

## New genotypes and molecular characterization of *Enterocytozoon bieneusi* in captive black bears in China

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### ABSTRACT

*Enterocytozoon bieneusi*, a common eukaryotic obligate intracellular parasite, can infect a wide range of hosts, including humans and domestic animals. There have been some reports of this organism in captive wildlife animals worldwide, but few studies have reported its detection in the captive black bears in Sichuan province of southwestern China. The present study was performed to determine the prevalence, genetic diversity, and zoonotic potential of *E. bieneusi* in captive Asiatic black bears from three farms in Sichuan province. Fecal specimens from Asiatic black bears in three farms were collected and analyzed for the prevalence of *E. bieneusi*. The overall prevalence of *E. bieneusi* was 18.7% (57/305) as determined by nested PCR amplification of the Internal Transcribed Spacer (ITS) gene on the rRNA of *E. bieneusi*, with the highest prevalence in the farm being 47.8% (44/92). Altogether, five genotypes of *E. bieneusi* were identified among the 57 *E. bieneusi*-positive samples, comprising three known genotypes (SC02, MJ2, and MJ5) and two novel genotypes named SCBB1 and SCBB2. Phylogenetic analysis showed that the genotypes SC02 and MJ2 were clustered into group 1 of zoonotic potential and that the genotypes MJ5, SCBB1, and SCBB2 were clustered into group 10. In conclusion, two known genotypes, SC02 and MJ2, were found to belong to the zoonotic potential group 1 and this evidence points to the fact that the *E. bieneusi* from these black bears could infect humans.

### 1. Introduction

Microsporidia are a group of emerging obligate intracellular pathogens. These species have been proven to be able to infect invertebrates and vertebrates and are responsible for causing diarrhea in a wide range of hosts (Andriole and Finch, 2016). The phylum Microsporidia comprises approximately 1300 species in 160 genera known to date, and at least 14 microsporidian species have been reported in humans (Shi et al., 2016). The fecal-oral route is the primary mode of infection by *E. bieneusi*. Generally, clinical manifestations caused by *E. bieneusi* in healthy individuals are self-limiting diarrhea and malabsorption. However, immunocompromised patients are more likely to be infected by *E. bieneusi* and are at risk of life-threatening diarrhea (Lin et al., 2013; Matos et al., 2012). Since 1985, when *E. bieneusi* was first reported in a Haitian patient with AIDS, this pathogen has been regarded as an emerging pathogen, attracting public health concern (Desportes et al., 2010).

The Internal Transcribed Spacer (ITS) region of the rRNA gene, which possesses considerable genetic variation, has been widely used for genotyping *E. bieneusi* isolates in humans and animals (Lin et al., 2013; Wagnerová et al., 2015). Based on sequence variations in the ITS

region, more than 474 different genotypes of *E. bieneusi* have been identified and the numbers are still growing (Li et al., 2019). Some genotypes considered to be zoonotic genotypes have been identified in humans and animals such as D, CAF1, EbpC, Type IV, and WL11 (Ma et al., 2015a; Qi et al., 2018; Md Robiul et al., 2014). Meanwhile, other genotypes are deemed to be host-specific or host-adapted (Li et al., 2019; Yue et al., 2014; Zhang et al., 2018).

In China, *E. bieneusi* has been found in humans, domestic animals, livestock, and wild animals (Deng et al., 2018; Yanxue et al., 2015) and there are only two literature sources citing *E. bieneusi* infections in captive Asiatic black bears (Deng et al., 2017). Due to the fact that Asiatic black bears are widely kept in farms for their economic and medical value, as well as owing to the presence of high-density feeding environments, infective *E. bieneusi* spores from infected black bears are easily spread between different individuals. This may increase the potential risk to the breeder and the public. In light of these circumstances, the aim of the present study was to determine the prevalence of *E. bieneusi* in captive Asiatic black bears in farms and evaluate its genetic diversity via ITS sequencing analysis to assess possible implications for public health.

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## 2. Methods

### 2.1. Ethics statement

The study protocol was reviewed and approved by the Research Ethics Committee and the Animal Ethical Committee of Sichuan Agricultural University. Appropriate permissions were obtained from farm managers before the collection of fecal specimens.

### 2.2. Specimen collection

In total, 305 fecal specimens were collected between August 2017 and November 2018 from Asiatic black bears in three different farms in the Sichuan provinces of southwestern China (Table 1). All animals were healthy, and the age, gender, and clinical signs were recorded. Fresh fecal specimens from each Asiatic black bear were collected immediately after defecation on the ground and transferred individually into clean 50-ml plastic containers. All fecal specimens were stored at 4 °C in 2.5% (w/v) potassium dichromate.

### 2.3. DNA extraction

Fecal specimens were processed by sieving; then, samples were concentrated and washed three times with distilled water by centrifugation for 10 min at 1500×g. Genomic DNA was extracted from approximately 200 mg of processed specimens, using an EZNA® RStool DNA kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer's protocol. DNA was eluted in 200 µl of absolute ethanol and stored at –20 °C until use for PCR analysis.

### 2.4. PCR amplification

The extracted DNA was examined for the presence of *E. bieneusi* by nested PCR amplification of a 389 bp nucleotide fragment of the rRNA gene containing 76 bp of the 3'-end of the Small Subunit (SSU) rRNA gene, 243 bp of the ITS region, and 70 bp of the 5'-region of Large Subunit (LSU) rRNA gene. The primers and cycling parameters employed for these reactions were as previously described (Sulaiman et al., 2003; Yaoyu et al., 2011). TaKaRa Taq™ DNA Polymerase (TaKaRa Bio, Otsu, Japan) was used for all PCR amplifications. A negative control with no DNA added was included in all PCR tests. All secondary PCR products were subjected to electrophoresis on a 1% agarose gel containing ethidium bromide.

### 2.5. Nucleotide sequencing and analysis

The secondary PCR products of anticipated size were directly sequenced by Life Technologies (Guangzhou, China), using a BigDye 1 Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA). Sequence accuracy was confirmed by two-directional

sequencing and by sequencing a new PCR product if necessary. Nucleotide sequences obtained in the present study were aligned with each other and reference sequences downloaded from GenBank using the Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>) and ClustalX 1.83 (<http://www.clustal.org/>) to determine the genotypes of *E. bieneusi*. The genotypes were assigned previously published names if they were found to be identical to known genotypes. Genotypes with single nucleotide substitutions, deletions, or insertions in the ITS gene region of the 243 bp sequence of *E. bieneusi* relative to known genotypes were considered novel genotypes and named according to the established nomenclature system (Santin and Fayer, 2010).

### 2.6. Phylogenetic analysis

To assess the genetic relationships between the *E. bieneusi* genotypes in the present study and reference sequences previously published in GenBank, phylogenetic analysis was performed by constructing a neighbor-joining tree using Mega 6 software (<http://www.megasoftware.net/>), which is based on evolutionary distances calculated using a Kimura 2-parameter model. The reliability of these trees was assessed using bootstrap analysis with 1000 replicates.

### 2.7. Statistical analysis

The  $\chi^2$ -test was performed to compare *E. bieneusi* infection rates between different sampling farms. A P-value of P < 0.05 when determining statistical significance in the differences between different sampling farms was considered significant. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were also calculated to explore the strength of the association between *E. bieneusi* positivity and each factor.

### 2.8. Nucleotide sequence accession numbers

Representative nucleotide sequences were deposited into the GenBank database under the following accession numbers: MK547515 to MK547519 for the rRNA gene ITS sequences of *E. bieneusi*.

## 3. Results

### 3.1. Prevalence of *E. bieneusi* in Asiatic black bears

In total, 57 of the 305 fecal specimens from Asiatic black bears (18.7%) were found to be positive for *E. bieneusi* via ITS-PCR amplification (Table 1). All three tested farms showed evidence of *E. bieneusi* infection, and the infection rate of *E. bieneusi* in the different farms ranged from 4.5% to 47.8%. The highest infection rate of *E. bieneusi* was observed in farm 3 (47.8%, 44/92), followed by farm 2 (7.2%, 9/125) and farm 1 (4.5%, 4/88). The differences in infection rate among the

**Table 1**  
Prevalence and genotypes of *E. bieneusi* in Asiatic black bears from farms in Sichuan province.

Sources	No. of tested	No. of positive	Prevalence (%)	95% C I	OR	P value	Genotype (n)
farm 1	88	4	4.5	1.1–9.1	Reference	< 0.01	SC02(2), MJ2 (1), SCBB1(1)
farm 2	125	9	7.2	3.2–12	0.61 (0.18–2.06)		SC02(1), MJ5 (1)
farm 3	92	44	47.8	37–58.7	19.3 (6.52–56.9)		SC02(33), MJ2 (3), MJ5 (7) SCBB2(1)
Total	305	57	18.7	14.8–23			SC02(38), MJ2 (4), MJ5 (13), SCBB 1 (1), SCBB 2 (1)

**Table 2**  
Factors associated with prevalence and genotype of *E. bienersi* in Asiatic black bears from farms in Sichuan province.

Category	No. of examined	No. of positive	Prevalence (%)	95% C I	OR	P value	Genotype (n)
Gender							
F	147	25	17.0	10.9–23.8	Reference	0.47	SC02(14), MJ2 (3), MJ5 (7) SCBB 1 (1)
M	158	32	20.3	13.9–6.6	1.24 (0.69–2.21)		SC02(24), MJ2 (1), MJ5 (6), SCBB 2 (1)
Total	305	57	18.7	14.8–23			SC02(38), MJ5 (13), MJ2 (4), SCBB 1 (1), SCBB 2 (1)
Age (year)							
0–0.5	26	4	15.4	3.8–30.8	Reference	0.69	SC02(2), MJ5 (1), SCBB2(1)
0.5–1.5	82	16	19.5	11–29.2	1.33 (0.40–1.41)		SC02(12), MJ2 (3), MJ5 (1)
1.5–5	68	15	22.1	13.2–32.4	1.56 (0.46–5.22)		SC02(10), MJ5 (5)
5–15	99	19	19.2	12.1–27.3	1.31 (0.40–4.24)		SC02(13), MJ5 (5), SCBB1(1)
15–20	30	3	10.0	0–23.3	0.61 (0.12–3.03)		SC02(1), MJ2 (1), MJ5 (1)
Total	305	57	18.7	14.8–23			SC02(38), MJ5 (13), MJ2 (4), SCBB 1 (1), SCBB 2 (1)

three farms were statistically significant ( $P < 0.01$ ) (Table 1). No statistically significant differences were noted with respect to the ages ( $P = 0.47$ ) or gender ( $P = 0.69$ ) of the bears (Table 2).

### 3.2. Genetic characterization and genotype distribution of *E. bienersi* in Asiatic black bears

Five genotypes were identified by sequence analysis of the ITS gene in 57 *E. bienersi*-positive specimens, including three known genotypes (SC02, MJ2, MJ5) and two novel genotypes. The two novel genotypes were named SCBB1 and SCBB2. Genotype SC02, which was identified in the largest number of *E. bienersi*-positive specimens (66.7%, 38/57), was present in all three farms, followed by genotype MJ5 (22.8%, 13/57), which was detected in farm 2 and farm 3 and genotype MJ2 (7%, 4/57), which was detected in farm 1 and farm 3.

One Single Nucleotide Polymorphisms (SNP) within the 243-bp region of the ITS gene sequence of *E. bienersi* was found in each of the two novel genotypes compared to the known genotypes. Genotype SCBB1 had one SNP (insertion: G) relative to genotype CHB1 (KU852466). Genotype SCBB2 had another SNP (substitution: G/C) relative to genotype MJ5 (MF522186).

### 3.3. Phylogenetic analysis

Phylogenetic analysis by the neighbor-joining method based on the ITS gene sequences of *E. bienersi* indicated that the five genotypes obtained in the present study belonged to three distinct groups: SC02 and MJ were clustered into group 1 and further classed into subgroup 1b while genotypes MJ5, SCBB1 and SCBB2 were clustered into group 2 (Fig. 1).

## 4. Discussion

In the present study, an overall infection rate of 18.7% (57/305) was observed in captive Asiatic black bears. The *E. bienersi* infection rate observed in this study was lower than the previously reported rates of 27.4% (29/106) and 19.75% (80/405) reported for captive black bears in zoos in southwest China and in the Yunnan province respectively (Wu et al., 2018). Moreover, it is lower than the previously reported rates of 27% and 29.8% reported for captive wildlife in the Bifengxia Zoo (Li et al., 2016) and the zoos in east China (Karim et al., 2015) respectively. Similarly, the infection rate was also significantly lower than the rate of 40% (2/5) observed in black bears in New York City [23]. However, the present infection rate is higher than the rate of 15.8% and 10.6% (21/198) reported for captive wildlife in the

Zhengzhou Zoo and Chengdu Zoo in China (Li et al., 2015, 2016). The observed differences in the infection rate of *E. bienersi* among different farms may be explained by variations in feeding density, geography, management system, sample size, and climate.

In the present study, three known genotypes (SC02, MJ2 and MJ5) and two novel genotypes named SCBB1 and SCBB2 were identified by analyzing the ITS gene sequences of *E. bienersi*. Genotype SC02 was the most prevalent genotype among the *E. bienersi*-positive isolates (66.7%, 38/57), which is in contrast with the results of a previous study in Yunnan province, where MJ2 was found to be the most dominant genotype (Wu et al., 2018). It is also not in agreement with the data on captive black bears in zoos in the Sichuan and Guizhou province, where CHB1 was found to be the most dominant genotype (Deng et al., 2017). These differences may be caused by regional disparity and the infection of the drinking water with parasitic spores. Genotype SC02 has a variety of hosts, such as Tibetan blue bears, sun bears, northern raccoons, horses, and squirrels, suggesting that the parasites from black bears can spread to other animals raised in the same farm or nearby (Deng et al., 2016; Li et al., 2016). Genotypes MJ2 and MJ5 were first found in black bears in the Yunnan Province (Wu et al., 2018). These genotypes seem to be specific to black bears.

A phylogenetic analysis based on a neighbor-joining tree of ITS gene sequences showed the genetic relationship of the five obtained genotypes of *E. bienersi* with the known genotypes. The two genotypes, SC02 and MJ2, belonged to group 1, which is composed of genotypes almost exclusively found in humans, indicating their potential for zoonotic transmission and risks to public health (Akinbo et al., 2013; Lin et al., 2013; Ma et al., 2015b). The genotype MJ5 was clustered into group 10 with the two new genotypes SCBB1 and SCBB2. Group 10 is a new group and most *E. bienersi* in this group have been detected in black bears, or to a lesser extent, in marsupials (Li et al., 2015; Zhang et al., 2018). Therefore, further molecular epidemiological studies are required in order to investigate the potential ability of the identified genotypes in group 10 to cause microsporidiosis in humans and other animals.

In conclusion, the present study demonstrated the prevalence rate of *E. bienersi* was 18.7% (57/305) in Asiatic black bears from the farms in Sichuan province. We also found three known genotypes of *E. bienersi*, SC02, MJ2 and MJ5, and two novel genotypes, SCBB1 and SCBB2, in Asiatic black bears. The previous identification of genotype SC02 in humans indicates that Asiatic black bears may represent a potential host for transmission of microsporidiosis to humans and animals. Our findings indicate the need for appropriate strategies to control and prevent the transmission of this pathogen from captive Asiatic black bears to humans and other animals.

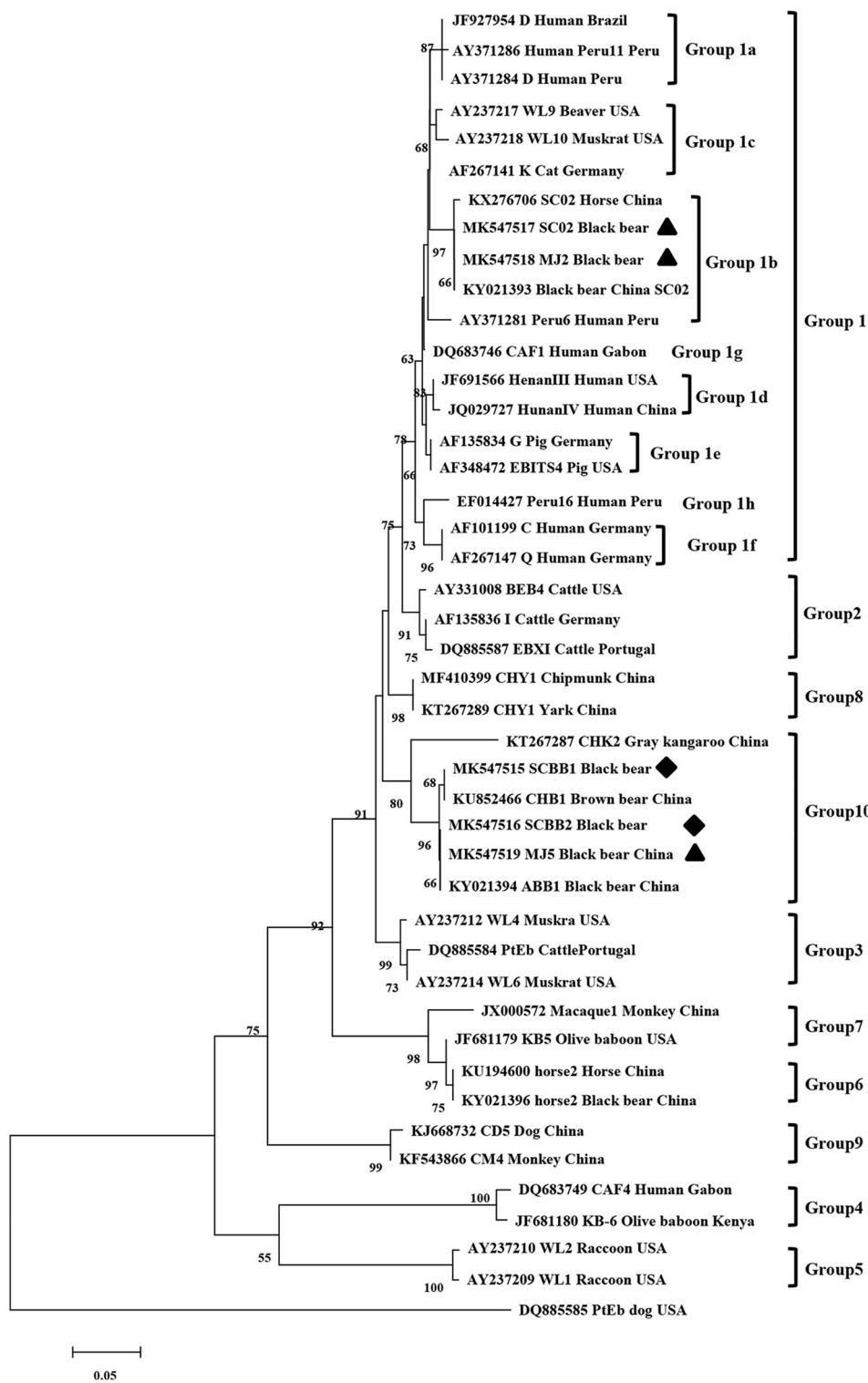


Fig. 1. Phylogenetic relationship among the *Enterocytozoon bieneusi* groups. The relationship between the *E. bieneusi* genotypes identified in this study and other known genotypes deposited in GenBank was inferred by neighbor-joining analysis of ITS sequences based on genetic distance using the Kimura-2-parameter model. The numbers on the branches represent percent bootstrapping values from 1000 replicates, with more than 50% shown in the tree. Each sequence is identified by its accession number, genotype designation, and host origin. Genotypes marked with black rhombuses and black triangles are novel and known genotypes identified in this study, respectively.

**Conflicts of interest**

The authors declared that they have no conflicts of interest to this work.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijppaw.2019.06.012>.

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