Genetic characterization of *Blastocystis* from wild animals in Sichuan Wolong National Natural Reserve, southwestern of China-Zoonotic potential

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Abstract

Background

Blastocystis, a highly prevalent eukaryotic parasite, has been identified in a wide range of hosts, including humans, domestic and wild animals. Many animals are potential sources of *Blastocystis* infection for humans, while few information about the prevalence of *Blastocystis* in wild animals have being documented. Therefore, the present study was designed to investigate the prevalence and subtypes of *Blastocystis* in wild animals of Sichuan Wolong National Natural Reserve, southwestern of China, so as to assess the zoonotic potential of these animals.

Methods

A total of 300 faecal samples were collected from 27 wildlife species in three areas of Sichuan Wolong National Natural Reserve in southwestern China. The subtype (ST) genetic characteristics and prevalence of *Blastocystis* were determined by PCR amplification of the barcode region (a fragment of ~600 bp) of the SSU rRNA gene, and phylogenetic analysis were further performed to determine the genetic characteristics of *Blastocystis* subtypes.

Results

30 of 300 faecal samples (10.0%) were *Blastocystis*-positive. The highest prevalence of *Blastocystis* was found in Yinchanggou (18.3%), which was significantly higher than that in Niutoushan (7.5%), and Genda (5.5%) ($P < 0.05$). Specifically, the highest prevalence of *Blastocystis* was found in primates (20.0%, 1/5), followed by rodentia 14.3% (1/7), artiodactyla 13.1% (26/198), carnivora 2.3% (2/87), galliformes 0% (0/3). Sequence analysis showed 5 subtypes (ST1, ST3, ST5, ST13, and ST14), with ST13 and ST14 as the predominant subtype (33.3%, 10/30), followed by ST1 (20.0%, 6/30).

Conclusions

To the best of our knowledge, this is the first molecular investigation on *Blastocystis* infection in wild animals in southwestern of China. ST1, ST3, and ST5 were identified in both humans and wild animals, suggesting that these wild animals may be potential reservoirs of *Blastocystis* for human infection.

Background

The enteric parasite *Blastocystis* (classified in stramenopiles) was the most common protist in humans[1]. Generally, the primary mode of transmission is through *Blastocystis* -contaminated water and food via the fecal-oral route[2]. There is an strong evidence to suggest that some human infections may be caused by zoonotic transmission of *Blastocystis*[3, 4]. The pathogenicity of *Blastocystis* remains

controversial, there are studies associating it with symptoms of a variety of gastrointestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS)[5–7]. However, few microbiome studies indicated that *Blastocystis* is a common commensal in the human gut and it can increase the bacterial diversity[8].

Based on sequence analysis of the small subunit ribosomal (*SSU*) RNA gene, at least 22 subtypes of *Blastocystis* have been identified in animals and humans worldwide[9]. Subtypes ST1-9 and ST12 have been found in humans with varying prevalence but ST1-4 are the most common ones, accounting for more than 90% of human *Blastocystis* infections[10, 11]. A accumulating evidence showed that the same subtype of *Blastocystis* can colonize a wide range of hosts, implying these subtypes lack host specific features.

In China, *Blastocystis* has been found in humans, domestic and captive wildlife animals (e.g., belonging to the orders Carnivora, Artiodactyla, Perissodactyla, Rodentia, and Primates)[2, 12–16], highlighting that these animals may be potential hosts for human infection with this pathogen. However, only limited studies have been conducted on *Blastocystis* isolated from wild animals in China, and its role as reservoirs of infection for humans and other animals is remain unknown.

Sichuan Wolong National Natural Reserve is the third largest nature reserve in China which covers an area of 200,000 hectares, makes it the largest reserve, with complex natural conditions and the largest number of rare animals in Sichuan Province (https://baike.so.com/doc/5376249-5612365.html). Due to the fact that with the conecetion to the outside world and the development of tourism, the chances of contact between animals and humans in the reserve have been greatly increased so that the risk of transmission of zoonoses is increasing. Therefore, the purpose of this study was to investigate the prevalence and subtypes of *Blastocystis* in wild animals and assess the zoonotic potential of *Blastocystis* colonizing in these animals.

Materials And Methods

Sample collection

Between March 2020 and December 2020, a total of 300 faecal samples were collected from three areas of Sichuan Wolong National Natural Reserve (Fig. 1). Specifically, 127 faecal samples were collected from rodentia, primates, artiodactyla, carnivora in Genda, and 93 specimens were collected from rodentia, primates, artiodactyla, carnivora in Yinchanggou, and 80 faecal samples were collected from rodentia, artiodactyla, carnivora, galliformesin in Niutoushan. All those faecal samples were collected by experienced mountain patrol staffs of the Sichuan Wolong National Natural Reserve during the mountain patrol and were strictly controlled to minimize potential contamination among animal species. Camouflaged video equipment was placed to identify the animal's species prior to sampling, based on its past range or nesting location. Briefly, stool samples were collected on tracks or in the vicinity of nests. The species was inferred in the field according to the shape, size and texture of the faecal samples as

well as presence of footprints, nearby nests and confirm with information from the video. Some animal feces are collected immediately after the animal is observed to defecate, for example galliformes grimates and some artiodactyla. Fecal samples that could not identify the species or feces samples more than two days were not included. All faecal samples were collected in sterilized plastic containers using disposable sterile gloves and preserved at -4 ℃ until DNA extraction.

DNA extraction

All faecal specimens were sieved and washed three times with distilled water by centrifugation at 3000×*g* for 10 min. Genomic DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) from approximately 250 mg according to the manufacturer's instructions and Positive and negative controls were included. DNA quality was verified by NanoDrop (Thermo Fisher Scientific, Carlsbad, CA, USA) measurements. DNA was eluted in 50 µl of nuclease-free water and stored at -20°C until PCR analysis.

PCR amplification

Use polymerase chain reaction (PCR) amplification of the barcode region (a fragment of ~600 bp) of the SSU rRNA gene to screening all DNA preparations for the presence of *Blastocystis* and the primers and cycling parameters were used as previously described by Scicluna *et al*.[17]. The Taq PCR Master Mix (Sangon Biotech Co., Ltd., Shanghai, China) was used for all PCR reactions. Reagents per 25 µl reaction were as follows: 12.5 µl Taq PCR Master Mix (Sangon Biotech Co., Ltd., Shanghai, China), 1 µl each primer (0.4 µM), 2 µl genomic DNA sample, 1.5 mM MgCl2, and nuclease-free water up to desired volume. All PCRs were performed in triplicate, positive and negative controls were included in all the PCR tests. PCR products were subjected to 1.5 % agarose gel (AddGene, Watertown, MA, USA) electrophoresis and visualized by staining with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific).

Nucleotide sequencing and analysis

PCR products with the predicted size (*Blastocystis* approximately 600 bp) were excised from the agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. All positive purified PCR products were bidirectionally sequenced on an ABI PRISMTM 3730 DNA Analyzer (Applied Biosystems, USA), using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster, CA, USA). Nucleotide sequences obtained in this study were subjected to BLAST searches (http://www.ncbi.nlm.nih.gov/blast/) and then aligned and analyzed with each other. Reference sequences were downloaded from the GenBank database using the program Clustal X 2.0 (http://www.clustal.org/) to determine the subtypes of *Blastocystis* isolates. The representative nucleotide sequences generated in present study have been deposited in GenBank under accession numbers MW404496, MW404497, MW404561, MW404583, MW404585, MW404588, MW404590.

Phylogenetic analysis

To assess the genetic relationships among the *Blastocystis* genotypes in the present study and those identified sequences obtained from GenBank in previous studies, phylogenetic analysis was performed

by constructing a neighbor-joining tree using Mega 6 software (http://www. megasoftware.net/), evolutionary distances were calculated by a Kimura 2-parameter model. Before phylogenetic analysis the undefined positions were removed from the alignment, and then the alignment was trimmed using MEGA 6 (http://www.megas oftwa re.net/). The reliability of these trees was assessed by bootstrap analysis with 1000 replicates.

Statistical analysis

The difference in *Blastocystis* prevalence between different Areas, and the order of animals was analyzed with the binary logit model, using SPSS 20.0 (IBM, Chicago, IL, USA). Each of these variables was included in the binary logit model as an independent variable by multivariable regression analysis. When the *P* value was less than 0.05, the results were considered statistically significant. The adjusted odds ratio (OR) and 95% confidence interval (CI) for each variable were calculated with binary logistic regression, and all risk factors were entered simultaneously.

Results

Prevalence of Blastocystis **in wild animals**

In the present study, 30 of 300 (10.0%) faecal samples collected from three areas of Sichuan Wolong National Natural Reserve, southwestern China were determined to be *Blastocystis*-positive by PCR amplification the barcode region of the SSU rRNA gene. Specifically, 17 of 93 (18.3%) samples from Yinchanggou, 6 of 80 (7.5%) samples from Niutoushan and 7 of 127 (5.5%) animals sampled from Genda were *Blastocystis*-positive (Table 1). The difference in *Blastocystis* prevalence were significant in three areas ($P < 0.05$). The prevalence of *Blastocystis* in nonhuman primates (NHPs) was 20.0%, which is higher than that in rodentia (14.3%), artiodactyla (13.1%), carnivora (2.3%), and Galliformes (0.0%). However, the difference of *Blastocystis* prevalence among different order animals was not significant ($P > 0.05$).

Table 1
Factors associated with the prevalence of *Blastocystis* in wild animals in China.

Factors	**No. of positive /Overall**	**Prevalence (95% CI)**	**OR (95% CI)**	***P* value**
Locations				
Genda	7/127	5.5 (1.5–9.5)	Reference	Reference
Yinchanggou	17/93	18.3 (10.4–26.1)	3.8 (1.5–9.7)	0.004
Niutoushan	6/80	7.5 (1.7–13.3)	1.4 (0.5–4.3)	0.6
Host				
Rodentia	1/7	14.3 (11.6–40.2)	Reference	Reference
Primates	1/5	20.0 (15.1–55.1)	1.5 (0.1–31.6)	0.8
Artiodactyla	26/198	13.1 (8.4–17.8)	0.9 (0.1–7.8)	0.9
Carnivora	2/87	2.3 (0.9–5.4)	0.1 (0.01–1.8)	0.1
Galliformes	0/3	0.0	0 (0)	1.0
Total	30/300	10.0 (6.6–13.4)		

In general, of the 27 species tested in this study, 10 (37.0%) were positive for *Blastocystis* (Table 2). Specifically, of the 16 species tested at the Genda, 4 (25.0%) were positive for *Blastocystis*. At the Yinchanggou, the prevalence of the parasite was 53.8% (7/13) and 21.1% (4/19) species at Niutoushan in the present study were shown to be infected with *Blastocystis*.

Table 2
Animal samples collected from various hosts from Nature reserve three different areas in Sichuan province, southwestern China.

Host	Scientific name	GD	YC	NT	No. of *Blastocystis*-positive/Overall
Primates					
Tibetan macaque	*Macaca thibetana*		3		0/3
Golden monkey	*Rhinopithecus*	2			1/2
Artiodactyla					
Sambar	*Rusa unicolor*	18	13	8	8/39
Sika deer	*Cervus nippon*		14		5/14
Long-tailed goral	*Naemorhedus griseus*		15	2	5/17
Crested deer	*Elaphodus cephalophus*	2	4	2	4/8
Chinese antelope	*Capricornis milneedwardsii*	1	1	1	1/3
Dwarf musk deer	*Moschus berezovskii*	12	1	6	0/19
Takin	*Budorcas taxicolor*	15	9	1	0/25
Blue sheep	*Pseudois nayaur*	34		8	3/42
Antelope	*gazelle*			1	0/1
Yak	*Bos mutus*			15	0/15
Wild ox	*Bison bison*	1			0/1
Goral	*Naemorhedus goral*	10			0/10
Wild pig	*Sus scrofa*	4			0/4
Rodentia					
Porcupine	*Hystrix hodgsoni*	5	1	1	1/7
Carnivora					
Sand badger	*Arctonyx collaris*		2	1	1/3
Lesser panda	*Ailurus fulgens*	2	8		0/10
Leopard cat	*Prionailurus bengalensis*	3	3	4	1/10

GD = Genda; YC = Yinchanggou; NT represents Niutoushan

Host	Scientific name	GD	YC	NT	No. of *Blastocystis*-positive/Overall
Giant panda	*Ailuropoda melanoleuca*	11	19	10	0/40
Stone Marten	*Stone Marten*			2	0/2
Masked civet	*Paguma larvata taivana*			1	0/1
Black bear	*Ursus thibetanus*			1	0/1
Jackal	*Cuon alpinus*	1			0/1
Snow leopard	*Panthera uncia*	6		13	0/19
Galliformes					
Blood Pheasant	*BloodPheasant*			1	0/1
Chinese Monal	*Lophophorus lhuysii*			2	0/2
Total		127	93	80	30/300
GD = Genda; YC = Yinchanggou; NT represents Niutoushan					

Interestingly, the prevalence of *Blastocystis* varies greatly among different species (Table 3). The highest of *Blastocystis* prevalence was observed in golden monkey (50.0%, 1/2), and crested deer (50.0%, 4/8). By comparison, blue sheep and leopard cat showed lower *Blastocystis* prevalence of *Blastocystis*, accounting for 7.1% and 10.0% respectively.

Table 3
The prevalence of *Blastocystis* among different species.

Species	Prevalence(No. of positive/Overall)	GD	YC	NT
Golden monkey	50.0(1/2)	ST5(1)		
Sambar	20.5(8/39)	ST13(3)	ST14(3)	ST14(2)
Sika deer	35.7(5/14)		ST1(4);ST3(1)	
Long-tailed goral	29.4(5/17)		ST1(1);ST5(2);ST13(2)	
Crested deer	50.0(4/8)	ST13(1)	ST13(1)	ST13(1);ST14(1)
Chinese antelope	33.3(1/3)		ST14(1)	
Blue sheep	7.1(3/42)	ST13(2)		ST14(1)
Porcupine	14.3(1/7)		ST1(1)	
Sand badger	33.3(1/3)			ST14(1)
Leopard cat	10.0(1/10)		ST14(1)	
Total	20.7(30/145)	ST5(1);ST13(6)	ST1(6);ST3(1) ST5(2);ST13(3);ST14(5)	ST13(1);ST14(5)
GD = Genda; YC = Yinchanggou; NT represents Niutoushan				

Subtype distributions of Blastocystis **in wild animals**

Among the 30 *Blastocystis*-positive samples, 5 subtypes were identified, including three zoonotic STs (ST1, ST3 and ST5) and two animal-specific STs (ST13 and ST14). ST13 (10/30) and ST14 (10/30) were the dominant subtypes in wild animals examined in the present study (Table 4), followed by ST1 (6/30), ST5 (3/30), ST3 (1/30). ST3 was only found in one faecal samples (Table 4). Notably, ST14 has the widest host range in wild animals, which detected in sambar, crested deer, chinese antelope, blue sheep, sand badger and leopard cat (Table 4). Meanwhile, ST13 was identified in four species of animals, including sambar, long-tailed goral, crested deer, and blue sheep (Table 4).

Table 4
Subtype distributions from different animal species.

Host	*Blastocystis* sp. STs					Sequences
	1	3	5	13	14	
Primates						
Golden monkey			1			1
Artiodactyla						
Sambar				3	5	8
Sika deer	4	1				5
Long-tailed goral	1		2	2		5
Crested deer				3	1	4
Chinese antelope					1	1
Blue sheep				2	1	3
Rodentia						
Porcupine	1					1
Carnivora						
Sand badger					1	1
Leopard cat					1	1
Total	6	1	3	10	10	30

Genetic characteristics of Blastocystis **subtypes**

The homology analysis of the SSU rRNA gene revealed that six sequences of ST1 isolates identified in sika deer, long-tailed goral, and porcupine were identical to those from pan troglodytes in Tanzania: Rubondo Island (HQ286905). Similarly, one ST3 sequences from sika deer showed 100% identity with GenBank sequences MW242639 (from red-bellied tree squirrel in China), three ST5 sequences had 100% similarity with that from sheep in China: Heilongjiang (MF974615).

In the case of ten ST13 isolates, two representative sequences were obtained from sambar, long-tailed goral, crested deer, and blue sheep. The sequence (MW404585) of ST13 isolates showed a homology of 99.43% to the sequence of ST13 isolated from a reindeer in China (MH325366), with three nucleotide substitutions. The remaining one sequences MW404588 showed a homology of 100% to the sequence (MF186700) of ST13. Similarly, In ten of ST14 isolates, has also been obtained two representative sequences from sambar, crested deer, chinese antelope, blue sheep, sand badger and leopard cat. The sequence (MW404561) and (MW404583) of ST14 isolates showed a homology of 99.42% to the

sequence of ST14 isolated from a sheep in Czech Republic (MT039559) and had 99.81% similarity with that from sika deer in China (MK357783), with three and one nucleotide substitutions respectively.

Phylogenetic analysis of Blastocystis

A total of 7 representative sequences were obtained from 30 *Blastocystis* isolates in the present study. The sequences obtained in the present study showed high identity with the reference sequences of *Blastocystis* in GenBank. Newly acquired sequences belong to ST1, ST3, ST5, ST13, and ST14. ST1 along with sequences originating from pan troglodytes and western lowland gorilla clustered together. ST3 grouped together with sequences mainly from cervus nippon. ST5 clustered together with sequences from sheep and sus scrofa domesticus. ST13 along with sequences isolated from reindeer and water deer grouped together. ST14 formed a clade with sequences from ovis arie, cattle, sheep, and goat (Fig. 2).

Discussion

Blastocystis is the most frequent parasite that has been reported in humans and a variety of animals with controversial pathogenicity[18, 19], previous studies have found that infection with *Blastocystis* is linked to nutritional and gastrointestinal disorders in both developing and developed countries[20]. However, recent microbiome studies have reported that the presence of *Blastocystis* may be an indicator of good intestinal health[21]. Zoonotic STs is supposed to be commonly transmitted between animals and humans, and some certain STs of animal origin are significant potential reservoirs for human infections[22–24].

Epidemiological studies have been conducted in domestic animals including pig, cattle, dairy cattle, sheep and goat but only few reported on captive wildlife has been documented in China[2]. The prevalence of *Blastocystis* in wild animals examined in this study was 10.0% (30/300), which was lower than that of captive wild animals from zoo animals in Western Australian (42%, 32/76)[3], in Qinling Mountains, China (40.2%, 200/497)[25], in zoo animals in Japan (39.0%, 46/118)[26], in wild animals in Brazil (34.4%, 115/334)[27], in zoo animals in United Kingdoms (34.2%, 79/231), and in various captive animals in French (32.2%, 99/307)[28] in captive wildlife in four zoos outhwestern China(15.7%, 66/420) [14] and in captive mammalian wildlife in Bangladesh National Zoo (15.5%,31/200)[24]. While in this study the *Blastocystis* prevalence in wild animals was higher than that in zoo animals in three cities in China (6.0%, 27/450)[29]. At this time, it is unclear how those factors contribute to affect the prevalence, between countries or within the same country, such as the conditions in which the animals were housed, animal species, size of examined samples, or management methods.

In the present study, the prevalence of *Blastocystis* in nonhuman primates (NHPs) was 20.0%, which is higher than that in rodentia (14.3%), artiodactyla (13.1%), carnivora (2.3%), and galliformes (0.0%) and five *Blastocystis* STs, including ST1, ST3, ST5, ST13 and ST14, were identified in 30 *Blastocystis*-positive samples from 4 orders wildlife (Table 4). To date, These STs have been documented from primates in Europe such as monkeys and macaque[28, 30–32] and Brazil[27]. ST4, ST5, and ST8 have also been

found in monkeys elsewhere[27, 30, 31, 33]. In the current study, sequences obtained from primates belonged to ST5. These isolates were infecting the golden monkey. The presence of ST5 has also been found sporadically in humans in close contact with animals, suggesting zoonotic transmission[34, 35].

Previous studies have confirmed that other STs, such as ST1-3, ST5, ST7, ST8, ST10, and ST17 has been identified in rodents[36, 37] and in this study, ST1 was identified in rodents (porcupine), corroborating previous data on rodents. Wistar rats infected with ST1 could cause moderate and severe degrees of pathological changes, This subtype indicated the potential pathogenicity, Hussein *et al.* Reported[38].

Five subtypes (ST1, ST3, ST5, ST13 and ST14) were identified in artiodactyla (long-tailed goral, sika deer, sambar, crested deer, chinese antelope and blue sheep) In this study. Previous studies reported many animals in the order of artiodactyla harboring the *Blastocystis*, such as cattle, pigs, sheep, deer, and goats[2, 39]. To date, the majority of STs such as ST1, ST3, ST5, ST13 and ST14 have been identified in artiodactyla[36, 40]. In artiodactyla ST1 was found in sika deer and long-tailed goral, ST3 was identified in sika deer, ST5 were found in long-tailed goral, ST13 was found in sambar, long-tailed goral, crested deer, blue sheep and ST14 was identified in sambar, crested deer, chinese antelope and blue sheep in present study. Many previous surveys have indicated that, in human the ST1 and ST3 have been reported as the two most common subtypes in different countries[41, 42]. Which proposed a hypothesis of the potential transmission of *Blastocystis* infection between human and artiodactyla. Therefore, the role of artiodactyla to transmit these subtypes should be further evaluated in the future study. As reported, ST5 was the most predominant subtype in pigs[43], and it also identified in a various animals, such as cattle, sheep, NHPs, and birds[39]. The surprise is that a rare ST (ST13) was also determined in Java mouse-deer in France[28], in a mouse deer in United Kingdoms[36]. Meanwhile, sheep in China[44], muntjac deer in the United Kingdoms[37] were found infected with ST14.

In the current study, sequences obtained from carnivora belonged to ST14. These isolates have infected the sand badger and leopard cat. ST14 is often reported in artiodactyls but is almost absent in the carnivore order[37].

Conclusions

To the best of our knowledge, this is the first molecular investigation on *Blastocystis* infection in sambar, long-tailed goral, crested deer, chinese antelope, blue sheep, sand badger, leopard cat in China, further broadening the host range of *Blastocystis*. The prevalence of *Blastocystis* was 10.0% (30/300) in wild animals in southwestern China. Furthermore, five *Blastocystis* subtypes (ST1, ST3, ST5, ST13, and ST14) were identified in this study. In which, ST1, ST3 and ST5 are considered as the zoonotic subtypes, suggesting that these wild animals may serve as natural reservoirs for human *Blastocystis* infections. The findings in the present study could provide preliminary data for the monitoring and investigating the transmission route of *Blastocystis*.

Abbreviations

STs: Subtypes; PCR: Polymerase chain reaction; SSU rRNA: Small subunit ribosomal RNA; IBD: Inlammatory bowel disease; IBS: Irritable bowel syndrome; ORs: Odds ratios; NHPs: Nonhuman primates.

Declarations

Ethical statement

This study was performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. Due to only fecal samples collected after the spontaneous defecation of the wild animals were analyzed. Consequently, this study did not require full Animal Ethics Committee approval in accordance with China law. No animals were harmed during the sampling process. Permission was obtained from reserve managers prior to collection of fecal specimens.

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Authors' contributions

This study was conceived and designed by GP . Experiments were performed by SC, WM, TH, ZZ (Ziyao Zhou), and LD. Fecal samples were collected by XS, YC, HL, YC, ZZ (Zhijun Zhong), and HF. Data were analyzed by LS, and KZ. All authors have read and approved the submitted version of this manuscript.

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Availability of data and materials

The nucleotide sequences generated in the present study have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/) under accession numbers MW404496, MW404497, MW404561, MW404583, MW404585, MW404588, and MW404590. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

Figure 1

Geographical distribution of the sampled sites (filled triangle) in Sichuan Province, Southwestern China. Note: The designations employed and the presentation of the material on this map do not imply the

expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

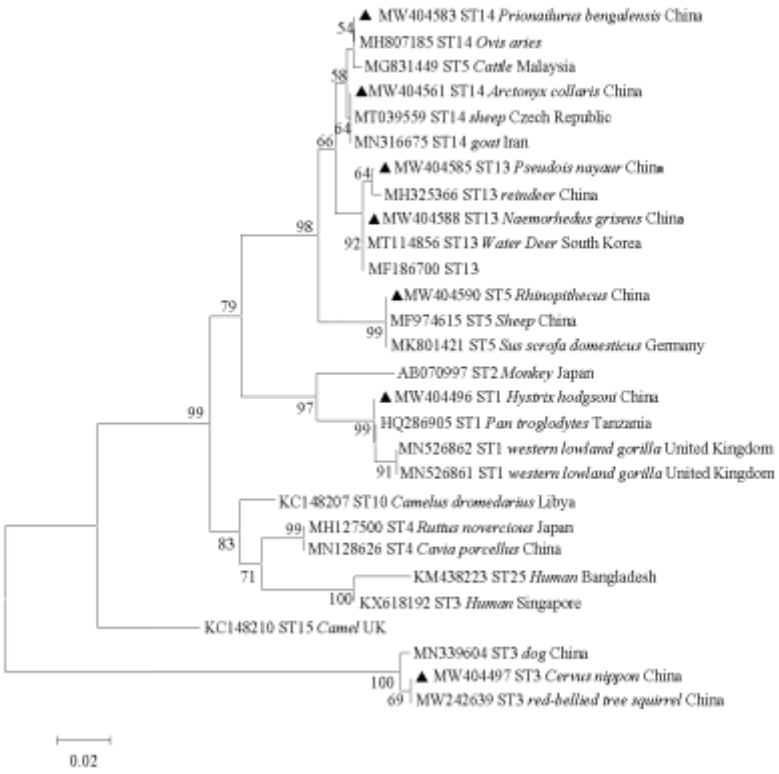

Figure 2

Phylogenetic relationships among nucleotide sequences of Blastocystis partial small subunit ribosomal RNA (SSU rRNA) genes. The neighbor-joining method was used to construct the trees by the Kimura-2-parameter model. The number on the branches are percent bootstrapping values from 1000 replicates, with values of more than 50% shown in the tree. Each sequence is identified by its accession number, subtypes, host origin, and country. Genotypes marked with black triangles are known genotypes identified in this study, respectively.

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