| *L. equatoriensis* | AF133837 | MCHO/EC/82/LSP1 | 394 | European Community | Cupolillo *et al*. 2000 |
| *L major* | AF303938 | MHOM/MQ/92/MAR1 | 394 | Martinique | Noyes *et al*. 2000 |
| *L. major* | EF524072 | *L.* sp. Ghana-2006 | 394 | Ghana | Villinski *et al*. 2008 |
| *L. major* | DQ295826 | MHOM/GH/04/HO-004 | 394 | Ghana | Fryauff *et al*. 2006 |
| *L. major* | DQ295827 | IPAP/EG/89/SI-177 | 394 | Egypt | Fryauff *et al*. 2006 |
| *Leishmania* sp. | GQ226033 | – | 394 | Thailand | Suankratay *et al*. 2010 |
| *L. mexicana* | GQ277631 | – | 395 | USA | Trainor *et al*. 2010 |
| *L. mexicana* | GQ277632 | – | 395 | USA | Trainor *et al*. 2010 |
| *Leishmania* sp. | GQ293225 | – | 394 | Thailand | unpublished data |
| *L. brasiliensis* | GQ332355 | – | 395 | – | de Almeida *et al*. 2009 |
| *L. donovani* | GQ332356 | – | 394 | – | de Almeida *et al*. 2009 |
| *L. chagasi* | GQ332357 | – | 394 | – | de Almeida *et al*. 2009 |
| *L. guyanensis* | GQ332358 | – | 395 | – | de Almeida *et al*. 2009 |
| *L. mexicana* | GQ332360 | – | 395 | – | de Almeida *et al*. 2009 |
| *L. major* | GQ332361 | – | 394 | – | de Almeida *et al*. 2009 |
| *L. panamensis* | GQ332362 | – | 395 | – | de Almeida *et al*. 2009 |
| *L. tropica* | GQ332363 | – | 394 | – | de Almeida *et al*. 2009 |
| *L. aethiopica* | GQ920678 | – | 394 | – | de Almeida *et al*. 2009 |
| *L. adleri* | M80291 | RLAT/KE/54/1433 | 395 | Kenya | van Eys *et al*. 1992 |
| *L. brasiliensis* | M80292 | – | 396 | – | van Eys *et al*. 1992 |
| *L. mexicana* | M80293 | – | 396 | – | van Eys *et al*. 1992 |
| *L. tropica* | M80294 | – | 395 | – | van Eys *et al*. 1992 |
| *L. donovani* | M80295 | – | 395 | – | van Eys *et al*. 1992 |
| *L. guanensis* | M81416 | – | 396 | – | van Eys *et al*. 1992 |
| *L. panamensis* | M81421 | – | 395 | – | van Eys *et al*. 1992 |
| *L. mexicana* | M81422 | – | 396 | – | van Eys *et al*. 1992 |
| *L. major* | M81427 | – | 395 | – | van Eys *et al*. 1992 |
| *L. aethiopica* | M81428 | – | 395 | – | van Eys *et al*. 1992 |

**Table I.** Continuation

| Species | GenBank accession number | WHO code | Sequence length (bp) | Origin | Reference |
|---|---|---|---|---|---|
| *L. infantum* | M81429 | – | 395 | – | van Eys *et al*. 1992 |
| *L. chagasi* | M81430 | – | 395 | – | Van Eys *et al*. 1992 |
| *L. tarentolae* | M84225 | – | 394 | – | Briones *et al*. 1992 |
| *L. herreri* | U50043 | MCHO/CR/74/Ch-97 | 391 | Costa Rica | Noyes *et al*. 1996 |
| Trypanosomatidae *G755* | U59491 | – | 398 | – | Noyes *et al*. 1997 |
| *L. hertigi* | U59492 | MCOE/XX/PA/LV42 | 400 | Panama | Noyes *et al*. 1997 |
| *L. amazonensis* | X53912 | MHOM/BR/73/M1845 | 395 | Brazil | Noyes *et al*. 1997 |
| *E. monterogeii* | X53911 | MCHO/CR/62/A9 | 394 | Costa Rica | Fernandes *et al*.1993 |
| *L. guyanensis* | X53913 | MHOM/BR/75/M4147 | 395 | Brazil | Ulinan *et al*. 1994 |
| *L. major* | X53915 | MHOM/SD/72/LV305 | 394 | Sudan | Ulinan *et al*. 1994 |
| *L. tarentolae* | X53916 | – | 394 | – | unpublished data |
| *L. donovani* | X07773 | – | 396 | Sudan | Looker *et al*. 1988 |
| *T. cruzi* | FJ555630 | – | 457 | USA | Marcili *et al*. 2009 |

dure, as described by Sambrook and Russell (2001). The *Leishmania* specific primers of SSU rRNA (Van Eys *et al*. 1992) R222 (5'-TATTGGAGATTATGGAGCTG-3') and R333 (5'-AAAGCGGGCGCGCGGTGCTG-3') were used to amplify SSU rRNA segments. The PCR protocols were 94°C for 4 min followed by 30 cycles of 94°C for 60 s, 50°C for 45 s, 72°C for 60 s, and then a final elongation step at 72°C for 5 min. The PCR products were purified on a 2.0% agarose gel stained with ethidium bromide, using a commercial DNA purification kit following the manufacturer's protocol. Purified productions of PCR were sent to commercial company for sequencing using the same PCR primers. The sequences have been deposited in GenBank under accession numbers HQ895845-HQ895863 (Table I).

*PCR amplification*

For SSU rRNA, primers R222 (5'-TATTGGAGATTATGG AGCTG-3') and R333 (5'-AAAGCGGGCGCGCGGTGC TG-3') were used to amplify a SSU rRNA fragment (Van Eys *et al*. 1992 ). The PCR protocols were 94°C for 4 min followed by 30 cycles of 94°C for 60 s, 50°C for 45 s, 72°C for 60 s, and then a final elongation step at 72°C for 5 min. For 7SL RNA, primers TRY7SL.For1.M13 (5'-GTAAAACGACGGCCA GTGCTCTGTAACCTTCGGGGGCT-3') and TRY7SL.Rev1. M13 (5'-CAGGAAACAGCTATGACGGCTGCTCCGTYNC CGGCCTGACCC-3') were used to amplify a 7SL RNA fragment (Zelazny *et al*. 2005). The PCR protocols were 95°C for 5 min followed by 34 cycles of 95°C for 60 s, 65°C for 60 s, 72°C for 60 s, and then a final elongation step at 72°C for 10 min. The PCR products were purified on a 2.0% agarose gel stained with ethidium bromide, using a commercial DNA purification kit following the manufacturer's protocol. Purified productions of PCR were sent to commercial company for sequencing using the same PCR primers. The sequences have been deposited in GenBank under accession numbers HQ895 845-HQ895863 for SSU RNA (Table I). As for 7SL RNA, the 9 sequences shared Genbank accession number JQ315203, because they have an identical sequence to each other.

*Sequence alignment and analysis*

For SSU RNA, a set of sequences were retrieved from Genbank, including 39 strains of *Leishmania*, 2 strains of *Trypanosoma* and one strain of *Endotrypanum monterogeii* (Table I). For 7SL RNA, a set of sequences were retrieved from Genbank, including 31 strains of *Leishmania*, 1 strains of *Trypanosoma* (Table II). The sequences were aligned using Clustal X (1.83) (Thompson *et al*. 1997) with default settings and refined manually. To generate a consensus tree, compositional heterogeneity was evaluated using Chi square ($\chi^2$) tests implemented in PAUP* 4.0b10 (Swofford 2002) and assessed using the software SeqVis v.1.3 (Ho *et al*. 2006) to visualize and to conduct matched-pairs tests of symmetry (Ababneh *et al*. 2006). The p-distance matrix was computed with MEGA v.4.1 (Tamura *et al*. 2007). And then phylogenetic analysis and tree: neighbour-joining (NJ) was performed by using the MEGA 4.0 program using the Kimura 2-parameter (K2P) model. The most appropriated model for Bayesian analysis was selected by combined utilization of the program PAUP and Modeltest 3.7 (Posada and Crandall 1998). Bayesian analyses were carried out using the program Mrbayes-3.1.2 (Ronquist and Huelsenbeck 2003). *Trypanosoma cruzi* and *Trypanosoma brucei* was used to root the trees.

## Results

*Sequence alignment and analyses*

In total, nineteen SSU RNA sequences and nine 7SL RNA sequences were obtained in this study. The sequences were

**Table II.** List of *Leishmania* strains, origin, and database accession numbers

| Species | Genbank accession no. | WHO code | Sequence length (bp) | Origin | Reference |
|---|---|---|---|---|---|
| *Leishmania* sp. | JQ315203 | canine isolate 10 | 138 | Sichuan, China | this study |
| *Leishmania* sp. | | MHOM/CN/85/GS4 | 138 | Sichuan, China | this study |
| *Leishmania* sp. | | MHOM/CN/54/#3 | 138 | Peking, China | this study |
| *Leishmania* sp. | | MHOM/CN/89/GS5 | 138 | Gansu, China | this study |
| *Leishmania* sp. | | MHOM/CN/90/SC10H2 | 138 | Sichuan, China | this study |
| *Leishmania* sp. | | MHOM/CN/83/GS2 | 138 | Gansu, China | this study |
| *Leishmania* sp. | | MHOM/CN/89/GS6 | 138 | Gansu, China | this study |
| *Leishmania* sp. | | MHOM/CN/89/SC7 | 138 | Sichuan, China | this study |
| *Leishmania* sp. | | MHOM/CN/84/SD1 | 138 | Shangdong, China | this study |
| *L. aethiopica* | AY722730 | MHOM/ET/72/L100 | 137 | Ethiopia | Zelazny *et al*. 2005 |
| *L. aethiopica* | AY722731 | MHOM/ET/67/L86 | 137 | Ethiopia | Zelazny *et al*. 2005 |
| *L. aethiopica* | AY722732 | clinical isolate | 137 | Ethiopia | Zelazny *et al*. 2005 |
| *L. amazonensis* | AY722721 | clinical isolate | 135 | Brazil | Zelazny *et al*. 2005 |
| *L. braziliensis* | AY781790 | MHOM/BR/75/M2903 | 139 | Brazil | Zelazny *et al*. 2005 |
| *L. braziliensis* | AY781791 | MHOM/BR/84/LTB300 | 139 | Brazil | Zelazny *et al*. 2005 |
| *L. braziliensis* | AY722724 | clinical isolate | 139 | Brazil | Zelazny *et al*. 2005 |
| *L. braziliensis* | AY722725 | clinical isolate | 139 | Panama | Zelazny *et al*. 2005 |
| *L. braziliensis* | AY722726 | clinical isolate | 139 | Venezuela | Zelazny *et al*. 2005 |
| *L. chagasi* | AY781793 | clinical isolate | 137 | Honduras | Zelazny *et al*. 2005 |
| *L. chagasi* | AY781794 | clinical isolate | 138 | Honduras | Zelazny *et al*. 2005 |
| *L. donovani* | AY722733 | MHOM/IN/80/DD8 | 138 | India | *Zelazny et al*. 2005 |
| *L. donovani* | AY722734 | MHOM/ET/62/L82 | 138 | Ethiopia | Zelazny *et al*. 2005 |
| *L. donovani* | AY722735 | clinical isolate | 138 | India | Zelazny *et al*. 2005 |
| *L. donovani* | AY781796 | clinical isolate | 138 | India | Zelazny *et al*. 2005 |
| *L. guyanensis* | AY722727 | MHOM/BR/75/M4147 | 139 | Brazil | Zelazny *et al*. 2005 |
| *L. guyanensis* | AY722728 | MHOM/BR/75/M4147 | 139 | Brazil | Zelazny *et al*. 2005 |
| *L. guyanensis* | AY722729 | clinical isolate | 139 | Surinam | Zelazny *et al*. 2005 |
| *L. infantum* | AY781792 | MHOM/TN/80/IPT1 | 138 | Tunisia | Zelazny *et al*. 2005 |
| *L. major* | AY722713 | MHOM/TN/80/IPT1 | 137 | Soviet Union | Zelazny *et al*. 2005 |
| *L. major* | AY722714 | clinical isolate | 137 | Israel | Zelazny *et al*. 2005 |
| *L. major* | AY722715 | clinical isolate | 137 | Niger | Zelazny *et al*. 2005 |
| *L. mexicana* | AY722716 | MNYC/BZ/62/M379 | 137 | Belize | Zelazny *et al*. 2005 |
| *L. mexicana* | AY722717 | clinical isolate | 137 | Guatemala | Zelazny *et al*. 2005 |
| *L. mexicana* | AY722718 | clinical isolate | 137 | Honduras | Zelazny *et al*. 2005 |
| *L. mexicana* | AY722719 | clinical isolate | 137 | Dominican Republic | Zelazny *et al*. 2005 |
| *L. mexicana* | AY722720 | clinical isolate | 137 | Dominican Republic | Zelazny *et al*. 2005 |
| *L. tarentolae* | AY781796 | clinical isolate | 138 | Algeria | Zelazny *et al*. 2005 |
| *L. tropica* | AY722722 | MHOM/Su/74/K27 | 137 | Soviet Union | Zelazny *et al*. 2005 |
| *L. tropica* | AY722723 | clinical isolate | 137 | Pakistan | Zelazny *et al*. 2005 |
| *L. tropica* | AY781789 | clinical isolate | 137 | Afghanistan | Zelazny *et al*. 2005 |
| *T. brucei* | M80262.1 | MHOM/BR/78SYLVIO | 139 | Brazil | Zelazny *et al*. 2005 |

deposited in GenBank under accession numbers HQ8958 45-HQ895863 for SSU RNA (Table I). As for 7SL RNA, the 9 sequences shared a Genbank accession number JQ315203, because they have an identical sequence to each other.

A total of 459 character sites in the matrix of the SSU rRNA were considered for analyses, with 113 variable sites and 40 phylogeny-informative sites. The range of sequences was from 353 bp (MHOM/CN/90/SC10H2) to 457 bp (*Trypanosoma cruzi*). The nucleotide frequency and all 4 bases

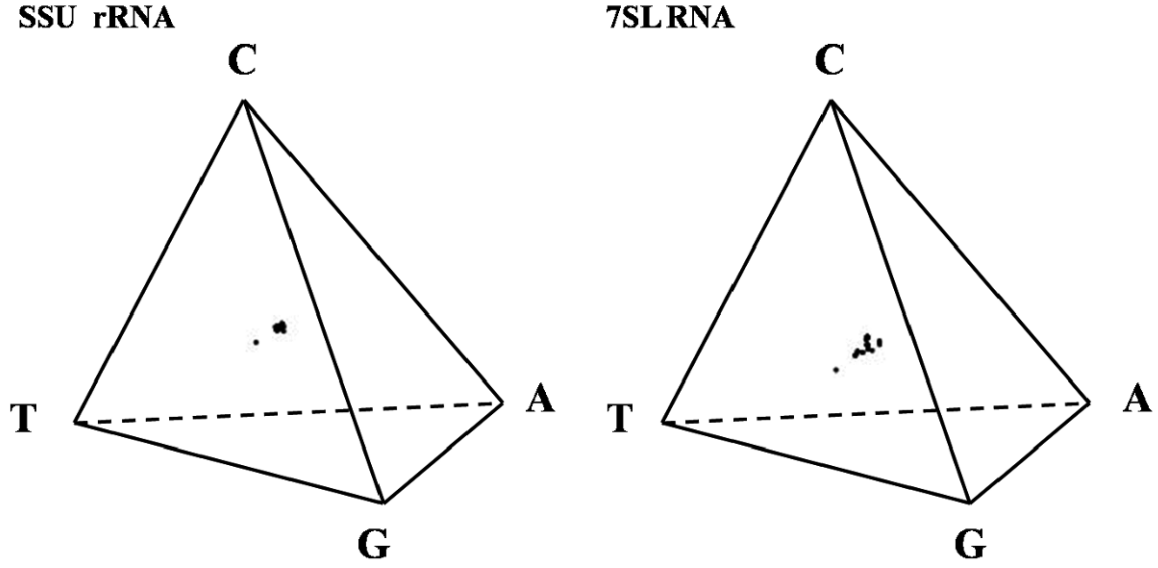


**Fig. 1.** Tetrahedral plots for cytochrome small subunit ribosomal RNA (SSU rRNA) gene dataset and 7SL RNA gene dataset, which were obtained using the Select Sites command from the View menu in the program SeqVis (Ho *et al*. 2006)

were identified. Frequency of the bases were A = 0.230, C = 0.253, G = 0.298 and T = 0.218, and the SSU rRNA genes of *Leishmania* parasites were revealed to be C+G greater than A+T. The overall transition/transversion was 1.989. Figure 1 showed the tetrahedral plot from the SSU rRNA gene. Clearly, there was no conspicuous compositional heterogeneity in the alignment. The implication of this plot is that these sites were likely to have evolved under the same stationary, reversible, and homogeneous. A $\chi^2$ test at the 5% level of significance for differences in base frequencies showed that there was no base compositional heterogeneity among sequences ($\chi^2 = 14.03$, df = 180, P = 1.00), which was known to adversely affect phylogenetic inference.

A total of 139 character sites in the matrix of the 7SL RNA were considered for analyses, with 31 phylogeny-informative sites. The sequences ranged from 135 bp (*L. amazonensis*) to 139 bp (*Trypanosoma brucei*). The nucleotide frequency and all 4 bases were identified. Frequency of the bases were A = 0.132, C = 0.253, G = 0.414 and T = 0.200. The 7SL RNA genes of *Leishmania* parasites were also C+G greater than A+T. Figure 1 also showed the tetrahedral plot from the 7SL RNA gene. There was no conspicuous compositional heterogeneity in the alignment, which indicated that these sites were likely to have evolved under the same stationary, reversible, and homogeneous. A $\chi^2$ test at the 5% level of significance for differences in base frequencies showed that there was no base

**Table III.** Pairwise genetic distances for SSU rRNA segments among *Leishmania* species in this study

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *L. braziliensis* complex | – | | | | | | | | | | | | | |
| *E. monterogeii* | 0.035 | – | | | | | | | | | | | | |
| unclassified *Leishmania* | 0.029 | 0.054 | – | | | | | | | | | | | |
| *L. hertigi* | 0.036 | 0.024 | 0.042 | – | | | | | | | | | | |
| *L. tropica* | 0.029 | 0.061 | 0.017 | 0.046 | – | | | | | | | | | |
| *L. donovani* complex | 0.031 | 0.064 | 0.021 | 0.049 | 0.01 | – | | | | | | | | |
| *L. major* | 0.023 | 0.055 | 0.014 | 0.040 | 0.005 | 0.008 | – | | | | | | | |
| *Leishmania* sp. | 0.037 | 0.069 | 0.025 | 0.052 | 0.014 | 0.021 | 0.016 | – | | | | | | |
| *Lizard Leishmania* | 0.025 | 0.055 | 0.016 | 0.040 | 0.008 | 0.010 | 0.003 | 0.015 | – | | | | | |
| *L. aethiopica* complex | 0.024 | 0.052 | 0.017 | 0.040 | 0.008 | 0.010 | 0.003 | 0.018 | 0.005 | – | | | | |
| *L. mexicana* complex | 0.023 | 0.055 | 0.014 | 0.040 | 0.005 | 0.008 | 0.007 | 0.015 | 0.003 | 0.003 | – | | | |
| *L. guyanensis* complex | 0.019 | 0.053 | 0.017 | 0.042 | 0.010 | 0.013 | 0.005 | 0.020 | 0.008 | 0.008 | 0.005 | – | | |
| Trypanosomatidae G755 | 0.068 | 0.072 | 0.079 | 0.085 | 0.081 | 0.075 | 0.075 | 0.093 | 0.077 | 0.077 | 0.075 | 0.069 | – | |
| *Trypanosoma cruzi* | 0.291 | 0.309 | 0.285 | 0.311 | 0.283 | 0.294 | 0.285 | 0.284 | 0.285 | 0.287 | 0.288 | 0.283 | 0.323 | – |

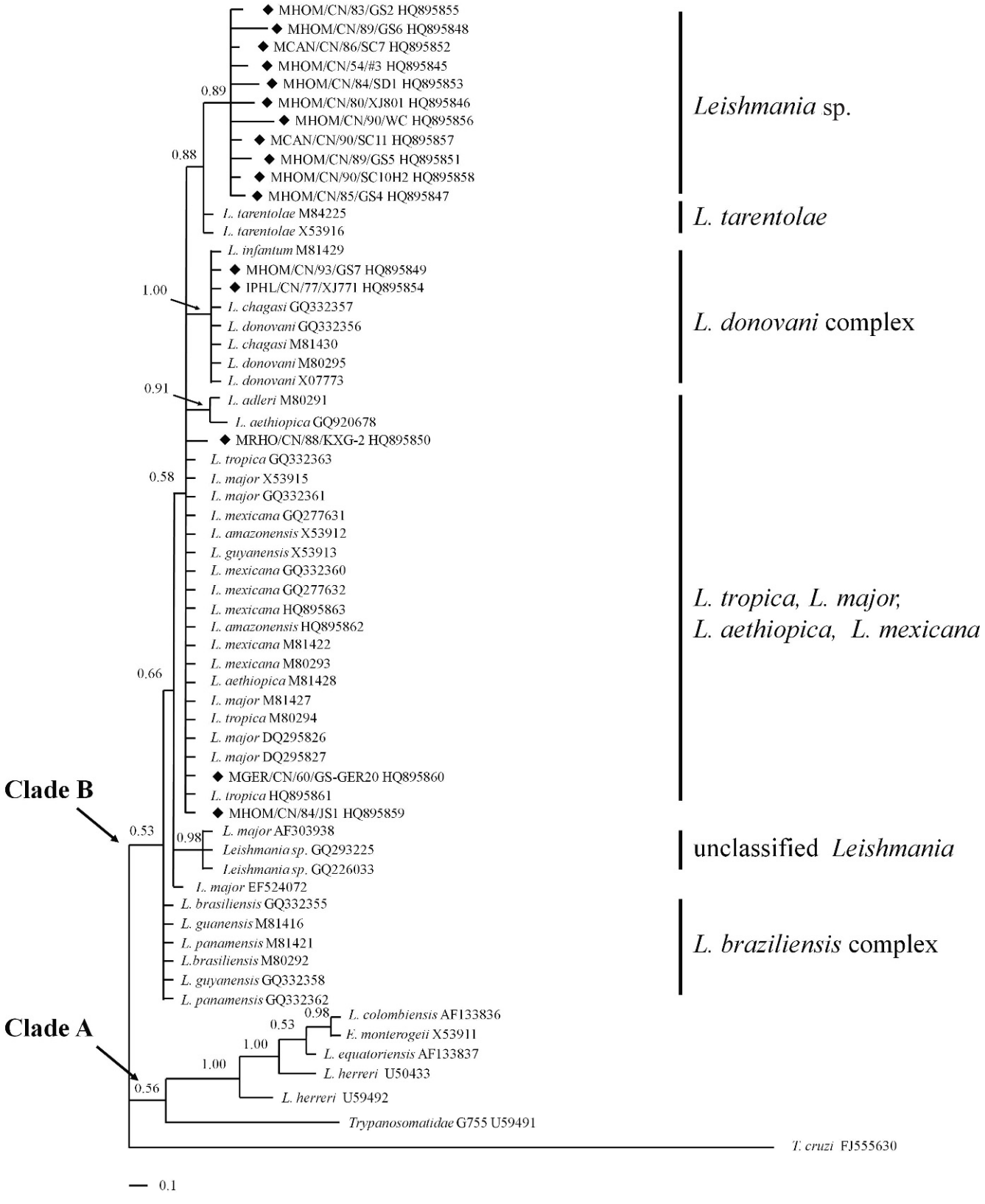


**Fig. 2.** The 50% majority-rule consensus tree inferred from Bayesian inference of small subunit ribosomal RNA (SSU rRNA) gene dataset by using MrBayes v. 3.1.2. Chinese isolates analyzed in this study were tagged with ◆. Numbers at nodes represent Bayesian posterior probabilities

**Table IV.** Pairwise genetic distances for 7SL RNA segments among *Leishmania* species in this study

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Leishmania* sp. | – | | | | | | | | | | |
| *L. aethiopica* complex | 0.145 | – | | | | | | | | | |
| *L. amazonensis* | 0.154 | 0.057 | – | | | | | | | | |
| *L. braziliensis* complex | 0.165 | 0.195 | 0.174 | – | | | | | | | |
| *L. donovani* complex | 0.108 | 0.057 | 0.065 | 0.174 | – | | | | | | |
| *L. guyanensis* complex | 0.151 | 0.160 | 0.147 | 0.044 | 0.131 | – | | | | | |
| *L. major* complex | 0.155 | 0.040 | 0.083 | 0.174 | 0.065 | 0.147 | – | | | | |
| *L. mexicana* complex | 0.155 | 0.048 | 0.008 | 0.175 | 0.065 | 0.147 | 0.075 | – | | | |
| *L. tarentolae* | 0.082 | 0.127 | 0.099 | 0.196 | 0.091 | 0.170 | 0.136 | 0.100 | – | | |
| *L. tropica* complex | 0.136 | 0.008 | 0.048 | 0.185 | 0.048 | 0.151 | 0.032 | 0.040 | 0.118 | – | |
| *L. guyanensis* complex | 0.375 | 0.315 | 0.326 | 0.419 | 0.354 | 0.403 | 0.328 | 0.314 | 0.325 | 0.328 | – |

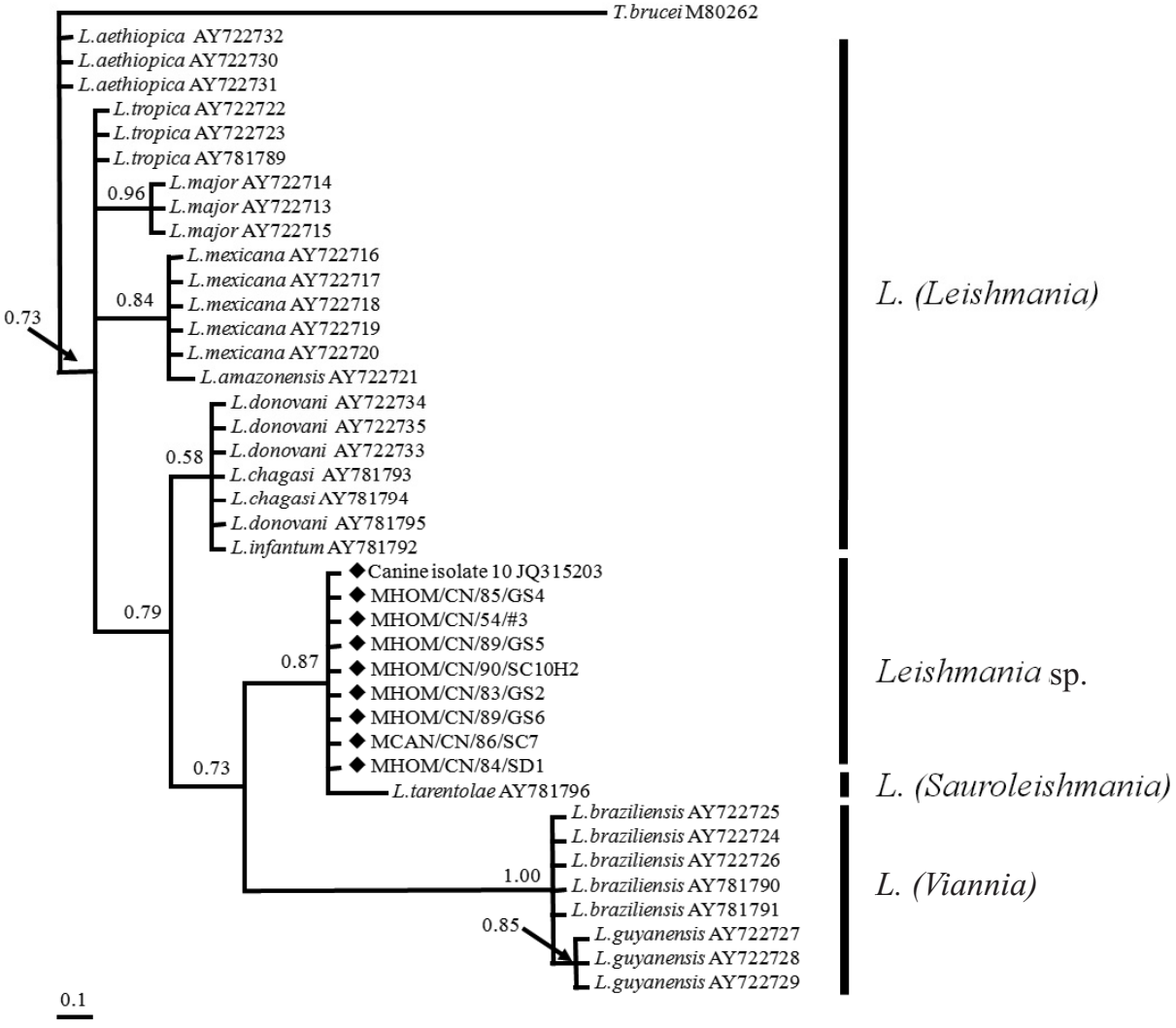


**Fig. 3.** The 50% majority-rule consensus tree inferred from Bayesian inference of 7SL RNA gene dataset by using MrBayes v. 3.1.2. Chinese isolates analyzed in this study were tagged with ◆. Numbers at nodes represent Bayesian posterior probabilities

compositional heterogeneity among sequences ($\chi^2$ = 18.18, df = 120, P = 1.00).

*Phylogenetic analyses*

Prior to the phylogenetic analyses, the most adequate models of nucleotide substitution, TVM+I+G for SSU RNA matrix and GTR+G for 7SL RNA matrix, were selected by Modeltest 3.7. P-distance within each matrix was also calculated by Mega 4.0 (Table III, Table IV).

For the aligned matrix of SSU RNA, the overall mean p-distance was 0.014. In the phylogenic tree inferred from this matrix (Fig. 3), two distance clade was observed, termed Clade A and Clade B. The isolates *L. colombiensis*, *L. equatoriensis*, *L. herreri*, *L. hertigi*, *E. monterogeii* and Trypanosomatidae G755 formed Clade A (PP = 0.56), with the other isolates formed Clade B (PP = 0.53). In Clade A, Chinese isolate IPHL/CN/77/XJ771 and MHOM/CN/93/GS7 clustered with *L. donovani* and *L. infantum* (PP = 1.00), while the isolate MHOM/CN/84/JS1, MRHO/CN/88/KXG-2 and MGER/CN/60/GS-GER20 formed a comb structure in the middle of the dendrogram. The other 11 Chinese *Leishmania* isolates (MHOM/CN/90/WC, MCAN/CN/90/SC11, MHOM/CN/80/XJ801, MHOM/CN/85/GS4, MHOM/CN/84/SD1, MCAN/CN/86/SC7, MHOM/CN/54/#3, MHOM/CN/83/GS2, MHOM/CN/90/SC10H2, MHOM/CN/89/GS6 and MHOM/CN/89/GS5) formed a clade with *L. tarentolae* (PP = 0.88), then clustered as a subclade (PP = 0.99).

For the aligned matrix of 7SL RNA, the overall mean p-distance was 0.110. In the phylogenic tree inferred from this matrix (Fig. 3), all 9 Chinese isolates clustered with *L. tarentolae*, supported by the PP 0.87. As the 9 Chinese isolates shared an identical 7SL RNA sequence to each other, they were grouped as *Leishmania* sp. to calculate p-distances with the other group. The p-distance of *Leishmania* sp. to the other *Leishmania* group ranged from 0.082 (*L. tarentolae*) to 0.165 (*L. braziliensis* complex).

## Discussion

The ribosomal RNA (rRNA) is one of the most conservative genes of eukaryotes, and they are universally distributed and are functionally equivalent in all known organisms (Sogin *et al.* 1986; Johnson and Baverstock 1989; Waters and McCutchan 1990). Meanwhile, the nucleotide sequence of the small subunit ribosomal RNA (srRNA) (16S-18S) is proved valuable for analyzing phylogenetic relationships among distantly related taxa because many regions remain conserved or semiconserved over large periods of time (Baverstock *et al.* 1989). SSUrRNA gene has been widely used to explore phylogenies in the Kinetoplastida (Noyes *et al.* 1997; Hughes and Piontkivska 2003; Stevens *et al*. 2001 ).

Lu *et al*. (1994) proposed that the isolate IPHL/CN/77/XJ771 was designated as *L. infantum* judging from the kinetoplast and nuclear DNA heterogeneity. The *L. infantum* assignment of the isolate XJ771 was challenged by ITS1 and COII data. In previous studies (Yang *et al*. 2010; Cao *et al*. 2011), the isolate IPHL/CN/77/XJ771 was corroborated as *L. donovani* instead of *L. infantum*. As for the isolate MHOM/CN/93/GS7, it was tentatively assigned to *L. donovani* based on the clinical manifestation, geographical and epidemiological criteria (Bu *et al*. 2000; Lu *et al*. 2001; Hu *et al*. 2002). Accordingly, the hypothesis that the isolate MHOM/CN/93/GS7 belongs to *L. donovani* corroborated. In this study, the IPHL/CN/77/XJ771 isolate and the MHOM/CN/93/GS7 isolate cluster with the *L. donovani* and *L. infantum* standard isolates supported by high posterior probability value (PP = 1.00). For the valid species of the genus *Leishmania*, Fraga *et al*. (2010) proposed new opinions based on the heat-shock protein 70 gene. They proposed that *L. donovani* was the sole species of the *L. donovani* complex, with only *L. donovani infantum* as a subspecies. As such, *L. infantum* would cease to exist as a separate species entity.

In the present study, *L. major*, *L. tropica*, *L. aethiopica*, *L. mexicana*, *L. gerbilli*, the isolate MRHO/CN/88/KXG-2, the isolate MGER/CN/60/GS-GER20 and the isolate MHOM/CN/84/JS1 form a comb structure. They cannot be accurately assigned by SSUrRNA gene. As described in Xu *et al*. (1984), the isolate MGER/CN/60/GS-GER20 is close to *L. major* and *L. tropica*. From Yang *et al*. (2010) and Cao *et al*. (2011) inferred that the isolate MGER/CN/60/GS-GER20 is *Leishmania gerbilli*. In this study, GER20 is closely to *Leishmania tropica* and *Leishmania major*. To our surprise, the isolate MHOM/CN/84/JS1, which was tentatively assigned as *L. donovani* on the basis of epidemiology by Lu *et al*. (2001), is neither in the *L. donovani* complex nor cluster with other Chinese *Leishmania* isolates in our study. In SSU rRNA gene analysis, the JS1 isolate is close to the K27 isolate, which may indicate that the genetic distance between them is close. As for the isolate MRHO/CN/88/KXG-2, it was identified as *L. turanica* (causative agent to monkey and man, causing cutaneous leishmaniasis) by MLEE (Guan *et al.* 1995), but clustered with *L. gerbilli* (Yang *et al.* 2010). However, it is close to *L. mexicana* complex and *L. tropica* complex, and cannot be accurately discriminated in this study. Eleven Chinese *Leishmania* isolates (except for the MHOM/CN/93/GS7 isolate, the IPHL/CN/77/XJ771 isolate, the MHOM/CN/84/JS1 isolate, the MGER/CN/60/GS-GER20 isolate and the MRHO/CN/88/KXG-2 isolate) formed a monophyletic group which was different to established *L. donovani* complex (based on phylogenetic topology and average genetic distance (0~0.323, Table III)) and the most relative species to this unclassified group is *L. tarentolae*. It implied that there may be an unclassified group in China, which was consistent with the study of Lu *et al*. (2001), Yang *et al*. (2010) and Cao *et al*. (2011). Based on comb structure, we cannot draw any fine conclusion on the phylogenetic relationships of Chinese *Leishmania* isolates. Combined the previous studies, we tentatively assume: (1)

the isolate JS1 is *L. tropica* instead of *L. donovani*; (2) the isolate MGER/CN/60/GS-GER20 is *L. gerbilli*; (3) *L. mexicana* (HQ 895863) and *L. amazonensis* (HQ 895862) share same haplotype, and they are the member of *L. mexicana* complex; (4) the *L. tarentolae* nested within the genus *Leishmania*.

In this study, *E. monterogeii* and *L. herreri* (U50043), *L. hertigi* (U59492), *L. colombiensis* (AF133836) plus *L. equatorensis* (AF133837) formed a group with high posterior probability value ( PP = 1.00). *E. monterogeii* belongs to the genus *Leishmania*. Our results are consistent with previous studies (Croan and Ellis 1996; Noyes *et al*. 1996, 1997; Croan *et al.* 1997; Fraga *et al*. 2010; Cao *et al.* 2011). Phylogenetic reconstruction studies of the various kinetoplastid lineages (Fernandes *et al*. 1993, Maslov and Simpson 1995) have shown that *Leishmania* and *Endotrypanum* are more closely related organisms to each other than either is to *T. cruzi*. Fernandes *et al*. (1993) proposed that the ancestor of *Leishmania* and *Endotrypanum* was common. *E. monterogeii* is very similar to *L. herreri* and *L. colombiensis* by MLEE and ITSrRNA analysis, while *Endotrypanum* sp. is the closest to *L. hertigi*/*L. deanei* (Noyes *et al.* 1997). As for *L. equatorensis* and *L. colombiensis*, they have been described which are known or suspected to infect human whose taxonomic status is not established (Grimaldi *et al*. 1992).

To further analyze this unclassified group in China, we employed partial 7SL RNA fragment as genetic marker. As described by Zelazny *et al*. (2005), this partial fragment of 7SL RNA allowed the unequivocal identification of *Leishmania* isolates to species or complex level. A total of 8 Chinese isolates which belongs to the unclassified group were used in 7SL RNA sequence analysis. The dendrogram constructed based on 7SL RNA shared a similar topology with the dendrogram constructed by Zelazny *et al*. (2005). To our surprise, the 8 isolates shared an identical 7SL RNA sequence, and formed a clade with *L. tarentolae* (0.87). This was congruent with our previous finding (Yang *et al*. 2010; Cao *et al*. 2011). More interestingly, the 7SL RNA sequence of Canine isolate 10 (JQ315203), which we recently obtained directly from the blood sample of an asymptomatic dog in Sichuan Province (unpublished data), also clustered with *L. tarentolae*. This finding provided indirect evidence that the unclassified *Leishmania* group may exist in China, as *L. tarentolae* was more adapted to promastigote stage and was nonpathogenic to mammals (Raymond *et al*. 2011).

Thus, the taxonomy of the genus *Leishmania* is very complex because species boundaries are difficult to define (Schönian *et al*. 2010). At present, we have applied four molecular markers (ITS1, COII, SSU rRNA and 7SL RNA) to identify Chinese *Leishmania* isolates. SSU rRNA gene phylogenetic analysis suggested that Chinese *Leishmania* isolates do not form a monophyletic group, among which 11 newly determined isolates form a monophyletic group, defined as *Leishmania* sp., while the isolate XJ771 and the isolate GS7 belong to *L. donovani* complex. Bayesian analysis and the genetic distance analysis provide further evidence that Chinese *Leishmania* isolates may be an unclassified pathogenic species epidemic in China, including isolates from hill foci, desert foci and plain foci. However, due to the low posterior probabilities at internal nodes in BI tree, the relationships among *L. tropica*, *L. major*, *L. gerbilli*, *L. mexicana*, *L. brasiliensis*, *L. guyanensis*, *L. aethiopica*, *L. tarentolae*, *L. donovani* complex and *E. monterogeii* were not resolved in this study. The phylogenetic analysis based on 7SL RNA also provided the evidence that an unclassified group may be exist in China. For this unclassified group, we further hypothesized that it may be a hybrid of sexual recombination between *L. tarentolae* and pathogenic species, as the sexual activity of *Leishmania* was demonstrated recently (Akopyants *et al.* 2009). However, more evidence should be sought to verify this hypothesis.

The strains in our analysis suggested that the small subunit rRNA (SSU rRNA) genes was useful for identification and classification of *Leishmania* isolates (Briones *et al*. 1992; Clark *et al*. 1995; Marche *et al*. 1995; Maslov *et al*. 1996; Bu *et al*. 2000; Hu *et al*. 2002). Although the small subunit rRNA (SSU rRNA) genes were too short (amplified in this study) and conservative to draw any conclusion, our results are still basically consistent with previous reports (Yang *et al*. 2010; Cao *et al*. 2011). It is not revealed accurately the status of Chinese *Leishmania* isolates in the phylogenetic tree employing this molecular marker. As SSU rRNA is quite conserved in nature, it cannot be expected to uncover the fine relationships among Chinese *Leishmania* isolates. It may be not a reliable marker for inferring deep level Chinese *Leishmania* isolates phylogeny. 7SL RNA may be a better marker than SSU rRNA, as it could clearly discriminate *Leishmania* isolates at species or complex level. However, accurate status of Chinese *Leishmania* isolates was not clearly revealed, as the 7SL RNA sequences of *Leishmania* were rare at present, especially *L. tarentolae*. Besides, some problem within the genus *Leishmania* remained unresolved based on 7SL RNA analysis. For example, the relationship between *L. donovani* and *L. infantum* was still unclear. Furthermore, species status should not be determined by just one gene, as various processes may lead to incongruence between the species tree and a gene tree (Snyder and Maddison 1997; Slowinski and Page 1999).

In conclusion, the results of this study enrich our understanding of the heterogeneity and relationships of Chinese *Leishmania* isolates. For better analysis of the phylogenetic relationships of Chinese *Leishmania* isolates, more molecular markers and more isolates from different epidemic foci of China should be employed to clarify the evolutionary history. The incongruences among different studies suggested that the isolates from different foci in China may have a more complex evolutionary history than previously assumed. More genes should be employed, alone or combined, to help us understand the classification and relationships among Chineses *Leishmania* isolates.

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