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Epidemiological and Molecular Investigation of Multilocus Genotype of *Enterocytozoon bieneusi* Using Nested Polymerase Chain Reaction Sensor

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The zoonotic risk and genetic diversity of *Enterocytozoon bieneusi* (*E. bieneusi*) and its infection in wildlife were investigated. A total of 70 fecal samples were collected from the South China Tiger Breeding Research Institute in Meihua Mountains and the Fuzhou Zoo of China in Fujian Province. *E. bieneusi* was assayed on the basis of the internal transcribed spacer (ITS) regions of the ribosomal RNA (rRNA) gene by nested polymerase chain reaction (PCR) with a complementary metal-oxide-semiconductor (CMOS) sensor. Four positive isolates of *E. bieneusi* were detected (5.7%) from three sika deer and one species of the Hylobatidae family (gibbons). Multilocus sequence typing (MLST) of ITS indicated two genotypes, namely, BEB6 and Type IV at MS1, MS3, MS4, and MS7. Type IV belongs to Group 1 with zoonotic potential. The amplification efficiency at the MS1, MS3, MS4, and MS7 sites was 50% (2/4). Among the four positive isolates, two positive isolates of sika deer were amplified simultaneously at the four sites. Nucleotide sequence analyses showed 1, 2, 1, and 2 nucleotide variant haplotypes at the MS1, MS3, MS4, and MS7 sites, respectively. In the 1, 2, 1, and 2 genotypes, two multilocus genotypes (MLGs) were formed. The comprehensive measures for determining the genetic diversity of *E. bieneusi* contribute to preventing or controlling the global spread of *E. bieneusi* in wildlife.

1. Introduction

Microsporidia are widely distributed and highly endemic organisms that infect a variety of invertebrates and vertebrates including humans.[1–3] There are more than 1400 species of microsporidia in 160 genera, and 14 species infect humans.[4] Microsporidia cause autoimmune diarrhea and immunodeficiency in immunocompromised hosts. In particular, AIDS patients

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suffer from fatal diarrhea given that microsporidia are considered to be important pathogens of zoonotic diseases.[5,6] Since the first discovery from an AIDS patient in Haiti in 1985, many cases of microsporidia infection have been reported in a variety of hosts worldwide, including humans, companion animals, livestock, birds, and wildlife.[2,7–9] The infection threatens public safety and causes considerable economic losses.

Significant advances have been made in developing genetic markers and genotyping of *Enterocytozoon bieneusi* (*E. bieneusi*), which allows the understanding of host specificity, transmission routes, and clinical picture of human microsporidiosis, along with the evolution of *E. bieneusi*. Currently, a high degree of genetic polymorphism in the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene of *E. bieneusi* was found in the sequence analysis of the ITS region, which is the standard method for species identification and genotyping of *E. bieneusi* isolates. The ITS sequence of the *E. bieneusi* rRNA is highly polymorphic between different isolates.[10,11] At present, the genotypes of *E. bieneusi* are divided into 11 groups according to ITS sequence differences. Group 1 has a zoonotic genotype that causes widespread concern in humans, whereas groups 2 to 11 are host-specific groups and do not have public health significance. However, the increased number of *E. bieneusi* genotypes makes it impossible to identify the source and route of the genotype of a single ITS gene, and whether the same ITS gene is polymorphic.[12]

rRNA gene sequencing is usually conducted by polymerase chain reaction (PCR) that enables DNA amplification. It uses a fluorescence detection system that counts the number of copied DNAs in a sample.

To accurately analyze the source and genetic evolution of *E. bieneusi* genotypes, Feng *et al*.[13] developed a multilocus sequence typing (MLST) method and identified microsatellite loci (MS1, MS3, and MS7) and one minisatellite locus (MS7) for ITS-positive samples of *E. bieneusi*. This method has high sensitivity and enables the comprehensive analysis of the genetic characteristics of the evolution of *E. bieneusi*.[4,14]

Fujian in China has abundant wildlife resources, where the survival of wild animals plays an important role in maintaining the balance and stability of the ecosystem. At present, the ecosystem is threatened by infection by *E. bieneusi*, which requires in-depth research. In this study, we investigated for the first time the infection by *E. bieneusi* and analyzed the risk in the zoonosis and genetic diversity of the wild animals in Fujian. The result provides basic information and knowledge of the infection by *E. bieneusi* for the prevention of related diseases and the effective protection of the wild animals.

2. Materials and Methods

2.1 Sample collection and preservation

Seventy fresh fecal samples from 28 species of wild animals (Table 1) were collected at the South China Tiger Breeding Research Institute of Meihua Mountain in Fujian and the Fuzhou Zoo from March to April 2018. Around 30 g of each sample was taken in a disposable plastic bag on which the animal species, location, and date of the collection were indicated. The samples were stored in 2.5% potassium dichromate solution at 4 °C for molecular detection.

Table 1
Species of wild animals from Fujian province in this study.

Animal groups	Number of species
Primates	Baboon (2), Macaque (2), Golden monkey (1), King Kong monkey (2), Black-capped capuchins (1), Ring-tailed lemur (1), Hamadryas baboon (1), Black langur (1), Red monkey (1), Golden-handed tamarin (1), Squirrel monkey (1), Gibbon (2), Black baboon (1), Golden monkey (1)
Carnivora	South China tiger (2), Meerkat (4), Red panda (3), Raccoon (4)
Birds	Gate chicken (6), Hornbill (3), Mandarin duck (1), Peacock (4)
Artiodactyla	Sika deer (17), Alpaca (1), Takin (1), Longhorn antelope (2), Hippo (1)
Perissodactyla	Rhinoceros (3)

2.2 Primary reagent

The fecal genome E.Z.N.A® DNA (OMEGA) extraction kit was used with the following reagents: rTaq DNAse, 10× rTaq buffer, 2.5 mM dNTP, and 25 mM $MgCl_2$ (Beijing Quanjin Biotechnology Co., Ltd.). Loading buffer (6×) and DL2000 DNA marker from Dalian Bao Bioengineering Co., Ltd were used with the primers synthesized by Shanghai Shenggong Biotechnology Co., Ltd.

2.3 Genomic DNA extraction from fecal samples

Two hundred milligrams of each sample were transferred into a 2 ml centrifuge tube. Then, 1 ml of double-distilled water was added and mixed with the sample. After the mixture was centrifuged at 12000 revolutions per minute (RPM) for 1 min, the supernatant was discarded. This process was repeated three times until the added potassium dichromate was washed away completely. Then, the centrifuge tube containing the sample was placed in a hot water bath at 100 °C for 5 min and frozen in an ultralow-temperature refrigerator at –80 °C for 5 min. After five times of repeated freezing and thawing, DNA extraction was carried out according to the instructions of the fecal genome E.Z.N.A® DNA extraction kit. The obtained fecal genomic DNA was stored at −20 °C until used in the experiment.

2.4 PCR amplification and sequencing

Nested PCR was used to amplify the ITS of wild animals' small subunit ribosomal RNA (SSU rRNA). *E. bieneusi* was identified after obtaining ITS-positive isolates by the method of Yue *et al*.[15] MLST was used to select multiple sites of MS1, MS3, MS4, and MS7 for ITS gene-positive samples based on the method of Feng *et al*.[13] The PCR amplification was carried out with a reaction system of 25 μl and repeated twice.

The procedure of nested PCR was as follows:

(1) pre-denaturation at 94 °C for 5 min for 35 cycles,

(2) denaturation at 94 °C for 45 s, annealing for 45 s, and extension at 72 °C for 1 min, and

(3) second extension at 72 °C for 7 min.

The primer sequences are shown in Table 2. After the above process, the amplification products were obtained by 1% agarose gel electrophoresis. The products were then sent to Guangzhou Qingke Biotechnology Co., Ltd. for bidirectional sequencing.

2.5 Sequential and phylogenetic analyses

The bioinformatics software Clustal X 1.83 was used to align the two positive and negative complementary nucleotide sequences obtained by sequencing. The similarity of the entire splice sequence was searched on the NCBI blast, and the homology was downloaded. The highest sequence was aligned with the obtained sequence to analyze sequence differences. With the Mega 7.0 software, the Kimura–2 parametric model in the neighbor-joining (NJ) method was used to construct a phylogenetic tree. The reliability of the phylogenetic tree was tested by bootstrap analysis for 1000 replicates. Gene polymorphisms of positive samples from three microsatellite loci and one minisatellite locus were also analyzed using the Mega 5.0 software.

3. Results and Discussion

3.1 Genotypic and phylogenetic analyses of *E. bieneusi*

Four positive samples of approximately 392 bp were successfully amplified (Fig. 1). The total infection rate was 5.7% (4/70), among which, three were fecal samples from sika deer and one from gibbons.

Table 2
Primers for nested PCR and annealing temperature.

Gene	Primer	Sequence (5'–3')	Annealing temperature (°C)	Fragment length (bp)
ITS	F1	GCTCTGAATATCTATGGCT	55	410
	R1	ATCGCCGACGGATCCAAGTG		
	F2	GGTCATAGGGATGAAGAG	55	392
	R2	TTCGAGTTCTTTCGCGCTC		
MS1	F1	CAAGTTGCAAGTTAGTGTTTGAA	58	843
	R1	GATGAATATGCATCCATTGATGTT		
	F2	TTGTAAATCGACCAAATGTGCTAT	58	675
	R2	GGACATAAACCACTAATTAATGTAAC		
MS3	F1	CAAGCACTGTGGTTACTGTT	55	702
	R1	AAGTTAGGGCATTTAATAAAATTA		
	F2	GTTCAAGTAATTGATACCAGTCT	55	537
	R2	CTCATTGAATCTAAATGTGTATAA		
MS4	F1	GCATATCGTCTCATAGGAACA	55	1066
	R1	GTTCATGGTTATTAATTCCAGAA		
	F2	CGAAGTGTACTACATGTCTCT	55	885
	R2	GGACTTTAATAAGTTACCTATAGT		
MS7	F1	GTTGATCGTCCAGATGGAATT	55	684
	R1	GACTATCAGTATTACTGATTATAT		
	F2	CAATAGTAAAGGAAGATGGTCA	55	471
	R2	CGTCGCTTTGTTTCATAATCTT		

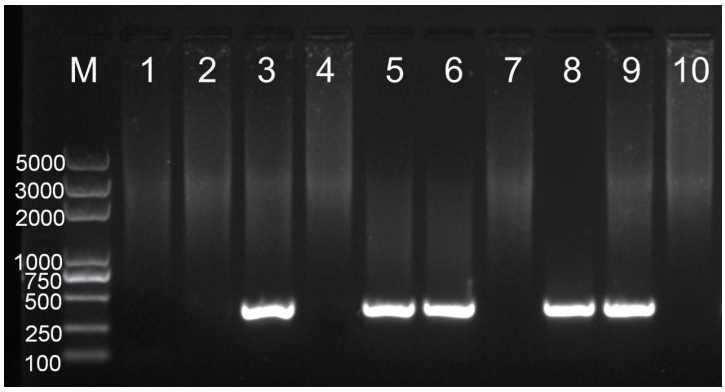

Fig. 1. TS sequence of *E. bieneusi* amplified by PCR through electrophoresis. M: DNA Marker DL 5000; 1–8: Fecal samples; 9: Positive control; 10: Negative control.

The positive PCR products were sequenced and analyzed. We identified two genotypes of *E. bieneusi*, which had a 100% similarity to the genotype Type IV nucleotide sequence (KX964628, KJ651436, and KF305582) and the genotype BEB6 nucleotide sequence (KX383616, KX383615, and KR815519) and a 99% similarity to the genotype CHN-H1 nucleotide sequence. The genotype Type IV was identified from one sika deer sample and one gibbons sample, and the BEB6 nucleotide sequence was identified from one sika deer sample. A different sika-deer-derived *E. bieneusi* was found in one of the sika deer samples with three base differences from the reported genotype CHN-H1. The peak map result confirmed that the similarity was 99%. The ITS sequences of three genotypes of *E. bieneusi*, which were identified from the wild animals in this study, were compared with other microsporidia genotypes reported in GenBank. The result showed that the homology was 91.5−100% (Fig. 2). The accession number was found to be MK357778-MK357781 when the obtained sequence was uploaded to NCBI.

A phylogenetic tree was constructed on the basis of the homology of the ITS sequences. Phylogenetic analysis results confirmed that one of the identified *E. bieneusi* was on the same evolutionary branch as the cat source Type IV isolate (KX964628), the other identified *E. bieneusi* from one sika deer sample as the deer source BEB6 isolate (KX383616), and the last one, a human CHN-H1 isolate (MG255733). The last one had three base differences but its isolate is regarded to be a new subtype of the genotype CHN-H1 and named FJL (Fig. 3).

As the microsporosis pathogens are small, their spores can only be identified by electron microscopy and staining techniques. As even experienced scientists may be affected by subjective factors and cause judgment errors, accurately identifying microsporidian species is not feasible.[16] The ribosomal ITS of *E. bieneusi* is highly polymorphic between different isolates. Thus, molecular detection based on ITS sequences has become the standard for the identification of *E. bieneusi*. According to the ITS sequence, more than 200 genotypes have been detected and divided into 11 groups, including humans, companion animals, livestock, birds, wild animals, and so on.[10]

Microspores of *E. bieneusi* were detected from wild animals in Fujian for the first time in this study. The results showed that the infection rate by *E. bieneusi* was 5.7% (4/70), and the infection

Percent Identity

Divergence

	1	2	3	4	5	6	7	
1		94.1	92.3	91.5	100.0	94.0	93.4	1
2	6.2		100.0	98.7	94.0	100.0	98.9	2
3	8.1	0.0		98.5	94.0	100.0	98.9	3
4	9.0	1.4	1.6		93.2	98.6	99.2	4
5	0.0	6.3	6.3	7.2		94.0	93.4	5
6	6.3	0.0	0.0	1.4	6.3		98.9	6
7	6.9	1.1	1.1	0.8	6.9	1.1		7
	1	2	3	4	5	6	7	

1 KX383616-red deer-BEB6.seq
2 KX964628-TypeIV-cat.seq
3 MF693832-004-52-sambar deer-.seq
4 MG255733-CHN-H1-Homo sapiens.seq
5 MK357778-sika deer-BEB6.seq
6 MK357780-Hylobatidae-TypeIV.seq
7 MK357781-sika deer-FJL.seq

Fig. 2. Homology (upper right corner) and genetic distance (lower left corner) between ITS sequences of *E. bieneusi* from Fujian wild animals and other mocrosporidians.

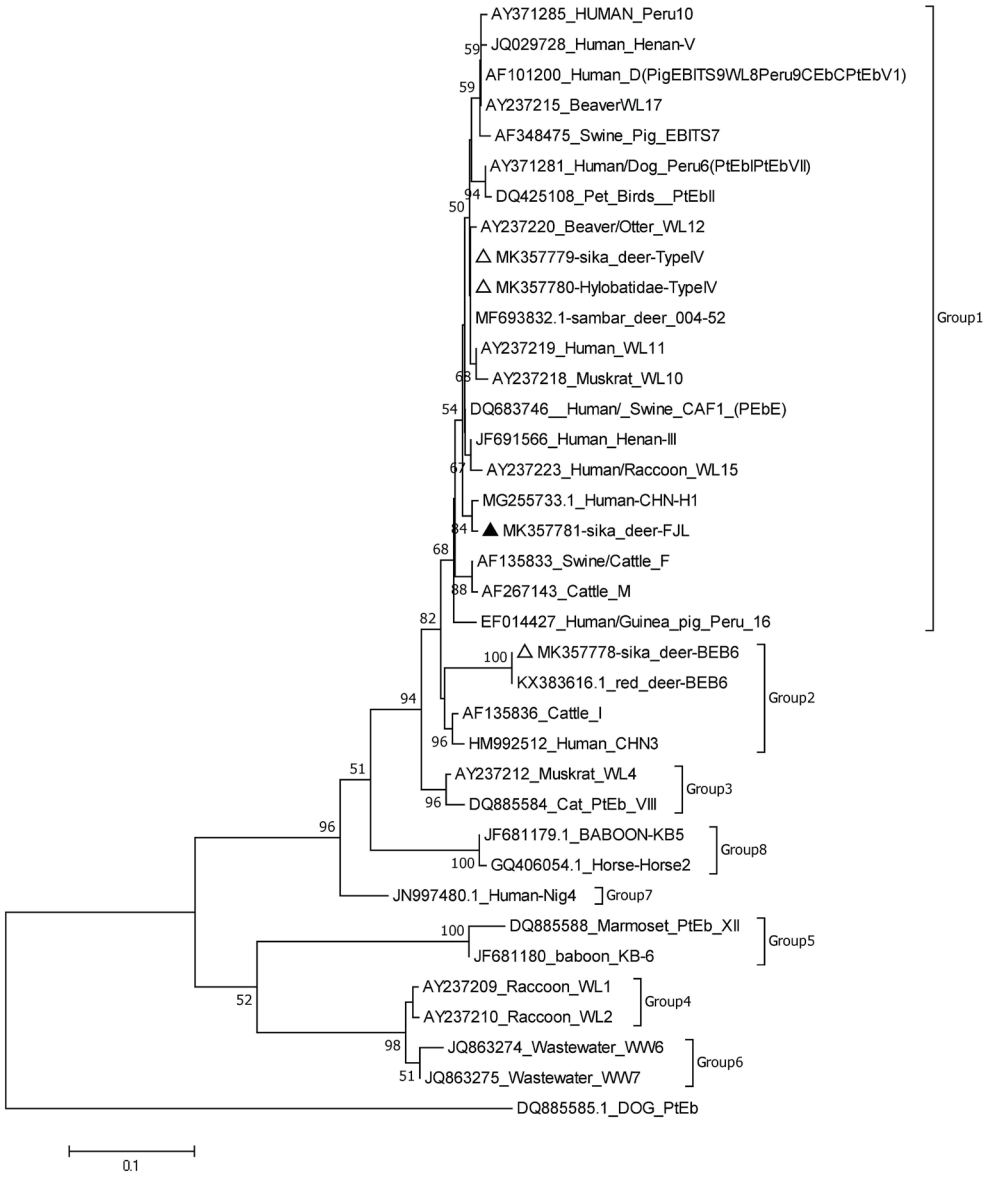

Fig. 3. Phylogenetic tree based on ITS sequence homology of microsporidians.

was found from sika deer and gibbons. Sequence alignment analysis of ITS loci identified two known genotypes, BEB6[1] and Type IV,[2] and a new sub-genotype CHN-H1.[1] FJL and Type IV from humans and BEB6 from animals belong to Group 1 in comorbid potential.[17,18] Li *et al*.[19] suggested the possibility of microsporidia transmission between nonhuman primates and humans from the finding that 29 samples of *E. bieneusi* (12.3% of the total 235 fecal samples) from primates in Kenya were positive. The sequencing analysis showed that the samples belonged to 10 genotypes (i.e., four known genotypes, namely, A, D, Peru7, and Peru11, and six new genotypes). The nucleic acid sequences and reference sequences of these 10 genotypes formed three phylogenetic groups in the phylogenetic analysis. In the 10 *E. bieneusi* genotypes, four genotypes were found in human infections as having a similar evolutionary relationship to the genotypes that can infect humans. Li *et al*.[20] studied the captive wildlife at Zhengzhou Zoo in China and found that the infection rate by *E. bieneusi* was 15.8% out of 32 specimens. Microsporidia infections were also found in cloven-like animals such as sika deer and primates such as gibbons, Hamadryas baboons, and macaques.

E. bieneusi has undergone a long-term clonal evolution as its stable genotypes are widely distributed in different parts of the world. Thus, *E. bieneusi* infection by already known and new genotypes was found in various hosts.[11,21–27]

3.2 MLST of *E. bieneusi*

Feng *et al*. found, by using the MLST technique, that four positive isolates of *E. bieneusi* were subjected to four sites of three microsatellite loci (MS1, MS3, and MS7) and one minisatellite locus (MS4).[13] The amplification results indicated that the amplification efficiency of the four ITS-positive isolates was 50% (2/4) at MS1, MS3, MS4, and MS7. Figures 4–7 show

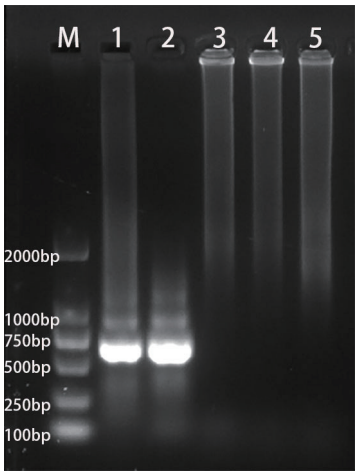

Fig. 4. PCR amplification of *E. bieneusi* ITS-positive samples based on MS1 gene. M: DL2000 Marker; 1: Cervus nippon No. 6 MS1; 2: Cervus nippon No. 9 MS1; 3: Hylobatidae No. 1 MS1; 4: Cervus nippon No. 1 MS1; 5: negative control.

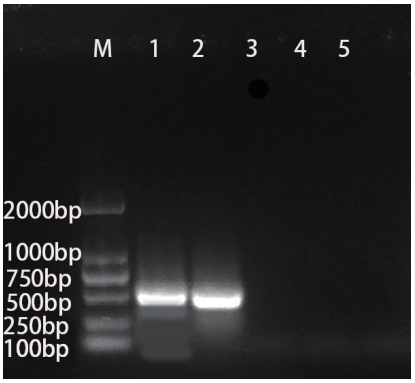

Fig. 5. PCR amplification of *E.bieneusi* ITS-positive samples based on MS3 gene. M: DL2000 Marker; 1: Cervus nippon No. 6 MS3; 2: Cervus nippon No. 9 MS3; 3: Hylobatidae No. 1 MS3; 4: Cervus nippon No. 1 MS3; 5: negative control.

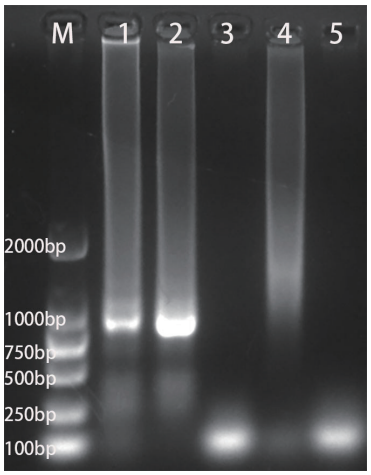

Fig. 6. PCR amplification of *E.bieneusi* ITS-positive samples based on MS4 gene. M: DL2000 Marker; 1: Cervus nippon No. 6 MS4; 2: Cervus nippon No. 9 MS4; 3: Hylobatidae No. 1 MS4; 4: Cervus nippon No. 1 MS4; 5: negative control.

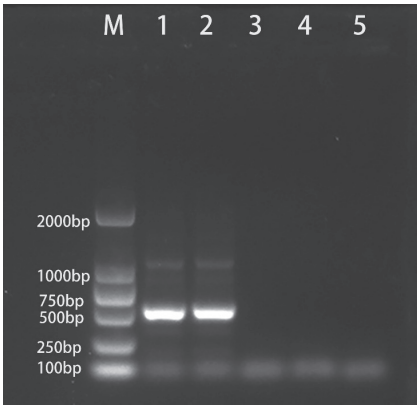

Fig. 7. PCR amplification of *E.bieneusi* ITS-positive samples based on MS7 gene. M: DL2000 Marker; 1: Cervus nippon No. 6 MS7; 2: Cervus nippon No. 9 MS7; 3: Hylobatidae No. 1 MS7; 4: Cervus nippon No. 1 MS7; 5: negative control.

the PCR amplification of ITS-positive samples of *E. bieneusi* based on MS1, MS3, MS4, and MS7. Four loci of MS3, MS4, and MS7 were amplified positive bands. The other two isolates were not amplified.

The identification of the ITS sequence proves that the genotype is susceptible to this host. The distribution of genotypes, the strength of infection, and the study of the population genetic structure are explained by the biological properties of *E. bieneusi*. The result of the nucleotide sequence analysis demonstrated that there were 1, 2, 1, and 2 nucleotide variant haplotypes at MS1, MS3, MS4, and MS7, respectively, and they formed two multilocus genotypes (MLGs). Among all the positive samples, two isolates of *E. bieneusi* were simultaneously amplified at three microsatellite loci (MS1, MS3, and MS7) and one minisatellite locus (MS4), and two MLGs were obtained (Table 3).

Feng *et al*.[12] used the MLST technique in the multisite sequence typing research and selected three microsatellite loci (MS1, MS3, and MS7) and one minisatellite locus (MS4) for nested PCR amplification. The results revealed the characteristics of pathogens through multiple biological tags. Du *et al*.[25] applied the MLST technique to the multisite sequence typing of *E. bieneusi* from wild animals in the Qinling area. They obtained seven positive isolates and found that MS1, MS3, MS4, and MS7 had genetic diversities with 3, 1, 2, and 2 different types, respectively. Wu *et al*.[24] found that MS1, MS3, MS4, and MS7 from Asian black bears had 4, 4, 5, and 10 nucleotide variant haplotypes, respectively, and identified a new multipoint genotype (MLG novel-ABB1). Zhang *et al*.[23] also showed that the haplotype polymorphisms existed in the microsporidia of *E. bieneusi* that infected domestic foxes in China. We used the MLST technique to find two positive ITS samples with 1, 2, 1, and 2 nucleotide variant haplotypes at MS1, MS3, MS4, and MS7, respectively. A total of two MLGs were formed, which shows a genetic polymorphism. These research results showed that not only do the microsporidia of *E.*

Table 3
Results of multilocus sequence typing of *E. bieneusi* in wild animals in Fujian.

Animal type	ITS genotype	MS1	MS3	MS4	MS7	MLGs
Deer 6	Type IV	Type I	Type I	Type I	Type I	MLG1
Deer 9	FJL	Type I	Type II	Type I	Type II	MLG2

bieneusi isolates from different animal sources exhibit different genetic polymorphisms, but the microsporidia of *E. bieneusi* from the same animal species also exhibit genetic diversity. For example, our research results showed that microsporidia of *E. bieneusi* isolates from the No. 6 sika deer and those from the No. 9 sika deer showed different genetic polymorphisms. Microspores were obtained from the No. 9 sika deer. Worm isolates showed the MLG1 gene subtype, whereas the microsporidia of *E. bieneusi* isolates from the No. 6 sika deer showed the MLG2 gene subtype (Table 3). The same ITS genotype also showed different MLG gene polymorphisms. For example, Zhang *et al*.[23] found that four different domestic fox microsporidian isolates of the NCF2 genotype showed 3, 2, and 3 MLG gene polymorphisms in MS1, MS3, and MS7 loci, respectively. It can be seen that the microsporidia of *E. bieneusi* have an extremely rich genetic polymorphism.

E. bieneusi is an important zoonotic opportunistic protozoan in the ecosystem and spreads in various water sources. Propagation hosts include almost all mammals including humans. It spreads mainly through sporulated oocysts that are resistant to adverse environmental conditions. When the host ingests water or food contaminated by oocysts, infection can occur.[2] Infected persons with normal immunity will have mild symptoms such as mild diarrhea, but immunocompromised patients, especially AIDS patients, may have severe diarrhea.[5,6] *E. bieneusi* infections have been reported worldwide, causing public health problems. *E. bieneusi* is considered a potentially dangerous microorganism by the National Institute of Allergy and Infectious Diseases. In several countries including Korea and Spain, *E. bieneusi* is regarded as a unicellular microsporidian fungal pathogen in a broad range of animal hosts, including wild and domestic animals and humans. These findings highlight the role of wildlife as a potential source of microsporidiosis for domestic animals and humans.[28–30] As shown in this study, *E. bieneusi* is genetically diverse, and comprehensive measures are needed to prevent or control the spread of *E. bieneusi* from wild animals worldwide.

4. Conclusions

A total of 70 fecal samples from 28 wild animals were collected at the South China Tiger Breeding Institute of Meihua Mountain in Fujian and the Fuzhou Zoo to identify *E. bieneusi*. We amplified the ITS of SSU rRNA by nested PCR that has been improved by the development of CMOS sensors and enables rRNA studies. As a result, four positive isolates of *E. bieneusi* were identified. The genotype of two isolates of *E. bieneusi* from sika deer and gibbons was Type IV and that of one isolate from a sika deer was BEB6. One isolate from a sika deer had the first identified genotype (FJL). The newly identified FJL and Type IV belong to the zoonotic Group 1, which suggests that the genotypes are spread from humans to wild animals in the Fujian area.

Multisite polymorphism analysis based on three microsatellite loci (MS1, MS3, MS7) and one minisatellite locus (MS4) in four positive samples showed that both isolates were amplified at four sites simultaneously, and nucleotide sequence analysis revealed 1, 2, 1, and 2 nucleotide variant haplotypes at MS1, MS3, MS4, and MS7, respectively. The results indicated genetic diversity. Therefore, comprehensive measures are needed to prevent the possibility of spreading *E. bieneusi* between wild animals and humans in Fujian.

Acknowledgments

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