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Phylogenetic analysis of HSP70 and cyt *b* gene sequences for Chinese *Leishmania* isolates and ultrastructural characteristics of Chinese *Leishmania* sp.

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Abstract Leishmaniasis is a worldwide epidemic disease caused by the genus *Leishmania*, which is still endemic in the west and northwest areas of China. Some viewpoints of the traditional taxonomy of Chinese *Leishmania* have been challenged by recent phylogenetic researches based on different molecular markers. However, the taxonomic positions and phylogenetic relationships of Chinese *Leishmania* isolates remain controversial, which need for more data and further analysis. In this study, the heat shock protein 70 (HSP70) gene and cytochrome *b* (cyt *b*) gene were used for phylogenetic analysis of Chinese *Leishmania* isolates from patients, dogs, gerbils, and sand flies in different geographic origins. Besides, for the interesting *Leishmania* sp. in China, the ultrastructure of three Chinese *Leishmania* sp. strains (MHOM/CN/90/SC10H2, SD, GL) were observed by transmission electron microscopy. Bayesian trees from HSP70 and cyt *b* congruently indicated that the 14 Chinese *Leishmania* isolates belong to three *Leishmania* species including *L. donovani* complex, *L. gerbilli*, and *L.* (*Sauroleishmania*) sp. Their identity further confirmed that the undescribed *Leishmania* species causing visceral Leishmaniasis (VL) in China is closely related to *L. tarentolae*. The phylogenetic results from HSP70 also suggested the classification of subspecies within *L. donovani* complex: KXG-918, KXG-927, KXG-Liu, KXG-Xu, 9044,

SC6, and KXG-65 belong to *L. donovani*; Cy, WenChuan, and 801 were proposed to be *L. infantum*. Through transmission electron microscopy, unexpectedly, the Golgi apparatus were not observed in SC10H2, SD, and GL, which was similar to previous reports of reptilian *Leishmania*. The statistical analysis of microtubule counts separated SC10H2, SD, and GL as one group from any other reference strain (*L. donovani* MHOM/IN/80/DD8; *L. tropica* MHOM/SU/74/K27; *L. gerbilli* MRHO/CN/60/GERBILLI). The ultrastructural characteristics of *Leishmania* sp. partly lend support to the phylogenetic inference that Chinese *Leishmania* sp. is in close relationship with reptilian *Leishmania*.

Keywords *Leishmania* · HSP70 · Cyt *b* · Ultrastructure · China

Introduction

Leishmaniases, one of the world's most neglected diseases, are caused by obligate protozoan parasite from genus *Leishmania* and transmitted via sandfly. More than 98 countries worldwide are currently threatened by leishmaniasis (WHO 2010). The clinical syndromes of this disease in human range from cutaneous Leishmaniasis (CL, most common form) to acute visceral Leishmaniasis (VL, kala-azar). These different clinical syndromes usually correlate with different pathogenic species. Thus, accurate species discrimination is crucially important for prognosis and treatment choice of the disease. It is now commonly accepted that genus *Leishmania* comprises three subgenera, i.e., *L.* (*Leishmania*) and *L.* (*Viannia*), and *L.* (*Sauroleishmania*) (Schönian et al. 2010). Thereinto, 31 *Leishmania* species are known to be parasites of mammals and 20 species are pathogenic for human beings (Akhoundi et al. 2016). *Sauroleishmania* was proposed as a subgenus for

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Leishmania that infects lizards (Saf'janova 1982), which was supported by researches on biological criteria and analyses of different *Leishmania* gene markers (Croan et al. 1997; Fraga et al. 2010; Noyes et al. 1997; Orlando et al. 2002). While there are still a few species within the subgenus *Sauroleishmania* without specific taxonomic positions.

In China, leishmaniasis is mainly endemic in the western and northwestern regions, such as Xinjiang Uygur Autonomous Region (Xinjiang province), Gansu province, and Sichuan province (Zheng et al. 2009). Previous researchers divided the epidemic focus of leishmaniasis in China into plain foci, hill foci, and desert foci according to different geographical origins, causative agents, and clinical manifestations (Lu et al. 1994). As a large country with a vast population and complex ecological environment, China gains an intricate taxonomy and evolution of *Leishmania*. By analyzing kDNA or nDNA, Hu et al. (1992) and Lu et al. (2001) suggested that the differences at genetic level did exist in *Leishmania* isolates from different areas in China. Then, our recent studies further revealed the heterogeneity of Chinese *Leishmania* isolates since several species, including *L. donovani* complex, *L. gerbilli*, *L. tropica*, and *L. turanica*, have been identified (Cao et al. 2011; Yang et al. 2010, 2013). Moreover, a novel and undescribed *Leishmania* species may exist in China (Cao et al. 2011; Guan et al. 2012; Sun et al. 2012). Nevertheless, the phylogenetic relationships and taxonomy of Chinese *Leishmania* have not been completely clarified, especially identification within species complex. In Xinjiang, although *L. donovani* and *L. infantum* have been confirmed as the causative agents of VL (Wang et al. 2010a), their distribution is still not clear. Considering that VL is the main form endemic in China, accurate identification of the causative agent is essential to effective control of the disease. Therefore, to get a more reliable phylogenetic inference of Chinese *Leishmania*, further studies are still necessary.

In order to discriminate *Leishnamia* species conveniently and accurately, various genetic markers have been developed, such as ribosomal DNA (rDNA) genes, kinetoplast DNA (kDNA) genes, and nuclear genes (Dávila and Momen 2000; Mahboudi et al. 2001; Victoir et al. 1998). In our previous studies, traditional viewpoints on taxonomy and cladistics hypotheses of Chinese *Leishmania* isolates have been challenged based on different molecular markers (Cao et al. 2011; Guan et al. 2012; Yang et al. 2010). More effective markers are still needed to clarify the species status and phylogeny of *Leishmania* in China. HSP70 gene, a *Leishmania* antigen gene on chromosome, is considered the optimal marker for species delimitation and phylogenetic inference of *Leishmania*, which showed a nearly perfect congruence with multilocus enzyme electrophoresis (MLEE) typing (widely accepted gold standard in *Leishmania* typing) (Fraga et al. 2010; Van der Auwera and Dujardin 2015). Cyt *b* gene, which is encoded on the kDNA maxicircle, is another widely used marker that has successfully

separated most tested species (Asato et al. 2009; Luyo-Acero et al. 2004). Considering different selection pressure and evolutionary rate on different genes, analyses combining gene markers from nucleus with kinetoplast would be reliable for phylogeny. The recent study (Zhang et al. 2013a) has constructed phylogenetic trees based on HSP70 and *lack* gene of some Chinese *Leishmania* strains; unfortunately, the analysis lacked the comparison with the representative *Leishmania* sequences from other regions of the world and the controversial Chinese *Leishmania* sp. was absent. Additionally, some researches have confirmed that more isolates and data would affect the phylogenetic results (Botilde et al. 2006; Kuwahara et al. 2009). Thus, more isolates from diverse regions would add to our understanding of species identification and phylogenetic relationships of Chinese *Leishmania* isolates.

Studies on morphology of *Leishmania* have been carried out extensively in the middle of twentieth century and was overlooked gradually with the booming up of molecular biology. Since all amastigotes and promastigotes look very alike by optical microscope (Bray 1974), results from electron microscopy seem to be more significant. Pioneering scientists did a lot of research on the ultrastructure of *Leishmania* and found that the differences on the number of subpellicular microtubules may be of value to differential taxonomy (Garnham 1962; Garnham 1971; Lewis 1975; Sanyal and Sen Gupta 1967). Since the Chinese *Leishmania* sp. without ascertainable taxonomic status, it is necessary to collect more comprehensive information that related to classification, including ultrastructural characteristics.

In this study, to further evaluate the phylogenetic relationships and obtain reliable species identification of Chinese *Leishmania*, 14 cyt *b* sequences and 10 HSP70 sequences of *Leishmania* isolates were obtained for phylogenetic analyses in conjunction with some representative *Leishmania* sequences retrieved from GenBank. In addition, for the interesting *Leishmania* sp. isolates in China, we further characterized the ultrastructure of three Chinese *Leishmania* sp. strains (MHOM/CN/90/SC10H2; SD; GL) by transmission electron microscopy in order to obtain additional information to help us to understand the novel species.

Materials and methods

Parasites and cultures

Fourteen Chinese *Leishmania* isolates and three WHO reference strains were used in this study and listed in Table 1. The promastigotes were cultivated in medium 199 supplemented with 15% heat-inactivated fetal bovine serum (HIFBS). For axenic cultivation, 100 U/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma) were added in and were put at 25 °C, pH 5.8.

Table 1 Details of Chinese strains and the WHO reference strains which were stored in our lab and sequenced in this study

Strain name	Species	Origin	Host	GenBank accession number	
				HSP70	cyt b
IMJW/CN/91/KXG-918	L. donovani	Karamay, Xinjiang, China	Phlebotomus major wui	KX061893	KX061904
IMJW/CN/87/KXG-65	L. donovani	Karamay, Xinjiang, China	Phlebotomus major wui	JX021437	KX061905
MHOM/CN/93/KXG-XU	L. infantum[a]	Karamay, Xinjiang, China	Homo sapiens	JX021433	KX061906
MHOM/CN/90/9044	L. donovani	Shandong, China	Homo sapiens	JX021428	KX061907
MHOM/CN/94/KXG-LIU	L. infantum[a]	Karamay, Xinjiang, China	Homo sapiens	JX021432	KX061908
MHOM/CN/90/SC10H2	Leishmania sp.	Sichuan, China	Homo sapiens	KX061896	KX061910
MCAN/CN/08/Cy	L. infantum	Wudu, Gansu, China	Canis lupus familiaris	KX061897	KX061911
MCAN/CN/90/WenChuan	L. infantum	Wenchuan, Sichuan, China	Canis lupus familiaris	KX061898	KX061912
MRHO/CN/81/EJNI-154	L. gerbilli	Ejina, Inner Mongolia, China	Rhombomys opimus	JX021435	KX061913
IMJW/CN/92/KXG-927	L. donovani	Karamay, Xinjiang, China	Phlebotomus major wui	KX061902	KX061915
MHOM/CN/86/SC6	L. donovani	Nanping, Sichuan, China	Homo sapiens	JX021429	KX061916
MHOM/CN/80/801	L. donovani[a]	Kashgar, Xinjiang, China	Homo sapiens	JX970993	KX061917
SD	Leishmania sp.	Shandong, China	Homo sapiens	KX061900	
GL	Leishmania sp.	Gansu, China	Homo sapiens	KX061895	KX061909
MRHO/CN/60/GERBILLI	L. gerbilli	Gansu, China	Rhombomys opimus	KX061901	KX061914
MHOM/IN/80/DD8	L. donovani	India		KX061894	EF579896
MHOM/SU/74/K27	L. tropica			KX061899	HQ908270

GenBank accession number starting with KX were uploaded in this study

[a] The species identification is disputed based on different technique and different gene markers applied

DNA extraction, amplification, and sequencing protocols

The promastigotes in logarithmic phase (approximately 1–5 × 10^9/mL) were harvested by centrifuging at 3300×g for 10 min at 4 °C and washed with PBS for three times. Genomic DNA was extracted using a standard sodium dodecyl sulfate-proteinase K procedure (Sambrook and Russell 2001). The cyt b fragment was amplified using primers LCBF1 and LCBR2 (Luyo-Acero et al. 2004). The PCR procedure were described as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1.5 min, and a final extension at 72 °C for 10 min. The primers HSP70sen and HSP70ant (Garcia et al. 2004) were used to amplify HSP70 segments. For HSP70, the PCR conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 60 s, 60 °C for 60 s, 72 °C for 1.5 min, with a final extension at 72 °C for 10 min. The PCR products were checked using 1.5% agarose gel electrophoresis and purified using a DNA purification kit (TIANGEN, Beijing, China) per the protocol of the manufacturer. Purified products were then sent to Tsingke Biological Technology Co., Ltd. (Chengdu, China) for gene sequencing using the same PCR primers on ABI 3730 automated sequencer (Applied Biosystems, Inc.). Obtained gene sequences were finally uploaded to GenBank (accession numbers are listed in Table 1).

Sequence alignment and phylogenetic analyses

A group of cyt b sequences were retrieved from GenBank, including 30 sequences of genus *Leishmania* and 1 sequence of *Trypanosoma brucei* (Tables 1 and 2). For HSP70, a total of 47 sequences of *Leishmania* with 1 sequence of *Trypanosoma cruzi* were retrieved from GenBank (Tables 1 and 2). The sequences were multiple-aligned using Clustal X v.1.83 (Thompson et al. 1997) with its default option. To generate a consensus tree, the base composition heterogeneity was evaluated using chi-squared (χ^2) tests executed in PAUP* 4.0b10 (Swofford 2002). Then, the phylogenetic trees reconstruction of *Leishmania* were implemented applying Bayesian inference (BI) with the MrBayes v.3.2 program (Ronquist and Huelsenbeck 2003). Gaps were treated as missing data. *Trypanosoma cruzi* and *Trypanosoma brucei* were used to root the trees. The most appropriate model (cyt b: GTR + I + G; HSP70: TrN + I + G) for Bayesian analyses was selected by combined utilization of the program PAUP and Modeltest 3.7 (Posada and Crandall 1998). Four Markov chains were proceeded for four million generations, and trees were sampled for every 200 generations. The first one million sample trees were discarded, and the remaining trees were used for construction of 50% majority-rule consensus tree and calculation of posterior probabilities of clades. The results of Bayesian analyses were visualized using Treeview v1.6.6 (Page 1996).

Table 2 The strains whose sequences were retrieved from GenBank

Strain name	species	Accession number	Strain name	species	Accession number
(A). cyt *b*					
MHOM/SD/62/2525M-C2-2M	*L. donovani*	AB095957	LEM695	*L. mexicana*	EF579906
MHOM/TN/80/IPT1	*L. infantum*	AB095958		*L. tarentolae*	M10126
MHOM/EC/88/INH-03	*L. braziliensis*	AB095967	MGER/CN/60/GS-GER20	*L. gerbilli*	HQ908259
MHOM/BR/74/PP75	*L. chagasi*	AB095959	MHOM/CN/93/GS7	*L. infantum*	HQ908261
MHOM/BR/71/LS94	*L. panamensis*	AB095968	MCAN/CN/60/GS1	*L. donovani*	HQ908262
MHOM/BR/79/M15065	*L. shawi*	AB433281	MRHO/CN/88/KXG-2	*L. turanica*	HQ908256
MPSA/SA/83/JISH220	*L. arabica*	AB434674	MHOM/CN/84/JS1	*L. tropica*	HQ908265
MRHO/SU/80/CLONE3720	*L. turanica*	AB434675	IPHL/CN/77/XJ771	*L. donovani*	HQ908267
MHOM/BR/00/LTB300	*L. braziliensis*	AB434681	MHOM/CN/89/GS6	*Leishmania* sp.	HQ908263
MHOM/SU/73/5ASKH	*L. major*	EF579914	MHOM/CN/84/GS3	*Leishmania* sp.	HQ908271
LEM235	*L. infantum*	EF579895	MHOM/CN/89/GS5	*Leishmania* sp.	HQ908269
LEM144	*L. aethiopica*	EF579899	MHOM/CN/83/GS2	*Leishmania* sp.	HQ908273
LEM391	*L. garnhami*	EF579903	MHOM/CN/84/SD1	*Leishmania* sp.	HQ908255
LEM690	*L. amazonesis*	EF579902	EATRO 427	*Trypanosoma. brucei*	M94286
LEM85	*L. guyanensis*	EF579905			
(B). HSP70					
TarII	*L. tarentolae*	AY423868	MHOM/PT/94/IMT202	*L. infantum*	HF586408
MHOM/PE/90/LCA08CL2	*L. peruviana*	EU599089	MHOM/PE/02/LH2312	*L. mexicana*	FN395038
MHOM/BR/73/M2269	*L. amazonensis*	EU599090	MHOM/BR/06/ICA	*L. braziliensis*	FN395043
MNYC/BZ/62/M379	*L. mexicana*	EU599091	MHOM/PE/02/LH2344	*L. lainsoni*	FN395049
MHOM/BR/75/M4177	*L. guyanensis*	EU599093	MDSA/BR/78/M5210	*L. naiffi*	FN395056
MHOM/ET/89/GERE	*L. aethiopica*	FN395018	MCAN/IL/97/LRC-L720	*L. infantum*	HF586393
MHOM/ET/72/L100	*L. aethiopica*	FN395021	TRE/CN/80/LIZRD	*L. donovani**	JX021434
MHOM/SD/2003/LCB33	*L. major*	HF586346	IAND/CN/90/KXG-R	*L. turanica*	JX021438
MHOM/AF/--/AMC153	*L. major*	HF586391	MCAN/CN/11/1101	*L. donovani* *	JX312705
MRHO/SU/95/T-9551R	*L. turanica*	HF586356	MCAN/CN/11/1102	*L. donovani* *	JX312712
MRHO/CN/90/KXG-57	*L. turanica*	JX021441	MCAN/CN/97/WDD23	*L. donovani* *	JX970994
MHOM/IN/79/DD7	*L. tropica*	FN395025	MHOM/CN/96/KS6	*L. donovani* *	JX970996
MHOM/KE/81/NLB_030B	*L. tropica*	FN395026	MHOM/CN/88/8801	*L. donovani* *	JX970997
MHOM/SD/68/1S	*L. donovani*	FN395027	IALE/CN/83/SELF-7	*L. gerbilli*	JX021431
MHOM/IN/00/Devi	*L. donovani*	FN395028	IWUI/CN/77/771	*L. donovani*	JX021425
MHOM/SD/82/Gilani	*L. donovani*	FN395029	MHOM/CN/83/GS2	*Leishmania* sp.	KJ667093
MHOM/SD/97/LEM3463	*L. archibaldi*	FN395030	IARP/CN/90/KXG-E	*Leishmania* sp.	JX021439
MHOM/MT/85/Buck	*L. infantum*	FN395031	MHOM/CN/85/GS4	*Leishmania* sp.	KJ667088
MHOM/PT/00/IMT260	*L. infantum*	FN395032	MHOM/CN/89/GS5	*Leishmania* sp.	KJ667090
MCHO/PA/00/M4039	*L. panamensis*	FN395055	MK-2015	*Leishmania* sp.	KR902347
			CL Brener	*Trypanosoma cruzi*	XM812645

*: the species identification is disputed based on different gene markers applied

Transmission electron microscopy

Besides the three undescribed *Leishmania* sp. isolates MHOM/CN/90/SC10H2, SD, and GL, three WHO reference strains (*L. donovani* MHOM/IN/80/DD8; *L. tropica* MHOM/SU/74/K27; *L. gerbilli* MRHO/CN/60/GERBILLI) were chosen for comparison of ultrastructural characteristics. The promastigotes in logarithmic phase, washed with PBS,

was prefixed in 3% glutaraldehyde solution. Then, the promastigotes was postfixed in 1% osmium tetroxide (OsO$_4$), dehydrated in acetone of gradient concentration, and embedded in epoxy resin. The ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (TEM; HITACHI, H-600IV, Japan). Electron micrograph were taken of clearly visible cells. The longitudinal sections were used for

ultrastructural observation. The transverse sections of each of six specimens were selected for counting the numbers of subpellicular microtubules.

Statistical analysis

The microtubule counts were analyzed by IBM SPSS Statistics 20.0 (IBM Corp., USA) using one-way analysis of variance, followed by Scheffe post hoc comparison. Any P value of less than or equal to 0.05 was regarded as statistical significance. All tables and pictures were created by using Microsoft Office 2013 (Microsoft Corp., USA).

Results

Sequence alignment and analyses

There were 14 cyt b gene fragments and 10 HSP70 gene fragments obtained through PCR. The GenBank accession numbers of these newly obtained sequences of Chinese isolates are listed in Table 1, of which KX061904-KX061917 were for cyt b and KX061893-KX061902 were for HSP70.

A total of 773 aligned sites in the matrix of cyt b, 324 were variable sites, with 193 parsimony-informative. Percentage of four DNA bases were as follows: A, 26.8; C, 7.8; G, 15.6; T, 49.8. The cyt b gene fragment of *Leishmania* is AT rich (76.6%). The heterogeneity test showed that there were no significant differences in base composition bias among cyt b data ($\chi^2 = 66.42$, $df = 150$, $P = 1.00$). For HSP70, 235 of the 1262 aligned characters were variable, including 116 that were parsimony-informative. Percentage of four DNA bases were as follows: A, 21.5; C, 30.4; G, 34.7; T, 13.5. The HSP70 gene fragment of *Leishmania* is CG rich (65.0%). The heterogeneity test of HSP70 data also showed that there were no significant differences in base composition bias ($\chi^2 = 15.83$, $df = 195$, $P = 1.00$).

Phylogenetic analyses

In the BI tree inferred from the aligned matrix of cyt b (Fig. 1), three clades were recovered with high support (PP = 0.97, 1.0, and 1.0, respectively), corresponding to the three subgenera *Vanina*, *Leishmania*, and *Sauroleishmania*. Chinese *Leishmania* isolates in this study were distributed in three subclades, i.e., *L. donovani* complex, *L. major* complex, and *Leishmania* sp. Ten isolates (KXG-918, KXG-927, KXG-Liu, KXG-Xu, 9044, SC6, KXG-65, 801, WenChuan, Cy) from different foci of China were clustered with five reference sequences of *L. donovani*/*L. infantum* and other three sequences from China (Yang et al. 2013), then together formed the monophyletic clade of *Leishmania donovani* complex (PP = 1.00). In this clade, however, *L. donovani* and

L. infantum were not reciprocally monophyletic groups. The strain Ejni-154 isolated from *Rhombomys opimus* in Inner Mongolia was clustered with the WHO *L. gerbilli* reference strain MRHO/CN/60/Gerbilli (PP = 1.00) and joined by some reference sequences of *Leishmania major* and *Leishmania turanica*, which formed a subclade *L. major* complex (PP = 1.0). The isolates GL and SC10H2, from Gansu and Sichuan respectively, were clustered with the other five Chinese *Leishmania* isolates which termed *Leishmania* sp. before (Yang et al. 2013) and formed a strong clade with one sequence of *L. tarentolae* (PP = 1.00).

The BI tree based on the aligned matrix of HSP70 (Fig. 2) showed that three subgenera *Vanina*, *Leishmania*, and *Sauroleishmania* were clearly divided and eight Chinese isolates in this study were distributed in three clades: *L. donovani* complex, *L. gerbilli*, and *Leishmania* sp. As well as cyt b, *L. donovani* and *L. infantum* were not reciprocally monophyletic groups. Nevertheless, the *L. infantum* strains were found as a separate subgroup within *L. donovani* complex in the BI tree (PP = 0.9). Two strains KXG-918 and KXG-927, which were isolated from *Phlebotomus major wui* in Xinjiang, were clustered with the WHO reference strain DD8 and other *L. donovani* strains. The other two strains Cy and WenChuan, which were isolated from canine in Sichuan and Gansu of China respectively, were clustered with other Chinese *Leishmania* isolates and FN395031, FN395032, HF586393, and HF586408 identified as *L. infantum* before (Fraga et al. 2010; Van der Auwera et al. 2013). The isolates SC10H2, SD, and GL were still clustered with other strains of *Leishmania* sp. and lizard *Leishmania* and then formed a clade with nonpathogenic *L. tarentolae* (PP = 1) instead of any other human pathogenic *Leishmania* species.

Ultrastructure characterization under transmission electron microscope

Through transmission electron microscopy, the promastigotes of three *Leishmania* sp. isolates showed roughly identical intracellular structures with other three reference strains, including a centrally located nucleus, a rod-like kinetoplast, branched mitochondrial system, and anterior flagellum. Unexpectedly, the Golgi apparatus has not been observed in the three isolates SC10H2, SD, and GL (Fig. 3b showed the Golgi absence in SC10H2). While, in the other reference strains (*L. donovani* MHOM/IN/80/DD8; *L. tropica* MHOM/SU/74/K27; *L. gerbilli* MRHO/CN/60/GERBILLI), the Golgi was well developed. The extensive membrane laminations and unequal-sized vesicles located near the kinetoplast could be observed (Fig. 3a showed the Golgi in DD8).

The plasma membrane of promastigote is double layered, and lying immediately beneath it are longitudinally arranged subpellicular microtubules. Table 3 shows the counting results of microtubules of the six strains. Then, the results were

Fig. 1 The 50% majority-rule consensus tree inferred from Bayesian inference of cyt *b* dataset using MrBayes v. 3.2. The *numbers at the nodes* represent Bayesian posterior probabilities; *brackets* indicate *Leishmania* species, complexes, and subgenera with reference to descriptions based on multilocus enzyme electrophoresis (Lainson et al. 1987; Rioux et al. 1990; Cupolillo et al. 1994; Cupolillo et al. 2000); *red-marked*: strains that genes were sequenced in this study; the strains that from China were tagged with *asterisk* (color figure online)
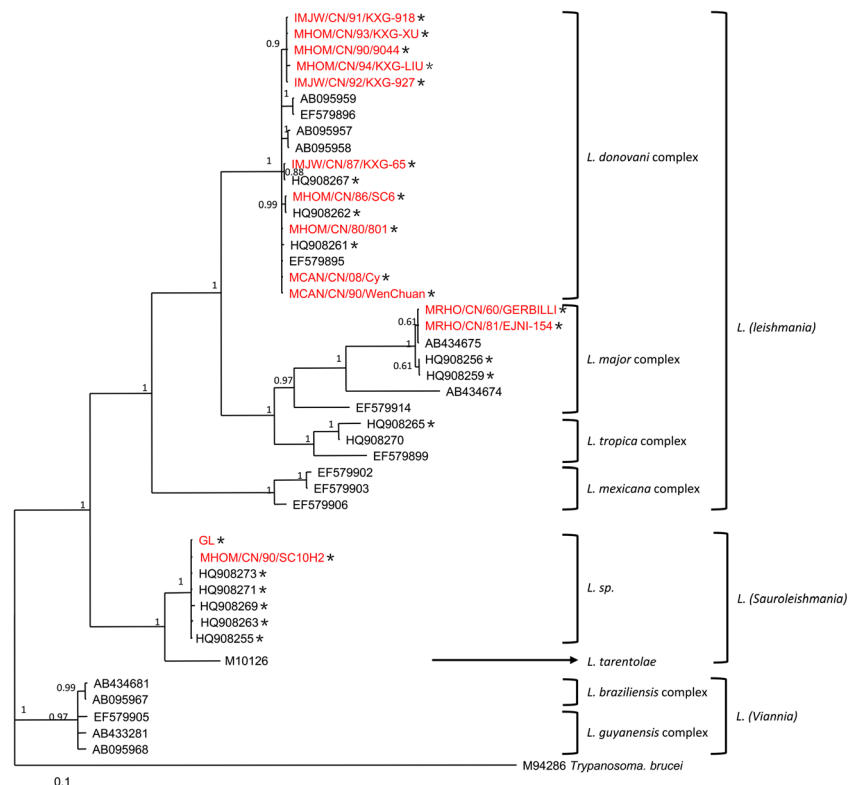
analyzed using one-way analysis of variance, and the calculated *F* value is 405.186, which suggested significant differences in the mean number of microtubules among the six strains ($P < 0.01$). Followed multiple comparisons test set SC10H2, SD and GL as one group ($P > 0.05$) which apart from DD8 ($P < 0.01$), K27 ($P < 0.01$) and GERBILLI ($P < 0.01$) respectively.

Discussion

With reconstruction of phylogenetic relationships of Chinese *Leishmania* based on HSP70 and cyt *b* gene sequences, the species identification of Chinese isolates were analyzed, compared, and correlated with their geographical origins. The ultrastructure of the interesting Chinese *Leishmania* sp. isolates were also observed to further understand the novel species. This study brings further insight into the taxonomic positions and phylogenetic relationships of Chinese *Leishmania*.

Using the cyt *b* gene, most of the strains analyzed in this study (KXG-918, KXG-927, KXG-Liu, KXG-Xu, 9044, SC6, KXG-65, 801, WenChuan, Cy), which were collected from the plain, hill, and desert foci of China, were clustered with other strains of *L. donovani* complex (Fig. 1). However, *L. infantum* cannot be discriminated from *L. donovani* according to cyt *b* data. While HSP70 gene is more powerful to identify *Leishmania* at the species, intra- and supra-species level (Fraga et al. 2010), which was confirmed in this study.

As can be seen in Fig. 2, the canine isolates Cy and WenChuan, along with other six Chinese isolates, 801, 8801, KS6, 1101, 1102, and WDD23 (accession numbers JX970993, JX970997, JX970996, JX312705, and JX312712), were clustered with other identified *L. infantum* strains from Europe (Fraga et al. 2010; Van der Auwera et al. 2013) and formed a subclade within the *L. donovani* complex. Although mentioned six Chinese isolates were classified as *L. donovani* according to *lack* gene analysis (Zhang et al. 2013b), the phylogenetic tree based on *lack* gene have not reflected a clear classification of subspecies within *L. donovani* complex. Therefore, the two canine isolates and the other six Chinese isolates are suggested to be *L. infantum* in this study. Since the canine isolate WenChuan was from Sichuan, this result indicated that the canine leishmaniasis (CanL) in Sichuan may be caused by *L. infantum*, which is consistent with the recent studies (Shang et al. 2011; Wang et al. 2011). Moreover, considering the infected dogs are the main reservoir host of human VL, *L. infantum* should be the causative agents of VL in Sichuan province. Likewise, Cy, 1101, 1102, and WDD23 were isolated from domestic dogs in Gansu, and 8801 from VL patients in Gansu as well. Thus, we inferred that *L. infantum* might be the pathogen that cause the VL in Gansu, and dogs are likely the principal source of infection to human. These inference was supported by the research of Zhang and Zhang (2010). While, about the identification of isolate 801, which are from VL patients in Kashi of Xinjiang, it remains controversial. That 801 are suggested

Fig. 2 The 50% majority-rule consensus tree inferred from Bayesian inference of HSP70 dataset using MrBayes v. 3.2. The *numbers at the nodes* represent Bayesian posterior probabilities; *brackets* indicate *Leishmania* species, complexes, and subgenera with reference to descriptions based on multilocus enzyme electrophoresis (Lainson et al. 1987; Rioux et al. 1990; Cupolillo et al. 1994; Cupolillo et al. 2000); *red-marked*: strains that genes were sequenced in this study; the strains that from China were tagged with *asterisk* (color figure online)
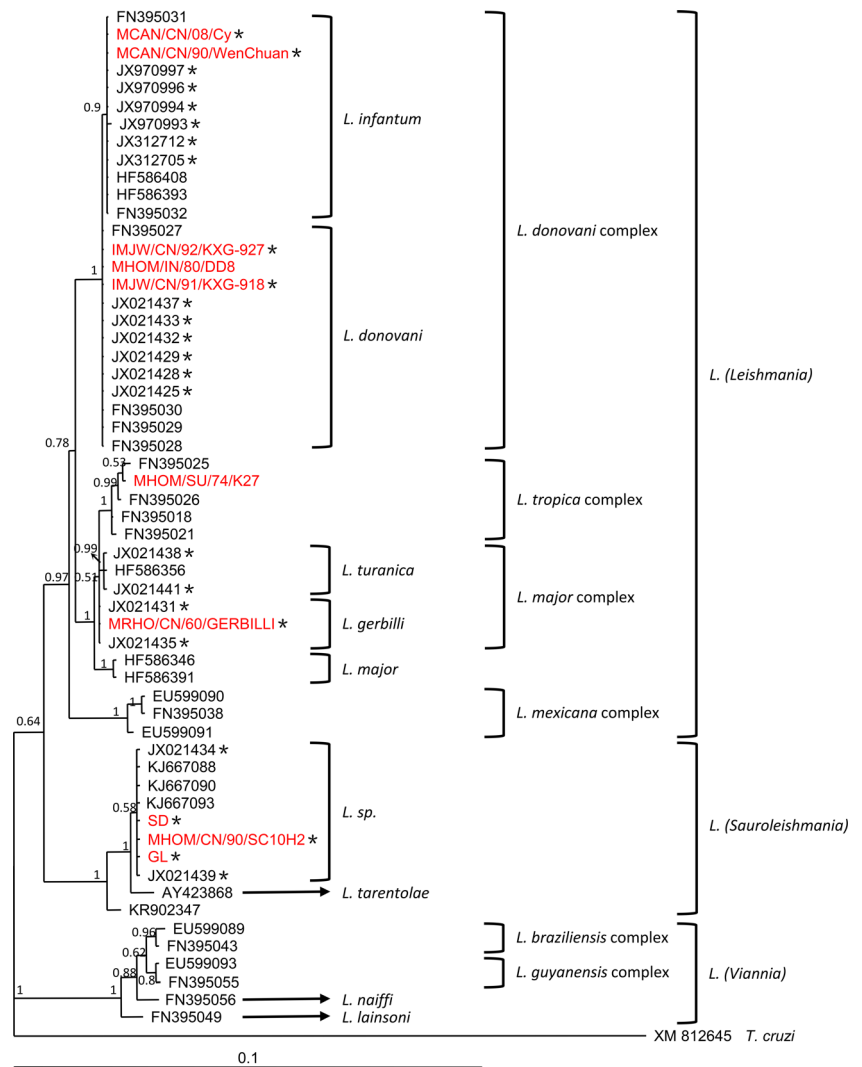

Fig. 3 Electron micrograph of *Leishmania* strains promastigote: presence/absence of Golgi apparatus. **a** DD8; **b** SC10H2. *K* kinetoplast, *Mi* mitochondrion, *G* Golgi apparatus, *N* nucleus
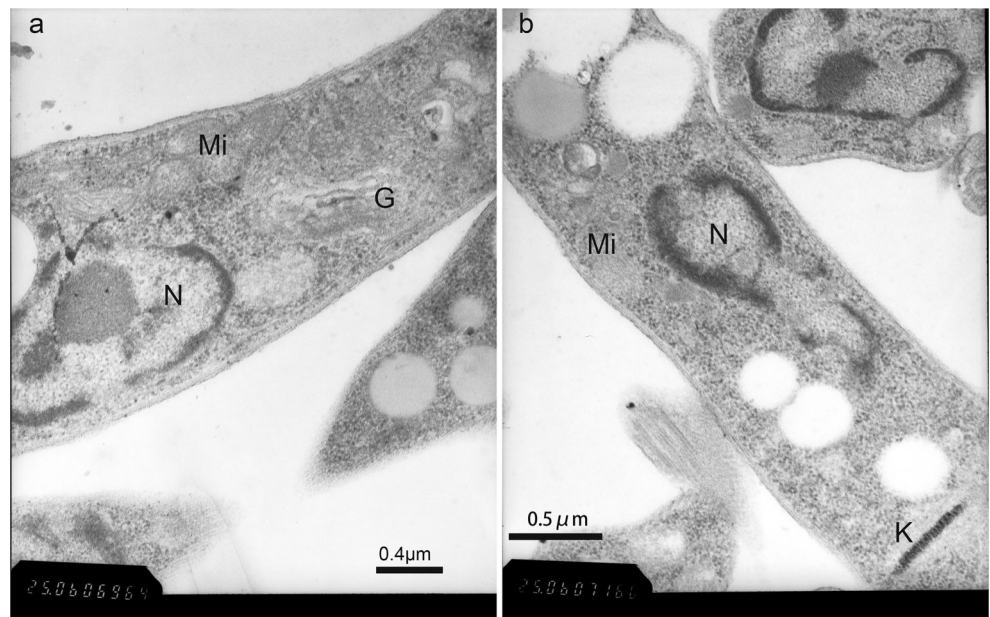
Table 3 Microtubules counting of promastigotes of *Leishmania* species

Strain	Mean no. of microtubules
DD8	89
K27	80
GERBILLI	72
SC10H2	105
SD	103
GL	106

to be *L. infantum* based on HSP70 analysis in this study is in accord with multilocus microsatellite typing (MLMT) investigation (Alam et al. 2014), but differs from the previous study of ITS1 sequences (Wang et al. 2010a; Yang et al. 2010) which considered that the isolate 801 belong to *L. donovani*. This divergence may be due to the short history that *L. infantum* has differentiated from *L. donovani* and/or the different efficacy of the two gene markers on phylogenetic analysis. It now appears that *L. infantum*, which is one of the causative agents of VL, is primarily distributed in western mountainous areas and plain of northwestern China, including Sichuan, Gansu, and Xinjiang provinces. This point is shared by the previous report (Lun et al. 2015).

In this study, two other strains, KXG-918 and KXG-927, which were isolated from *Phlebotomus major wui* in Karamay, Xinjiang, along with the strain KXG-65(accession number JX021437) also isolated from *Phlebotomus major wui* in the same area, were clustered with the reference strain DD8 and identified as *L. donovani*. Meanwhile, we found that the strains KXG-XU and KXG-LIU (HSP70 gene sequence JX021433 and JX021432), isolated from CL patients in Karamay, Xinjiang, also belong to *L. donovani* according to HSP70 BI tree, which was in accord with the study based on ISSR-PCR by Wang et al. (2010b). Accordingly, since the five strains KXG-918, KXG-927, KXG-65, KXG-XU, and KXG-LIU were all isolated in Karamay, Xinjiang, and grouped together based on HSP70 data, it can be inferred that *L. donovani* is the pathogen of CL in Karamay and *Phlebotomus major wui* is the vector. Whereas this phylogenetic inference challenged the previous determination that the *L. infantum* is the pathogen of CL in Karamay based on gene hybridization and animal inoculation (Guan et al. 1994; Wang et al. 1996; Yang et al. 1997). Generally, *L. donovani* cause VL and there have been few reports of CL caused by *L. donovani* except in Sri Lanka, where CL by *L. donovani* has been prevalent recently (Sanayaka et al. 2014). In China, cutaneous leishmaniasis has not been reported except in the single isolated endemic site, i.e., Karamay in Xinjiang. Given that Xinjiang is the major epidemic region and has an intricate prevalence of leishmaniasis, to be clear about the types of endemic *Leishmania*, more isolates would be needed, especially from Kashi and Karamay.

Using transmission electron microscope, there were no significant differences in the basic ultrastructure of the six strains,

but a clear Golgi apparatus had not yet been observed in the three isolates SC10H2, SD, and GL. Few studies have reported the *Leishmania* species which lack of Golgi apparatus before. Nevertheless, according to the ultrastructural study on *Leishmania* promastigotes from reptiles by Lewis (1975), the Golgi apparatus in *L. adleri* and *L. agamae* were not well developed and had not been found in *L. hoogstraali*. The three *Leishmania* strains, *L. adleri*, *L. agamae*, and *L. hoogstraali*, all belong to *L. (Sauroleishmania)* (Akhoundi et al. 2016). Kazemi et al. (2008) compared ultrastructure of promastigotes of lizard *Leishmania* and *Leishmania major* and found that there was no Golgi apparatus for lizard *Leishmania* promastigotes. Lewis also considered the true biologic dissimilarities must be reflected by the differences of development of the Golgi apparatus among strains, since his previous study suggested a positive correlation between the development degree of Golgi and the ability for promastigotes to invade and get into host cells (Lewis 1974). Additionally, we also analyzed the microtubule counts of the six strains, and found that the three isolates SC10H2, SD, GL formed one group and showed significant differences comparing with any other reference strain ($P < 0.01$). Previously, Gardener et al. (1977) found that the number of microtubules are various among different *Leishmania* species. Lewis (1975) considered that the microtubule counts in classification would be valuable to judge to which species the *Leishmania* strains does not belong rather than the opposite. Those above leaded us to speculate that the three isolates SC10H2, SD, and GL may be distinct from the other strains in this study (DD8, K27, and GERBILLI), and close to the reptile-infecting *Leishmania* that belong to *Sauroleishmania*. This conjecture was just in accord with the phylogeny inferred from cyt *b* and HSP70 gene data. Of course, we cannot rule out the possibility that the Golgi in the three strains has been changed in form or so underdeveloped that it has not been observed. The study of microtubule counts was also restricted by the amount of samples. Thus, to obtain a relatively accurate assessment, a wide range of sample sizes would be necessary.

Based on HSP70 and cyt *b* analyses, the three strains SD, GL, and SC10H2 formed a clade which was most closely related to the nonpathogenic *L. tarentolae* with several other Chinese isolates. The topological structure is consistent with our anterior studies on ITS1 (Yang et al. 2010), COII (Cao et al. 2011), SSU rRNA, and 7SL RNA (Guan et al. 2012). Meanwhile, the ultrastructural characteristics of three *Leishmania* sp. isolates were also inclined to the results of phylogenetic analyses in this study. However, the three strains were all isolated in VL patients from Shandong, Gansu, and Sichuan provinces, respectively, and the strain SC10H2 was confirmed to share high homology with the pathogens isolated from CanL in Beichuan County, Sichuan, China (Sun et al. 2012). These seem to be contradictory with former studies which indicated that *Sauroleishmania* is not pathogenic to

human (Belova 1971). Thus, our results further confirmed that a novel species of *Leishmania* causing CanL and kala-azar does exist in China and closely related to *L. tarentolae*. Although a growing number of information about Chinese *Leishmania* sp. has been obtained, however, the epidemiology and distribution of the *Leishmania* sp. are still unclear. It is known that sandfly is the key to *Leishmania* natural cycle. A recent study has found that *Leishmania* DNA obtained from *Sergentomyia dentate* is highly similar to *Leishmania* sp. (KJ667095, 99.3%) and *L. tarentolae* (98%) based on HSP70 gene analysis, which may be an evidence for natural cycle of *Sauroleishmania* agents in western Turkey (Özbel et al. 2016). Considering that Chinese *Leishmania* sp. gene segments have been detected in canines in Sichuan (Sun et al. 2012), it is necessary to investigate the *Leishmania* infection rate of sandflies in these endemic areas to track new evidence to understand the natural cycle of *Leishmania* sp. In addition, more recent *Leishmania* sp. isolates, valid *L. tarentolae* type strains, and abundant gene information of Chinese *Leishmania* sp. would add to our understanding of its definite taxonomic status and evolution.

In conclusion, based on cyt *b* and HSP70 sequence data, the 14 Chinese *Leishmania* isolates were found to belong to three *Leishmania* species including *L. donovani* complex, *L. gerbilli*, and *L. (Sauroleishmania)* sp. This result further confirmed that the undescribed *Leishmania* species causing visceral Leishmaniasis (VL) in China is closely related to *L. tarentolae*. The HSP70 results also suggested the classification of subspecies within *L. donovani* complex: KXG-918, KXG-927, KXG-Liu, KXG-Xu, 9044, SC6, and KXG-65 belong to *L. donovani*; Cy, WenChuan, and 801 were proposed to be *L. infantum*. Meanwhile, the ultrastructural characteristics of *Leishmania* sp. isolates partly lent support to the phylogenetic inference that Chinese *Leishmania* sp. is in close relationship with reptilian *Leishmania*. There are still many unknowns about Chinese *Leishmania* to be resolved. Further study on a wide range of epidemiological survey about patients, sandflies, and other reservoirs would be needed to get the whole picture of *Leishmania* in China.

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