Prevalence and Genetic Characterizations of *Enterocytozoon Bieneusi* in Captive Red Pandas (*Ailurus Fulgens*) In Sichuan Province, Southwestern China

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Research Article

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Abstract

Background: *Enterocytozoon bieneusi* (*E. bieneusi*) can infect a broad range of animals, and also the major pathogen for human microsporidiosis. The risk of zoonosis is uncertain because of limited research on red pandas. In addition, the semi-free range breeding enables the red panda direct contact with tourists. It is essential to investigate the prevalence and genotypes and to evaluate the safety of this breeding mode.

Methods: Based on nested PCR, 198 fecal specimens were sampled from 6 zoos in Sichuan province from July 2020 to December 2020, to identify positive samples by amplifying the internal transcribed spacer (ITS) region of ribosomal RNA with specific primers. The correlation analysis of infection rate was carried out between different breeding modes (captive and semi-free-range). To cluster the identified genotypes with related genotypes to deduce zoonotically potential by phylogenetic analysis. In addition, Multilocus genotypes (MLGs) in ITS-positive samples were performed using the Multilocus Sequence Typing (MLST) tool.

Results: The Polymerase Chain Reaction (PCR) results showed that 12.1% (24/198) samples were positive for *E.bieneusi*. The infection rates varied from 0% to 18.0% in different zoos and were significantly different in different breeding methods (χ^2=5.442, P=0.0197). Genotypes D, SC02, and SCR1(novel) were clustered in zoonotic group 1, while genotype PL2 is clustered in group 2-like with uncertain risk by phylogenetic analysis. Furthermore, 3 distinct multilocus genotyping were formed in ITS-positive isolates.

Conclusions: These results revealed the circulating of *E. bieneusi* in zoo red pandas, indicating that red pandas may be a source of human microsporidiosis and that semi-free range breeding mode as a risk factor increased the *E. bieneusi* infection rate and potential cross-species transmission.

Background

Microsporidia is a group of opportunistic obligated intracellular parasites, which are currently classified as fungi and their host range is very wide, including most invertebrates, vertebrates, and humans [1–3]. At least 1500 microsporidia species, which belong to 200 genera, have been formally described till now, 17 of which were reported to infect humans [4, 5]. *E. bieneusi* is one of the most common pathogens of human microsporidiosis, accounting for 90% of human cases of the disease [6, 7]. *E. bieneusi* infection can cause acute to chronic diarrhea, malabsorption, inflammation of the respiratory tract, and acalculous cholecystitis in people, and even deadly severe diarrhea in people with immunosuppression [8–10].

Through sequencing analysis of the ITS region of the ribosomal RNA (rRNA), more than 500 genotypes were identified and divided into 11 groups based on cluster analysis [11]. Group 1, including most species, was mainly comprised of genotypes with a wide host range and have higher zoonotic potential, including more than 40 genotypes identified in both humans and animals [12]. Group 2, the second-largest, was previously thought to only infect cattle, but with the discovery of a wider host range for some

genotypes in this group, suggesting potential public health issues to an extent [5]. The majority of the genotypes from groups 3–11 appear to be more host-specific, and therefore, lead to a slight or unknown threat to public health [5]. However, the single ITS loci cannot differentiate *E. bieneusi* isolates that are genetically closely related [13]. A higher resolution tool, MLST, was developed and used for sub genotyping [14]. To date, MLST tools are already used in more than 167 ITS genotypes to study the genetic characters by distinguishing the repeat sequence and single nucleotide polymorphisms (SNPs) [14, 15].

In recent years, the zoonotic genotypes of *E. bieneusi* have been found in many animal groups, including birds, non-human primates, domestic and captive wild animals [16–27]. Past studies have shown Genotypes D and EbpC occurred in Chinese red pandas [13, 19, 28]. At the same time, these two genotypes have been frequently reported in the Chinese population as well, suggesting the risk of red pandas as a potential reservoir of zoonotic pathogens.[2, 29]. The captive populations of red pandas in China are mostly concentrated in zoos in southwest China, especially in Sichuan. However, no regional large-scale epidemiological investigation has been carried out yet in these regions. Furthermore, some zoos breed red pandas in semi-free-range mode, the safety for both humans and animals is uncertain; the zoonotic potential in different regions and breeding modes should be assessed. Therefore, we carried out this study.

Methods

Sample collection

We collected 198 fresh fecal specimens from 6 zoos or breeding sites of 5 regions in Sichuan province, between July 2020 and December 2020 (Fig. 1, Table 2). Most animals are kept with a barbed wire fence nearly 1 meter high separating the animals from visitors. We divided the animals into two groups depending on the range of movement and the level of human connection. Group i) captive red panda groups which live alone and do not interact with each other, only contact with keepers; Group ii) semi-free-range, which means red panda groups can travel freely within certain limits and may interact with other groups or visitors. All samples were collected by sterile gloves within 24 hours after defecation and immediately transported in a cold box to the clinic veterinary laboratory of Sichuan Agricultural University, then stored at -20℃ till DNA extraction.

DNA extraction and PCR amplification

All samples were cryogenically centrifuged at 3000 rpm for 5 min after washing and filtering out impurities. Extracting genome DNA from 200mg pretreated feces using E.Z.N.A.® stool DNA Kit (OMEGA, Biotek Inc. USA) following the recommended procedures. Add 200 µl kit Solution Buffer into each extracted DNA sample and stored at -20 ° until use.

The positive samples were screened by an approximately 390 bp amplified fragment in the ITS region with nested PCR. 25µl PCR mixtures were composed of 12.5µl Premix *Taq*™ (TaKaRa Bio, Otsu,

Japan),8.5μl ddT H_2O, 2μl primers and 2μl genomic DNA. To increase the detection of positive isolates, use two pairs of different ITS primers (ITS1, ITS2) to amplify the specimens. The primers and reaction temperatures of PCR amplification were referenced from previous articles [14, 30, 31](Table 2). All PCR reactions arranged positive and negative controls. Secondary PCR products were stained by GoldView™ and visualized in 1.0% ethidium bromide-stained agarose gel by electrophoresis. To further identify sub-genotype characteristics, positive ITS specimens were amplified by MLST in loci MS1, MS3, MS4, and MS7[14].

Sequencing and phylogenetic analysis

To ensure accuracy, the nucleotide sequences of positive specimens were sent to TsingKe Biological Technology (Chengdu, China) for bi-directional sequencing. Using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.ni-h.gov/BLAST/) to identify the genotypes of *E. bieneusi*. Then Aligned the obtained sequences and the reference sequences from GenBank with Clustal X 1.83 (http://www.clustal.org/). The phylogenetic trees were reconstructed by the MEGA 7.0 program (version 7.0.26), specifically choosing the neighbor-joining method, Kimura 2-parameter model, and 1000 bootstrap replicates. The genotype names followed previously published ones if the obtained sequences are identical to known genotypes. Novel genotypes were identified according to the single nucleotide substitutions, deletions, or insertions in the 243bp region of the ITS gene and named by the nomenclature rules of the previous study [32].

Statistics analyses.

The chi-square test and exact probability test were used for significant differences of *E. bieneusi* infection among different regions and different groups based on SPSS 26.0 software (https://spss.en.softonic.com/). Set 95% confidence intervals (CIs) and odds ratios (ORs)dang. When P value ≤ 0.05, the differences in results were considered significant.

Nucleotide sequence GenBank accession numbers All ITS and MLST sequences obtained in this study were submitted to the GenBank database. Acquired the accession number MW880217-MW880236, MW880238-MW880241, and MW922590-MW922622.

Results

Occurrence and genotype of E. bieneusi in red pandas

Among 198 fecal samples detected by nested PCR, 24 tested positives for *E. bieneusi*, the overall infection rate reached 12.1% (24/198) (Table 1). The infection rates of *E. bieneusi* were 18.0% (18/100), 7.1% (3/42), 7.1% (1/14), 9.1% (1/11),0% (0/18),7.7% (1/13) in Chengdu Research Base of Giant Panda Breeding(CDBGPB), Sanlang Resort(SLR), Bifengxia Zoo(BFXZ), Chengdu Zoo(CDZ), Wolong China Giant Panda Garden(WCGPG), Panda Valley(PV), respectively. The differences among different regions were not significant ($\chi^2 = 7.364$, df = 5, P = 0.1949 > 0.05). In groups, Group i was 5.9% (5/85), and Group ii

reached up to 16.8% (19/113). There was a significant difference between in different groups (χ^2 = 5.442, df = 1, P = 0.0197 < 0.05).

Table 1
Sampling information and epidemic results of *E. bieneusi* in red pandas in this study.

Group	Region	Site	Breeding mode	No. of samples	No. of positive (%)	ITS genotypes
□	Chenghua	CDZ	Captive	11	1 (9.1%)	D
	Wenchuan	WLCGPG	Captive	18	0 (0.0%)	
	Chongzhou	SLR	Captive	42	3 (7.1%)	SCR1
	Ya'an	BFXZ	Captive	14	1 (7.1%)	D
□	Chenghua	CDRBGPB	Semi-free-range	100	18 (18.0%)	PL2
	Dujangyan	PV	Semi-free-range	13	1 (7.7%)	SC02

Note: CDBGPB: Chengdu Research Base of Giant Panda Breeding; SLR: Sanlang Resort; BFXZ: Bifengxia Zoo; CDZ: Chengdu Zoo; WCGPG: Wolong China Giant Panda Garden; PV: Panda Valley;

Phylogenetic analysis and MLST genotyping of E. bieneusi

All 24 positive isolates were successfully sequencing. observed Four genotypes, including 3 described genotypes (D, PL2, and SC02) and one novel genotype SCR1. The obtained ITS gene sequences of genotypes D, PL2, and SC02 were identical with reference sequences KY950534, MT497891, and KU852476 from GenBank, respectively. The homology was 98.6% between novel genotype SCR1 and genotype FJL (MK357781), with one single nucleotide substitution and one insertion. Among them, genotype PL2 (n = 18) was dominant and genotype SCR1 (n = 3) was ranked second. Both SC02 and D only identified one sample.

Table 2
The primers and annealing temperatures of PCR amplification.

Gene	Primer sequences (5'-3')	AT (°C)	Size (bp)	Reference
ITS1	F1: GATGGTCATAGGGATGAAGAGCTT	55	392	[31]
	R1: AATACAGGATCACTTGGATCCGT			
	F2: AGGGATGAAGAGCTTCGGCTCTG	55		
	R2: AATATCCCTAATACAGGATCACT			
ITS2	F1: GGTCATAGGGATGAAGAG	57	390	[14]
	R1: TTCGAGTTCTTTCGCGCTC			
	F2: GCTCTGAATATCTATGGCT	55		
	R2: ATCGCCGACGGATCCAAGTG			
MS1	F1: AAGTTGCAAGTTCAGTGTTTGAA	58	676	[14]
	R1: GATGAATATGCATCCATTGATGTT			
	F2: TTGTAAATCGACCAAATGTGCTAT	58		
	R2: ACATAAACCACTAATTAATGTAAC			
MS3	F1: CAAGCACTGTGGTTACTGTT	55	537	[14]
	R1:AAGTTA GGGCATTTAATAAAATTA			
	F2: GTTCAAGTAATTGATACCAGTCT	55		
	R2: CTCATTGAATCTAAATGTGTATAA			
MS4	F1: GCATATCGTCTCATAGGAACA	55	885	[30]
	R1: GTTCATGGTTATTAATTCCAGAA			
	F2: CGA AGTGTACTACATGTCTCT	55		
	R2: GGACTTTAATAAGTTACCTATAGT			
MS7	F1: GTTGATCGTCCAGATGGAATT	55	471	[14]
	R1: GACTATCAGTATTACTGATTATAT			
	F2: CAATAGTAAAGGAAGATGGTCA	55		
	R2: CGTCGCTTTGTTTCATAATCTT			

Note: AT: Annealing temperatures; F1:Forward primer of primary PCR; R1: Reverse primer of the primary PCR; F2:Forward primer of the second PCR; R2:Reverse primer of the second PCR.

Phylogenetic analysis results (Fig. 2) showed that genotype D and SCR1 belonged to subgroup1a in Group 1. SC02 is clustered in subgroup 1b of Group 1, and PL2 formed a separate clade related to Group 2 [33]. All genotypes were human-pathogenic [31]. To further analyze the gene polymorphism and the sub-genotype by using the MLST (Table 3). The amplification efficiencies in loci MS1, MS3, MS4, and MS7 were 25.0%(6/24), 45.8%(11/24),16.7%(4/24), and 50.0%(12/24), respectively. Only 3 genotype PL2 specimens tested positive in these five loci. Correspondingly generated 1, 2, 4, and 1 types in MS1, MS3, MS4, and MS7 locus based on different nucleotide repeats and SNPs. 3 distinct MLGs were formed in genotype PL2, and some degree of sequence polymorphism was shown.

Table 3
Multilocus genotypes of positive *E.bieneusi* isolates from red pandas in this study.

Sampling sites	Sampling no.	ITS genotype	Multilocus genotypes				
			MS1	MS3	MS4	MS7	MLGs
CDRBGPB	3	PL2					
	5	PL2		type1		type1	
	10	PL2		type1		type1	
	11	PL2	type1	type1	type1	type1	MLG1
	12	PL2	type1	type1			
	15	PL2					
	16	PL2				type1	
	18	PL2					
	19	PL2	type1		type1	type1	
	21	PL2					
	22	PL2		type1			
	40	PL2					
	43	PL2					
	47	PL2		type1		type1	
	63	PL2		type1		type1	
	86	PL2	type1	type1	type2	type1	MLG2
	93	PL2	type1			type1	
	95	PL2	type1	type1	type3	type1	MLG3
SLR	122	SCR1					
SLR	136	SCR1		type1		type1	
SLR	145	SCR1					
BFXZ	174	D		type2			
PV	185	SC02				type1	
CDZ	211	D					

Note: CDBGPB: Chengdu Research Base of Giant Panda Breeding; SLR: Sanlang Resort; BFXZ: Bifengxia Zoo; CDZ: Chengdu Zoo; WCGPG: Wolong China Giant Panda Garden; PV: Panda Valley;

This study sampled 6 zoos in 5 different regions and demonstrated the prevalence and genotype characterization of *E. bieneusi* among captive red pandas in Sichuan province, southwest China. In this study, red pandas in Group ii have a significantly higher prevalence of infection than Group i. The difference may be related to the larger range of red pandas and the more frequent contact between different animal populations and between animals and humans under the semi-free-range breeding mode. Meanwhile, higher prevalence indicated that the safety of semi-free-range breeding mode needs to be carefully regulated, due to zoonotic transmission potential.

The overall prevalence of this study was 12.1%, which is close to the previous report of red pandas in Shaanxi province, northwestern China, 11.1%-13.9% [13, 28]. In the present study, the prevalence of Chengdu Zoo and Bifengxia Zoo was 7.1% (1/14) and 9.1 % (1/11), both were lower than former prevalence data (10.6% and 29.7%)[19]. The differences in *E. bieneusi* prevalence between studies may be influenced by geographical region, sample size, sampling time, animal health condition, breeding mode, and population density[34–36]. The role of those factors can be further examined. As the closest phylogenetic relationship animal with red panda [37], the American raccoon (*Procyon lotor*), also possessed a higher prevalence (27.3%, 15/55) [27, 31]. Red pandas have a low overall prevalence compared to other common zoo species with a sampling size of more than 20 isolates (Table 4). Ignoring the interference of other factors, the adaptation of *E.bieneusi* to the red panda may be in the middle level.

Table 4
The prevalence of *E.bieneusi* in some species of Chinese zoos.

Family	Species	No. of tested	No.of positive (%)	Reference
Ailuridae	Red panda (*Ailurus fulgens*)	198	24(12.1%)	This study
Cercopithecidae	Golden snub-nosed monkey (*Cercopithecus kandti*)	160	74(46.2%)	[26]
	Rhesus macaque (*Macaca mulatta)*	304	60(19.7%)	[16, 33]
	Hamadryas baboon (*Papio hamadryas*)	21	6 (28.6%)	[42]
	Cynomolgus monkey (*Macaca fascicularis*)	80	47(58.8%)	[16, 18]
Cebidae	Squirrel monkey (*Saimiri* sp.)	43	17(39.5%)	[16]
	Black-capped capuchin (*Cebus apella*)	22	6 (27.3%)	[42]
Ursidae	Giant panda (*Ailuropoda melanoleuca*)	200	69(34.5%)	[16]
	Asiatic black bears (*Ursus thibetanus*)	106	29(27.4%)	[42]
Bovidae	Golden takin *(Budorcas taxicolor bedfordi)*	191	28(14.7%)	[42]
Anatidae	Whooper swans (*Cygnus cygnus*)	467	35(7.5%)	[42]
Moschidae	Musk deer (*Moschus berezovskii*)	223	38(17.0%)	[42]
Lemuridae	Ring-tailed lemur (*Lemur catta*)	45	11(24.0%)	[42]
Hominidae	Bornean orangutan (*Pongo pygmaeus*)	23	4 (17.4%)	[16]
Macropodidae	Red kangaroo (*Macropus rufus*)	38	14(36.8%)	[38]
	Grey kangaroo (*Macropus fuliginosu*)	23	0 (0.0%)	[38]

Four observed genotypes in this study are completely different from genotypes previously identified in red panda (EBPC, CHB1)[13, 19, 28]. As the dominant genotype, PL2 was only found in masked palm civets (*Paguma larvata*) and formed a clade related to Group 2 in phylogenetic analysis. [33, 39]. All positive isolates from CDRBGPB were PL2, which revealed red panda is a new host of genotype PL2, which expands the host range of this genotype. The high prevalence in CDRBGPB indicates that red pandas may occur inter-species transmission and be an adaptive host of PL2, but the infection route is unknown and needs more future research. Long-term monitoring and further study are also required to ascertain whether the semi-free-range breeding mode has increased the transmission of *E. bieneusi* within the red panda populations and the potential risk of cross-specific transmission. Both Chengdu Zoo and Bifengxia Zoo observed only 1 genotype D isolates. Considering that genotype D has previously been discovered in

the above-mentioned zoos, pathogens originated from other animal hosts are possible [26, 40]. Genotype SC02 was once detected in humans and various animals such as Asian black bears, Tibetan blue bears, sun bears, raccoons, horses, and squirrels [19, 41, 42]. The exchange and recombination in ITS genes of *E.bieneusi* may have resulted in genetic diversity and the new genotype SCR1 [43].

MLST analysis showed that amplification efficiency is different in various ITS genotypes, which is consistent with previous studies [11]. We found 3 MLGs, which showed higher genetic diversity in PB isolates than the other 5 sampling sites; however, the influence of sample size cannot be excluded. MLST tools revealed higher resolution and genetic diversity than ITS sequence genotyping, which is according to the conclusions of previous studies[18, 41, 42, 44]. The amplification efficiency of locus MS4 was higher than MS1, MS4, and MS7, which was inconsistent with previous reports[14, 45].

Conclusions

In conclusion,this study clear up the *E.bieneusi* prevalence and genotypes of captive red pandas in Sichuan zoos. Resultantly, captive red pandas are new hosts for identified genotypes, and the semi-free range breeding mode can be a significant risk factor in *E.bieneusi* prevalence. Genotype D, SC02, and SCR1 phylogenetically clustered in Group 1 indicated the red panda with nonnegligible zoonotic risk and can be a potential source of human-pathogenic microsporidia. Zoos should continue to monitor the epidemic of red pandas *E.bieneusi* infection and take necessary measures to reduce the infection. The risk of zoonosis in different breeding modes also needs to be further studied to ensure the safety of animals and human beings.

List Of Abbreviations

ITS: Internal Transcribed Spacer; MLGs: Multilocus genotypes; MLST: Multilocus Sequence Typing; PCR: Polymerase chain reaction; rRNA: ribosomal RNA; BLAST: Basic Local Alignment Search Tool; AT:Annealing temperatures; CI: Confidence Intervals; ORs: Odds Ratios; CDBGPB: Chengdu Research Base of Giant Panda Breeding; SLR: Sanlang Resort; BFXZ: Bifengxia Zoo; CDZ: Chengdu Zoo; WCGPG: Wolong China Giant Panda Garden; PV: Panda Valley;

Declarations

Ethics approval and consent to participate

This study Collect fecal samples of captive red pandas with the permission of the owner and/or administrator. All procedures are strictly followed by the requirements of the Procedures and Guidelines for Animal Ethics of the People's Republic of China.

Consent for publication

Not applicable.

Availability of data and materials

The datasets collected and/or analyzed during the current study are available

from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Yang-Yang Zeng, Wan-Yu Meng, and Song-Rui Liu are equally contributed to this study.

Competing interests

All authors related in this study have no conflicts interests and competing interests.

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Figures

Figure 1

The sampling sites of captive red pandas in Sichuan province, southwest China.

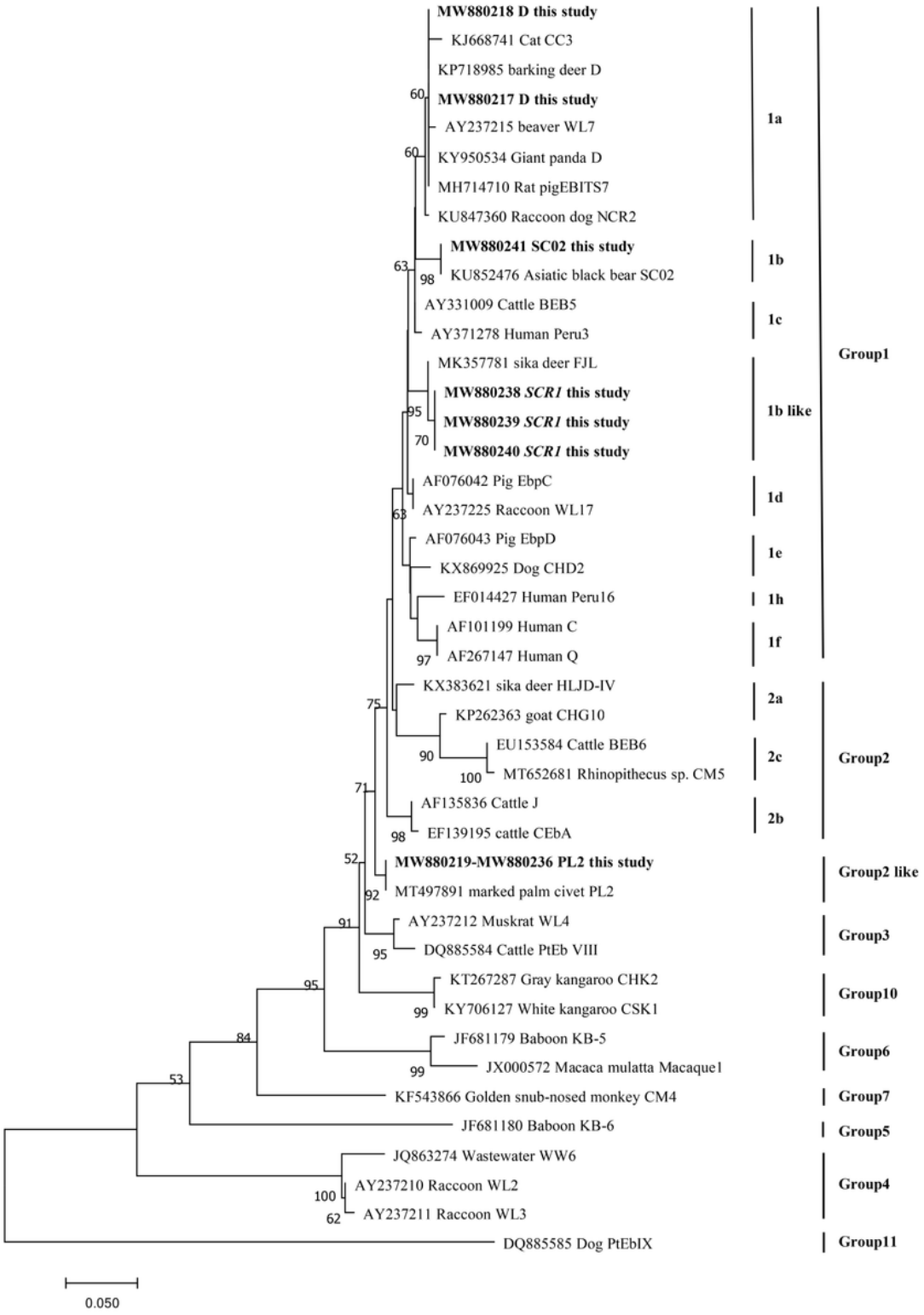

Figure 2

Phylogenetic tree of Enterocytozoon bieneusi genotypes based on ITS sequences analyzing. Phylogenetic relationships between obtained sequences in the present study and other sequences downloaded in GenBank. The known and novel E. bieneusi genotypes identified in this study are shown in bold and the novel genotypes are shown in italic. Genotype PtEb IX (DQ885585) from dogs was used as an outgroup.