Occurrence and Potentially Zoonotic Genotypes of *Enterocytozoon Bieneusi* in Wild Rhesus Macaques (*Macaca Mulatta*) Living in Nanwan Monkey Island, Hainan, China: A Public Health Concern

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Abstract

Background

Enterocytozoon bieneusi, a microsporidian species, is a zoonotic pathogen found in both humans and animals. Here, we determined the prevalence, explored the different genotypes of *E. bieneusi* in wild rhesus macaques (*Macaca mulatta*) (Hainan Island of China), and assessed their zoonotic potential.

Methods

We collected 173 fecal specimens from wild *M. mulatta* living in Nanwan Monkey Island, Hainan, China. Subsequently, we identified and genotyped *E. bieneusi* using nested PCR analysis amplification of the internal transcribed spacer region (ITS) of the rRNA gene. Lastly, a neighbor-joining tree was built based on gene sequences from the ITS region of *E. bieneusi*.

Results

Of the 173 specimens from wild *M. mulatta*, 26 (15%) were infected with *E. bieneusi*. We identified six genotypes of *E. bieneusi*, of which five were known: PigEBITS7 (n = 20), D (n = 2), Type IV (n = 1), Peru6 (n = 1), Henan-III (n = 1), and a novel genotype: HNM-IX (n = 1). From the phylogenetic analysis, the six genotypes identified here were all categorized into zoonotic group 1.

Conclusion

Based on the results taht the novel genotype falling under zoonotic group 1 and all the known genotypes found in humans, we conclude that the wild *M. mulatta* infected with *E. bieneusi* have a public health significance.

Background

Enterocytozoon bieneusi is a typical human-pathogenic microsporidia species that contaminates the enterocytes of the small intestine [1]. Although its general infection is described by chronic diarrhea, and malabsorption or no clinical signs in immunocompetent humans, it can result in enhanced increased fatality via chronic diarrhea in individuals with immunodeficiency, such as patients with Acquired Immune Deficiency Syndrome (AIDS) [2]. Different studies have shown that it appears in several animals (mammals, birds, and reptiles) and some environmental samples (water, soil, and food)[3]. Most human infections result from the zoonotic transmission of spores through either infected food or water[4].

Recent surveys incorporated genotype information of *E. bieneusi* and elaborated genotype distribution among human populations and animal hosts [3]. Different studies have observed substantial genetic

diversity within this species through sequencing the single internal transcribed spacer (ITS) region of the rRNA gene [5]. To date, scientists have detected approximately 500 *E. bieneusi* ITS genotypes. Among these genotypes, 49 were found in both animals and humans [3]. All genotypes of *E. bieneusi* could be categorized into 13 clades [6]. Here, two large groups (1 and 2) that are composed of genotypes common in animals and humans are termed zoonotic. The remaining 11 groups (3 to 13) contain genotypes from specific hosts or wastewater [3]. Furthermore, *E. bieneusi* is generally detected in various wildlife, either in captive or free populations, with a wide variety of genotypes, both host-adapted and host-free [3, 7]. Thus, wildlife is also an ecological resource for several human/animal infections. Therefore, the primary focus of epidemiological surveys should involve the genotyping of *E. bieneusi* isolates from under-sampled animal hosts with human contact to expand our knowledge regarding human microsporidiosis epidemiology and support *E. bieneusi* population analysis.

Rhesus macaques (*Macaca mulatta*) are prevalent in Southeast Asia, where their geographic range overlaps extensively with that of humans [8]. We carried out this study in the Nanwan Monkey Island, Nanwan peninsula, Lingshui county, south coast of Hainan, China. Globally, this is the only island-type nature reserve for *M. mulatta* and is home to over 2,500 monkeys. This island has a primitive natural environment, which makes it a perfect place for monkeys. Since its establishment in 1965, it has become a popular tourist destination. However, there is a lack of published studies on *E. bieneusi* infection in the *M. mulatta* living in the Nanwan Monkey Island. Therefore, this study aimed at investigate the incidence and different genotypes of *E. bieneusi* present in the wild *M. mulatta*.

Results

Infection rates of *E. bieneusi* in wild *M. mulatta*

Twenty-six of 173 specimens from wild *M. mulatta* were positive for *E. bieneusi* since they amplified the ITS region of the rRNA gene, with an average infection rate of 15.0%. Besides, the infection rate of *E. bieneusi* in monkeys less than one year of age (19.4%; 14/72) was higher than those animals older than one year (11.9%; 12/101). Meanwhile, out of all the positives, 14.2% (17/120) were females, and 17.0% (9/53) were males. However, as illustrated in Table 1, the infection rates difference were not statistically significant either by age or by gender.

Table 1
Prevalences of *E. bieneusi* and distributions of genotypes in Macaca mulatta by age and gender.

Gender	Positive no./Examined no.(%)-genotype (n)			
	Less than one years of age	Over one years of age	Total	[a]Statistics value
Male	5/24(20.8)-PigITS7(5)	4/29(13.8)-PigITS7 (3); Type IV (1)	9/53 (**17.0**)-PigITS7 (8); Type IV (1)	$\chi^2 = 0.23$, $P = 0.63$
Female	9/48(18.8)-PigEbITS7(7); D(2)	8/72(11.1)-PigITS7(5); Peru 6 (1); HNM-IX (1); Henan-III (1)	17/120 (14.2)-PigITS7(12); D(2); Peru 6 (1); HNM-IX (1); Henan-III (1)	
Total	14/72(**19.4**)-PigEbITS7(12); D(2)	12/101(11.9)-PigITS7(8); Peru 6 (1); Type IV (1); HNM-IX (1); Henan-III (1)	26/173 (15.0)-PigITS7(20); D(2); Peru 6 (1); Type IV (1); HNM-IX (1); Henan-III (1)	
[b]Statistics value	$\chi^2 = 1.88$, $P = 0.17$			

[a] Statistics value = Male vs Female, [b]Statistics value = Less than one years of age vs Over one years of age, Bold = the values higher than that in the same group were shown in bold.

Genotype distribution of *E. bieneusi* by gender and age

Six genotypes were identified in the wild *M. mulatta* through sequencing and multiple sequence alignment. They included five known genotypes (PigEbITS7, Type IV, Peru 6, D, and Henan-III) and one novel genotype (HNM-IX). Among them, genotype PigEbITS7 was dominant, and was found in 76.9% (20/26) of *E. bieneusi* isolates. All the remaining genotypes were at a lower frequency: 7.7% (2/26) for genotype D, and 3.8% (1/26) each for genotypes Peru 6, Type IV, Henan-III, and HNM-IX. Subsequently, two genotypes (PigEbITS7 and D) were detected in the less than one-year-old animals, whereas five genotypes (PigEbITS7, Peru 6, Type IV, Henan-III, and HNM-IX) in the more than one-year-old animals. As demonstrated in Table 1, two genotypes (PigEbITS7 and Type IV) were predominant in males, whereas five (PigEbITS7, D, Peru 6, Henan-III, and HNM-IX) in females.

Genetic relationships of ITS genotypes

Novel genotype HNM-IX had one single nucleotide polymorphism (SNP), with genotype EbpC (AF076042) having it at nucleotide site 51 of the ITS region. As illustrated in Fig. 2, phylogenetic analysis revealed that all genotypes belonged to zoonotic group 1. They were further sub-divided into different genotype sub-groups such as PigEbITS7 and D in subgroup1a; genotypes Peru 6 in subgroup 1b; genotype Type IV in subgroup 1c; and genotypes Henan-III and HNM-IX in subgroup 1d.

Discussion

Non-human primates (NHPs) are known to possess a high genetic relationship with humans, which makes them useful biomedical research models. NHPs might be vulnerable to human diseases, thereby acting as zoonotic reservoirs [8, 10]. In 1997, the first case of transference of *E. bieneusi* infection was recorded between a human (afflicted with AIDS) and a rhesus monkey (afflicted with simian immunodeficiency virus) [11]. However, until 2011, there was a lack of studies on the occurrence of *E. bieneusi* in non-human primates at the genotype level [10]. Zhao et al., summarized 16 studies on the infection of *E. bieneusi* in NHPs from seven countries [8, 12]. Among them, seven studies included *M. mulatta*, and they were all from China, with a prevalence range from 4.2 to 31.1% [12–18]. For the first time, our study has detected *E. bieneusi* in wild *M. mulatta* from the Hainan Province of China, with a prevalence of 15.0%. Generally, *E. bieneusi* has been found to be more prevalent in wild *M. mulatta* here than other wild NHPs, such as baboons from Kenya (12.3%) [10], chimpanzees from Cameroon (4.5%) and Kenya (2.6%) [21], gorillas from the Central African Republic (4.0%) [20], orangutans from Indonesia (2.0%) [19], and five captive species of wild NHPs from the Qinling Mountains of China [17]. Our study showed that *E. bieneusi* was more prevalent in *M. mulatta* than farm monkeys from Henan (6.8%), Guangxi (8.5%), Sichuan (10.5%), and zoo monkeys from Henan (12.5%) in China [14–16, 22]. However, the prevalence of *E. bieneusi* in monkeys from Rwanda (18.0%) and some cities in China, like Shanxi (18.2%), Shanghai (26.7%), Hebei (27.0%), and Beijing (29.2%) was higher than that observed in our study [14–16, 20, 22]. Additionally, there are two more studies that identified *E. bieneusi* infection in laboratory macaques in Beijing (25.6%) and Guangxi (18.5%), China, which were both higher compared with this study [13, 23]. In fact, in Hainan, two studies were reported on captive long-tailed macaques infected with *E. bieneusi*, which were also more than that observed in our study [8, 24]. Similar to humans and farm animals, age substantially increases the risk *E. bieneusi* infection in NHPs [8]. Here, we identified a elevated *E. bieneusi* infection rate in young *M. mulatta* compared with adults, which agreed with the results of captive long-tailed macaque and laboratory macaques from Hainan, China and North China, respectively [8, 14]. In addition to age, the health of the hosts, the detection methods, sample size, the experimental design, animal practices, etc. could cause the increase in prevalence.

Among the five known genotypes in our study, the genotype PigEbITS7 was detected in 76.9% (20/26) of *E. bieneusi* isolates, which shows predominance in the investigated wild *M. mulatta*. This genotype was initially detected in pigs from the USA [7] and it has been confirmed to have a broad host range, even in humans [3]. In China, PigEBITS7 was detected in some patients, including AIDS and hospitalized children, and several animals such as rodents, NHPs, and urban wastewater [5, 8, 25–27].

Additionally, previous studies have reported the presence of four other genotypes (Type IV, Peru6, D, and Henan-III) in humans and animals around the world, of which genotypes D and Type IV are commonly found in *E. bieneusi*- induced microsporidiosis in humans [3, 28]. Both genotypes D and Type IV have been detected in infants, HIV-positive patients, and HIV-negative patients in China [25, 29–33]. Meanwhile, they have been found in NHPs, pigs, dogs, snakes, cats, hippopotamus, Pere David's deer, chinchillas, Siberian tiger, lions, Fischer's lovebird, red foxes, wastewater, and lake water [3].

Genotypes Peru 6 (syn. PtEbI, PtEbVII) (from Peru and Portugal) and Henan-III (from Malaysia and China) have been spread across limited geographical area as well as small number of *E. bieneusi*-infected human cases compared with genotypes D and Type I [34–36]. Meanwhile, genotype Peru 6 has been identified in sheep, goats, reindeers, and wastewater [37–40], whereas genotype Henan-III has been found in NHPs, pet snakes, pigs, and birds in China [41–44]. Therefore, the above shreds of evidence suggest the possible zoonotic transmission of these genotypes from the wild *M. mulatta* to humans.

In this study, the novel genotype HNM-IX was genetically closely related to the human-pathogenic genotype EbpC which was commonly found in humans from Iran, Czech Republic, Peru, China, Thailand, and Vietnam [29, 45–48]. It was also found in more than 15 animal species and in environmental samples [3, 49]. From the phylogenetic analysis, the six genotypes identified here were all categorized into group 1. Group 1 had almost all human-pathogenic genotypes and possessed 94% of the known *E. bieneusi* ITS sequences [3]. Therefore, the genotypes in wild macaques investigated including the novel one could have a sizeable zoonotic possibility.

Conclusions

This study is the first report to detect *E. bieneusi* infection in wild *M. mulatta* from Hainan, China. Human-pathogenic genotypes PigEbITS7, D, Type IV, Peru6, and Henan-III in these animals support a zoonotic nature for *E. bieneusi*. Here, the results of the phylogenetic analysis of the novel genotype falls into group 1, which suggests a zoonotic possibility. Thus, visitors, veterinary workers, and the management of the wild *M. mulatta* should be educated and informed to minimize the risk for transmission of *E. bieneusi* from those animals.

Methods

Fecal sample collection

We obtained 173 stool samples from *M. mulatta* in Nanwan Monkey Island, located on the Nanwan peninsula, Lingshui county, south coast of Hainan, in the southernmost province of China. It is geographically located at 109°48' east longitude and 18°29' north latitude (Fig. 1). In this study, we used sterile disposable latex gloves to collect fresh fecal samples, which were then placed in marked plastic cups and stored at 4°C.

DNA extraction

We sieved the fecal specimens, concentrated the filtrates, washed them thrice with distilled water, and centrifuged (10 min, 1500g). Then, we used a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) to extract genomic DNA from washed fecal specimens (180–200 mg) following manufacturer's guidelines. Finally, DNA was eluted in 200 µL of AE buffer and stored at -20°C.

PCR amplification

We analyzed all DNA preparations for *E. bieneusi* using nested PCR amplification. This amplification contained a nucleotide fragment (389 bp) containing 3’ end small subunit (SSU) (76 bp), ITS region (243 bp), and 5’ region of the large subunit (LSU) (70 bp) from *E. bieneusi* rRNA gene. Primers and cycle parameters were designed by Buckholt et al. (2002) [9]. All PCR tests used Taq DNA polymerase as well as a negative control (no DNA). Finally, all PCR products were separated via 1.5% agarose gel electrophoresis, followed by ethidium bromide staining.

Nucleotide Sequencing

All appropriately sized PCR products were purified using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on an ABI PRISM 3730 XL DNA Analyzer (Sinogeno- max Biotechnology Co. Ltd., Beijing, China), followed by direct sequencing using PCR primers. We performed bidirectional sequencing to verify sequence accuracy.

Sequence analysis

We determined *E. bieneusi* genotypes. Here, we aligned the nucleotide sequences with each other, and used the BLAST and Clustal X 1.83 to access the reference sequences. In particular, the first published names corresponded to the sequences who had a 100 percent resemblance to those from known genotypes. Otherwise, they were described as novel genotypes. Finally, the nomenclature was established by naming all genotypes according to the 243 bp of the ITS gene region of *E. bieneusi* [5].

Phylogenetic analysis

Here, we studied the genetic association between the novel and known genotypes. Next, we used the Mega X software (http://www.megasoftware.net/) to compare the ITS region of all identified nucleotide sequences with those of the reference sequences. The neighbor-joining tree was built based on the evolutionary distances calculated using a Kimura 2-parameter model and bootstrap analysis of 1,000 replicates.

Abbreviations

AIDS: acquired immune deficiency syndrome; SNP: single nucleotide polymorphism; NHPs: non-human primates; BLAST: Basic Local Alignment Search Tool; ITS: internal transcribed spacer; SSU: small subunit LSU: large subunit

Declarations

Acknowledgements

Not applicable.

Ethics approval and consent to participate

The Research Ethics Committee and the Animal Ethics Committee of Hainan Medical University approved the study protocol. All animal experiments complied with the guidelines provided by the Regulations for the Administration of Affairs Concerning Experimental Animals.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article. The identified nucleotide sequence of the novel genotype was submitted to the GenBank database (accession# MW551790).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WZ, FT and GLFT and GL

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Figures

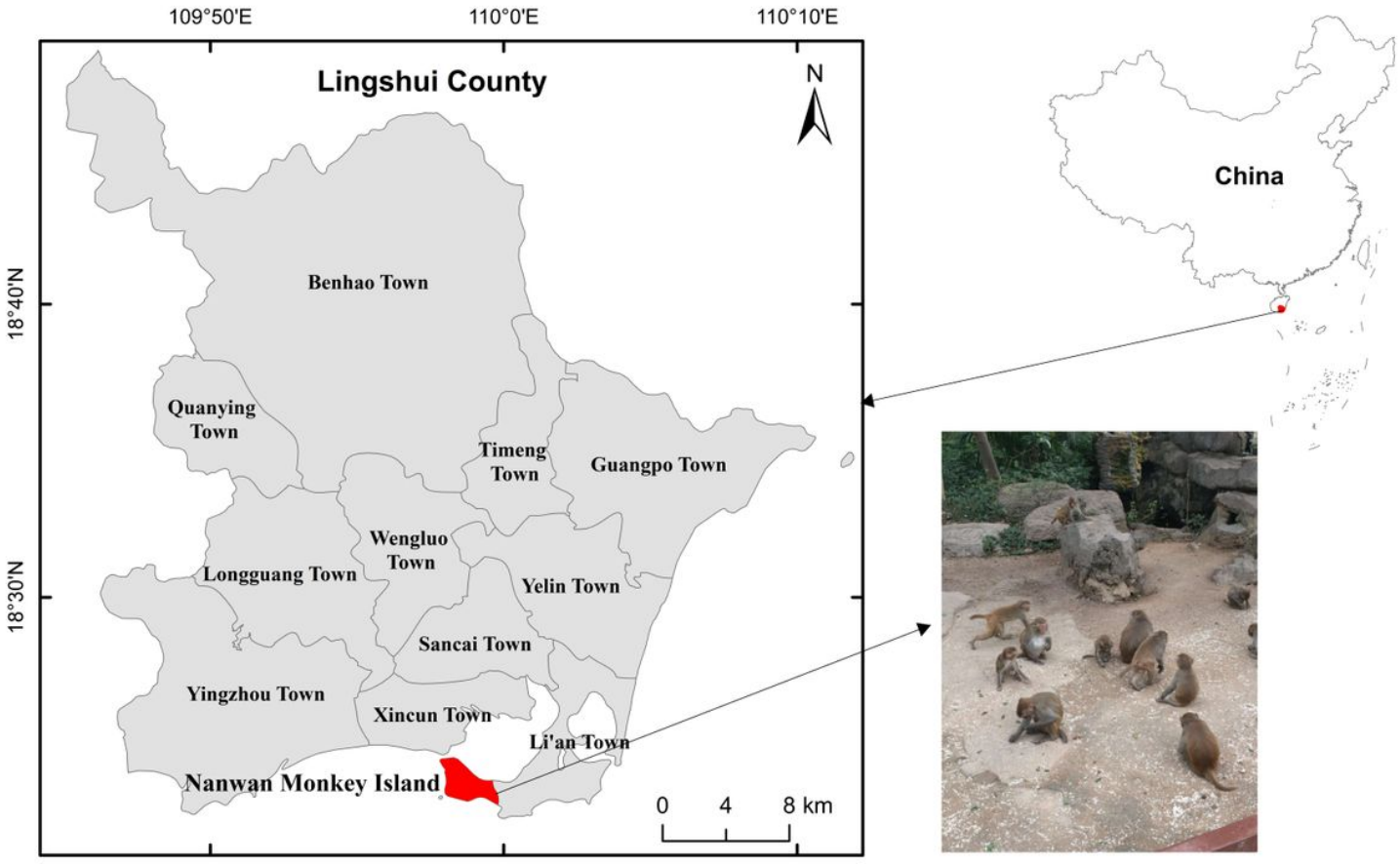

Figure 1

The location of the Nanwan Monkey Island, Hainan of China where the location of sample collection. 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.

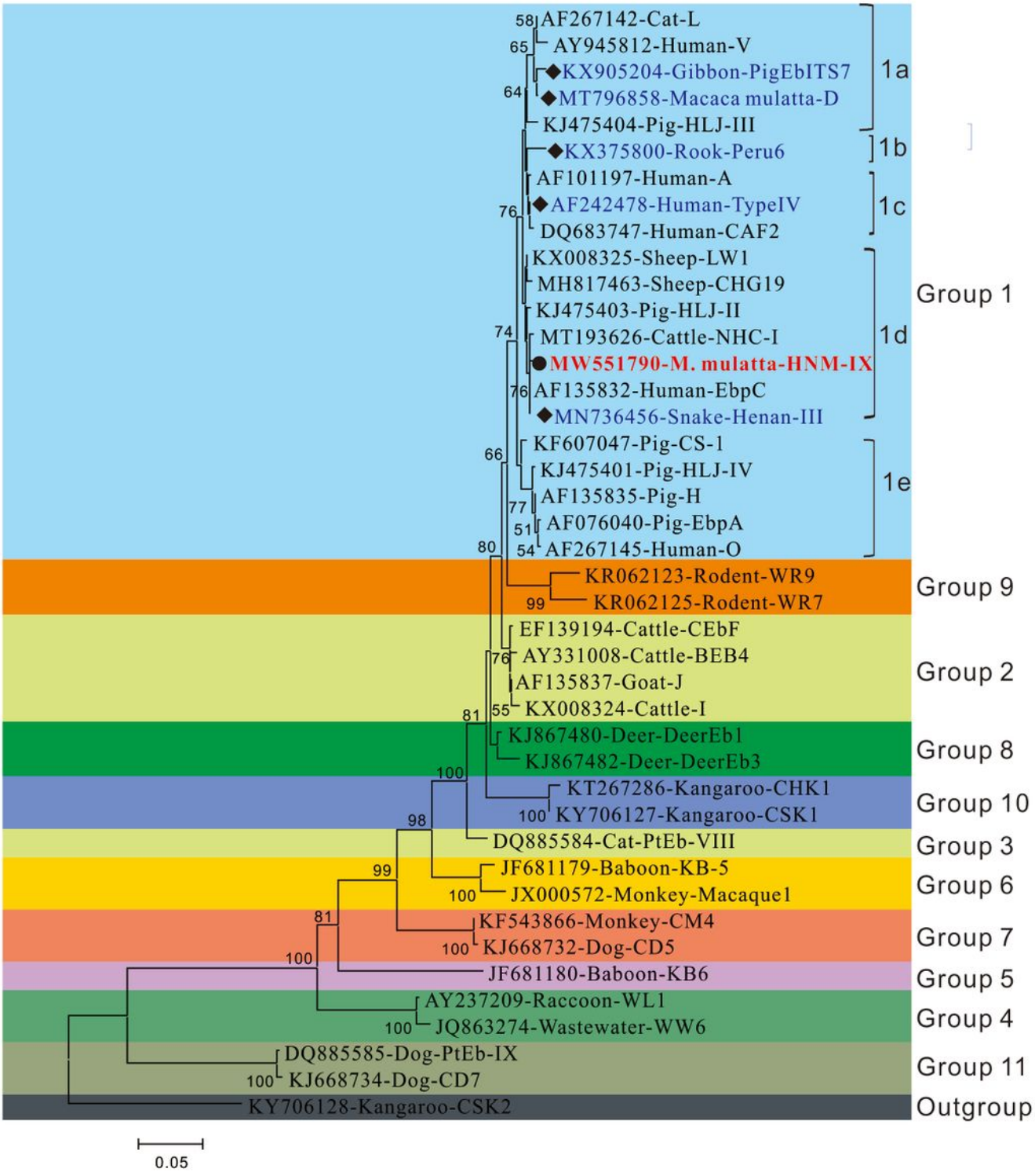

Figure 2

Phylogenetic relationship of Enterocytozoon bieneusi genotype groups. The relationship of Enterocytozoon bieneusi genotypes identified in the present study and other known genotypes deposited in the GenBank was inferred by neighbor-joining ITS sequences analysis based on the genetic distance using the Kimura two-parameter model. The numbers on the branches are percent bootstrap values from 1,000 replicates. Each sequence is identified by its accession number, host origin, and genotype designation. The group terminology for the clusters is based on Zheng et al. [49]. The squares and circles filled in black indicate novel and known genotypes identified in this study, respectively.