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Molecular characterization of *Cryptosporidium* spp., *Giardia* spp. and *Enterocytozoon bieneusi* in eleven wild rodent species in China: Common distribution, extensive genetic diversity and high zoonotic potential

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A R T I C L E I N F O

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A B S T R A C T

Cryptosporidium spp., *Giardia* spp. and *Enterocytozoon bieneusi* are common zoonotic pathogens in humans and animals. Although rodents are important parts of the ecosystem and common hosts for these pathogens, little is known of the distribution, genetic diversity and zoonotic potential of these pathogens in wild rodents. A total of 442 fecal samples were collected from eleven wild rodent species in three provinces of China, and analyzed for these pathogens by PCR and DNA sequencing. The infection rates of *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi* were 19.9% (88/442), 19.8% (75/378) and 12.2% (54/442), respectively. Altogether, 23 known *Cryptosporidium* species/genotypes were identified and their distribution varied among different sampling locations or rodent species. Subtyping of the zoonotic *Cryptosporidium* species identified two novel subtype families XVe and XVf in *C. viatorum*, the subtype family XIIh and a novel subtype family XIIj in *C. ubiquitum*, and the subtype family IId in *C. parvum*. Three *Giardia* species were identified, including *G. microti* ($n = 57$), *G. muris* ($n = 15$) and *G. duodenalis* ($n = 3$), with *G. duodenalis* assemblages A and G identified in brown rats in urban areas of Guangdong. In addition, 13 *E. bieneusi* genotypes including eight known and five novel ones were identified, belonging to Groups 1, 2, 10, 14 and 15. Within nine genotypes in the zoonotic Group 1, common human-pathogenic genotypes D, Type IV, PigEbITS7 and Peru8 were detected only in brown rats and Lesser rice-field rats in urban areas of Guangdong. Apparent host adaptation and geographical differences were observed among *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi* genotypes in wild rodents in the present study. Furthermore, the zoonotic *Cryptosporidium* species and *E. bieneusi* genotypes commonly found here suggest a high zoonotic potential of these pathogens in wild rodents, especially in brown rats in urban areas. Hygiene and One Health measures should be implemented in urban streets and food stores to reduce the possible direct and indirect transmission of these rodent-related pathogens.

1. Introduction

Cryptosporidium spp., *Giardia* spp. and *Enterocytozoon bieneusi* are important zoonotic pathogens in humans and various domestic and wild animals [1,2]. Humans can be infected by these pathogens through direct contact with infected individuals or consumption of contaminated water or food, developing diarrhea and other clinical manifestations [1,3]. Because of the broad host range of these pathogens, zoonotic transmission plays an important role in human cryptosporidiosis, *Giardia*sis and microsporidiosis.

Abbreviations: bg, *β*-giardin gene; *gdh*, glutamate dehydrogenase gene; *gp60*, 60 KDa glycoprotein gene; ITS, internal transcribed spacer; SSU rRNA, small subunit rRNA gene; tpi, triosephosphate isomerase gene.

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Rodents are the largest order of mammals. Among them, wild rodents are widely distributed worldwide and adapted easily to various habitats, including both wild and anthropogenically modified habitats. They frequently reside in human-populated areas particularly in areas with less than desirable hygiene conditions. As wild rodents harbor numerous pathogens that are transmissible to humans, they are considered a major threat to public health [4,5]. Among the parasitic pathogens, *Cryptosporidium* spp., *Giardia* spp., and *E. bieneusi* might be transmitted from wild rodents to humans.

The identification of genetic diversity in *Cryptosporidium* spp., *Giardia* spp., and *E. bieneusi* has accelerated in recent years. To date, over 47 *Cryptosporidium* species and approximately 120 genotypes have been recognized [2,6,7,8]. Rodents are among the most important reservoirs of *Cryptosporidium* spp., with 28 known *Cryptosporidium* species and 41 genotypes being identified in rodents [6,7,8,9]. Among them, 20 zoonotic *Cryptosporidium* species and genotypes have been reported in rodents, with *C. parvum*, *C. hominis*, *C. viatorum* and *C. ubiquitum* as the major ones [9,10]. Within nine valid *Giardia* species, *G. microti*, *G. cricetidarum*, *G. muris* and *G. duodenalis* are commonly found in rodents, and *G. duodenalis* is the only zoonotic species infecting humans and most vertebrates [3]. Among the eight assemblages of *G. duodenalis*, assemblage A-B are zoonotic and assemblage C—H are commonly host-adapted [11,12]. At present, over 500 genotypes of *E. bieneusi* have been identified belonging to 15 phylogenetic groups, including the zoonotic Group 1 and host-adapted Groups 2–11 [13,14,15]. More than 60 *E. bieneusi* genotypes have been detected in rodents belonging to Groups 1–3, 6 and 9, with 25 genotypes found in humans as well [16,17,18,19].

In recent years, the infection of *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi* in wild rodents has attracted some attention in China. The data generated, however, are fragmented in nature, with most studies being limited to confined animal species, geographic areas or individual pathogen [20,21]. To better understand the transmission characteristics and zoonotic potential of the three pathogens in rodents, this study was conducted to examine the distribution and genetic identity of *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi* in eleven species of wild rodents in three provinces of China.

2. Material and methods

2.1. Ethics approval and consent to participate

The research protocol was approved by the Research Ethics Committee of the South China Agricultural University. The wild rodents used for the study were handled in compliance with the regulations of the Chinese Laboratory Animal Administration Act of 2017.

2.2. Fecal sample collection

This study was performed from October 2018 to November 2021, using samples collected from Guangzhou City in Guangdong Province, Mianyang City and Aba Tibetan and Qiang Autonomous Prefecture in Sichuan Province, and Urumqi City in Xinjiang Uygur Autonomous Region, China (Fig. 1). A total of 442 fecal samples were collected from eleven rodent species, including 99 South China field mice (*Apodemus draco*), 97 brown rats (*Rattus norvegicus*), 66 voles (*Microtus arvalis*), 66 long-tailed ground squirrels (*Spermophilus undulatus*), 42 Coxing's white-bellied rats (*Niviventer coninga*), 41 Yunnan red-backed voles (*Eothenomys miletus*), 26 Lesser rice-field rats (*Rattus losea*), 2 greater bandicoot rats (*Bandicota indica*), 1 Chestnut white-bellied rat (*Niviventer fulvescens*), 1 common Chinese zokor (*Myospalax fontanieri*) and 1 Pallas's squirrel (*Callosciurus erythraeus*) (Table 1). Guangzhou is one of the largest cities with highest population density in China, so we trapped wild rodents from urban areas in Guangzhou City where humans frequently interacted, such as city streets and food stores. In contrast, due to the relatively low population density and diverse ecological environments in Mianyang City, Aba Tibetan and Qiang Autonomous Prefecture and Urumqi City, we trapped wild rodents from rural areas in these regions where their habitats had less overlap with human activities, including farmland, forests and other natural environments. The trapped rodents were euthanized by CO_2 inhalation, and approximately 500 mg of each fecal sample was collected directly from the rectal content of each rodent and stored in 2.5% potassium dichromate at 4 °C before DNA extraction.

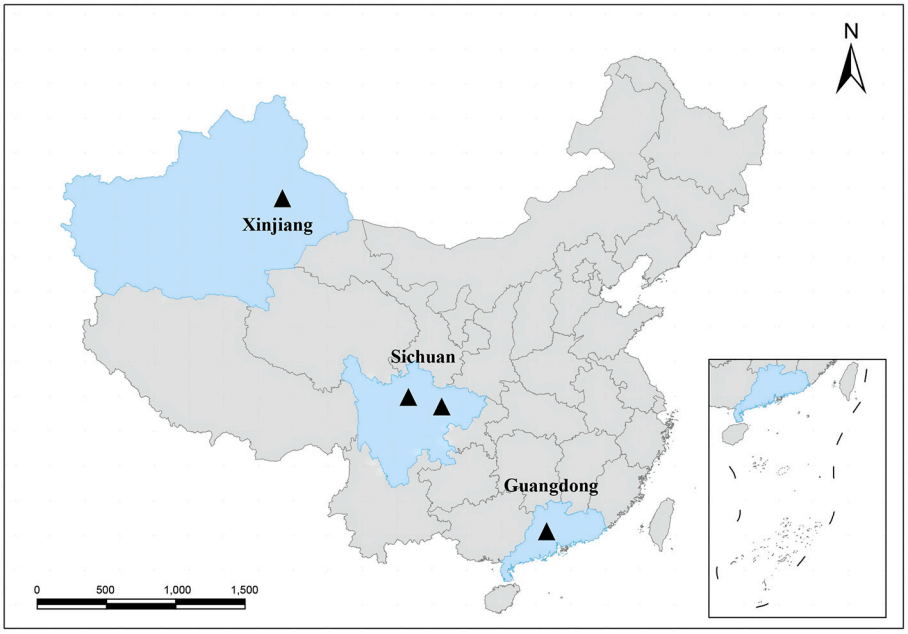

Fig. 1. Geographical locations (triangles) of wild rodents examined in the present study in three provinces of China (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Infection and distribution of *Cryptosporidium* spp. in wild rodents in four regions of China.

Samples	No. positive/ no. examined (%)	Species/Genotypes (n)	Subtypes (n)
By sampling locations			
Guangdong	30/125 (24.0)	rat genotype IV (10), *C. muris* (5), Rat genotype III (4), *Cryptosporidium* sp. MT561515 (3), *C. tyzzeri* (2), w19 (3), *C. parvum* (1), *C. baileyi* (1), *Cryptosporidium* sp. KY483983 (1)	*C. parvum*-IIdA20G1 (1)
Sichuan	38/185 (20.5)	*C. viatorum* (14), *C. occultus* (6), Vole genotype II (4), *C. microti* (2), *C. muris* (2), *C. ubiquitum* (2), Bear genotype (3), Vole genotype 1 (2), W25 (1), *Cryptosporidium* sp. KY644567 (1), *C. sciurinum* (1)	*C. viatorum*-XVeA4 (4), *C. viatorum*-XVeA6 (2), *C. viatorum*-XVfA4 (2), *C. viatorum*-XVfA5 (2), *C. viatorum*-XVeA5 (1), *C. ubiquitum*-XIIh (1), *C. ubiquitum*-XIIj (1)
Xinjiang	20/132 (15.2)	chipmunk genotype III (7), sw1 (7), *C. alticolis* (3), vole genotype V (3)	/
By rodent species (Common Name)			
Rattus norvegicus (Brown rat)	21/97 (21.6)	rat genotype IV (7), *C. muris* (4), W19 (3), *Cryptosporidium* sp. MT561515.1 (3), rat genotype III (2), *C. parvum* (1), *C. baileyi* (1)	*C. parvum*-IIdA20G1 (1)
Rattus losea (Lesser Rice-field rat)	7/26 (26.9)	rat genotype III (2), *C. tyzzeri* (2), *C. muris* (1), rat genotype IV (1), *Cryptosporidium* sp. KY483983 (1)	/
Niviventer coninga (Coxing's white-bellied Rat)	11/42 (26.2)	*C. viatorum* (6), *C. occultus* (5)	*C. viatorum*-XVeA4 (1), *C. viatorum*-XVeA5 (1), *C. viatorum*-XVeA6 (1), *C. viatorum*-XVfA4 (1), *C. viatorum*-XVfA5 (1)
Bandicota indica (Greater Bandicoot Rat)	2/2 (100.0)	rat genotype IV (2)	/
Niviventer fulvescens (Chestnut White-bellied Rat)	1/1 (100.0)	*C. occultus* (1)	/
Apodemus draco (South China field mouse)	15/99 (15.2)	*C. viatorum* (8), bear genotype (3), *C. muris* (2), *C. ubiquitum* (1), *C. sciurinum* (1)	*C. viatorum*-XVeA4 (3), *C. viatorum*-XVeA6 (1), *C. viatorum*-XVfA4 (1), *C. viatorum*-XVfA5 (1), *C. ubiquitum*-XIIj (1)
Myospalax fontanieri (Common Chinese zokor)	0/1 (0)	/	/
Microtus arvalis (Vole)	13/66 (19.7)	SW1 (7), vole genotype V (3), *C. alticolis* (3)	/
Eothenomys miletus (Yunnan red-backed vole)	10/41 (24.4)	vole genotype II (4), *C. microti* (2), vole genotype 1 (2), *Cryptosporidium* sp. KY644567 (1), W25 (1)	/
Callosciurus erythraeus (Pallas's squirrel)	1/1 (100.0)	*C. ubiquitum* (1)	*C. ubiquitum*-XIIh (1)
Spermophilus undulatus (Long-tailed ground squirrel)	7/66 (10.6)	chipmunk genotype III (7)	/
Total	88/442 (19.9)	*C. viatorum* (14), rat genotype IV (10), *C. muris* (7), sw1 (7), chipmunk genotype III (7), *C. occultus* (6), vole genotype II (4), rat genotype III (4), *C. alticolis* (3), vole genotype V (3), bear genotype (3), w19 (3), *Cryptosporidium* sp. MT561515 (3), *C. microti* (2), *C. tyzzeri* (2), *C. ubiquitum* (2), vole genotype 1 (2), *C. sciurinum* (1), w25 (1), *C. parvum* (1), *C. baileyi* (1), *Cryptosporidium* sp. KY644567 (1), *Cryptosporidium* sp. KY483983 (1)	*C. viatorum*-XVeA4 (4), *C. viatorum*-XVeA6 (2), *C. viatorum*-XVfA4 (2), *C. viatorum*-XVfA5 (2), *C. viatorum*-XVeA5 (1), *C. parvum*-IIdA20G1 (1), *C. ubiquitum*-XIIh (1), *C. ubiquitum*-XIIj (1)

2.3. DNA extraction

Each fecal sample was washed twice with distilled water to remove potassium dichromate by centrifugation at room temperature at 2000 $\times g$ for 10 min. Genomic DNA was extracted from approximately 200 mg of each fecal sample using the Fast DNA Spin Kit for Soil (MP Biomedical, Santa Ana, CA, USA). The extracted DNA was stored at −20 °C until PCR amplification.

2.4. PCR amplification

Cryptosporidium spp. were examined by nested PCR amplification of the small subunit (SSU) rRNA gene [22]. Subtypes of the three human-pathogenic *Cryptosporidium* species, *C. parvum*, *C. ubiquitum* and *C. viatorum*, were determined by PCR and sequence analysis of the 60-kDa glycoprotein (*gp60*) gene [23,24,25]. *Giardia* spp. were detected by nested PCR targeting the b-giardin (*bg*), triosephosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*) genes [26,27,28]. *Enterocytozoon bieneusi* was identified by nested PCR amplification of the internal transcribed spacer (ITS) of the rRNA gene [29]. Two replicates were used in PCR analysis of each target for each sample. Genomic DNA of *C. bovis* from cattle, assemblage D from dogs, and genotype PtEb IX from dogs were used as positive controls in PCR analyses of *Cryptosporidium* spp., *Giardia.* spp., and *E. bieneusi*, respectively, while *C. hominis* DNA from crab-eating macaques was used as the positive control in *Cryptosporidium* subtyping. Reagent-grade water was used as the negative control.

2.5. Sequence and phylogenetic analyses

All positive secondary PCR amplicons were sequenced bi-directionally on an ABI 3730 instrument by the Sangon Biotech (Shanghai, China). Generated sequences were assembled using ChromasPro 2.1.6 (http://technelysium.com.au/ChromasPro.html) and aligned using ClustalX 2.0.11 (http://clustal.org) with the reference sequences from GenBank (https://www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using maximum likelihood (ML) implemented in MEGA 7.0.14 (https://www.megasoftware.net/) with the General Time Reversible model. Bootstrap values were calculated by analyzing 1000 replicates.

2.6. Statistical analysis

The Chi-square test implemented in SPSS 20.0 (IBM Corp., New York, NY, USA) was used to assess differences in infection rates of *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi* between sampling locations or rodent species. The results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Infection of Cryptosporidium spp

Of the 442 fecal samples collected from wild rodents, 88 (19.9%) were positive for *Cryptosporidium* spp. based on PCR analysis of the SSU rRNA gene (Table 1). The infection rate of *Cryptosporidium* spp. was 24.0% (30/125) in Guangdong, 20.5% (38/185) in Sichuan, and 15.2% (20/132) in Xinjiang, respectively (Table 1). By rodent species, Lesser rice-field rats had the highest infection rate (26.9%, 7/26) of *Cryptosporidium* spp., followed by Coxing's white-bellied rats (26.2%, 11/42), Yunnan red-backed voles (24.4%, 10/41), brown rats (21.6%, 21/97), voles (19.7%, 13/66), South China field mice (15.2%, 15/99) and long-tailed ground squirrels (10.6%, 7/66). The few greater bandicoot rats, chestnut white-bellied rats and Pallas's squirrels sampled were all positive for *Cryptosporidium* spp., while the common Chinese zokor sampled was negative for *Cryptosporidium* spp

3.2. Cryptosporidium species and genotypes

A total of 23 known *Cryptosporidium* species and genotypes with different zoonotic potential were identified by sequence analysis of the SSU rRNA PCR products and phylogenetic analyses (Table 1 and Fig. 2). They included *C. viatorum* ($n = 14$), rat genotype IV ($n = 10$), *C. muris* ($n = 7$), sw1 ($n = 7$), chipmunk genotype III ($n = 7$), *C. occultus* ($n = 6$), vole genotype II ($n = 4$), rat genotype III ($n = 4$), *C. alticolis* ($n = 3$), vole genotype V ($n = 3$), bear genotype ($n = 3$), w19 ($n = 3$), *Cryptosporidium* sp. MT561515 ($n = 3$), *C. microti* ($n = 2$), *C. tyzzeri* ($n = 2$), *C. ubiquitum* ($n = 2$), vole genotype 1 ($n = 2$), *C. sciurinum* ($n = 1$), w25 ($n = 1$), *C. parvum* ($n = 1$), *C. baileyi* ($n = 1$), *Cryptosporidium* sp. KY644567 ($n = 1$), and *Cryptosporidium* sp. KY483983 ($n = 1$). Among these *Cryptosporidium* species and genotypes, nucleotide sequences generated from *C. baileyi*, *C. muris*, *C. parvum*, *C. tyzzeri*, *C. ubiquitum*, sw1, *C. sciurinum*, and rat genotype III were identical to GenBank sequences KY448456, GU319781, MF671870, OQ826430, MH794165, HM015872, MZ726453, and JX294368, respectively. In addition, nucleotide sequences from *C. viatorum*, rat genotype IV, chipmunk genotype III, *C. occultus*, vole genotype II, *C. alticolis*, vole genotype V, bear genotype, w19, *Cryptosporidium* sp. MT561515, *C. microti*, vole genotype 1, w25, *Cryptosporidium* sp. KY644567, and *Cryptosporidium* sp. KY483983 had 1–5 single nucleotide polymorphisms (SNPs) compared with the GenBank reference sequences (Fig. 2).

The distribution of *Cryptosporidium* spp. varies among different provinces or species of rodents (Table 1). By sampling locations, rat genotype IV was dominant in Guangdong; *C. viatorum* was dominant in

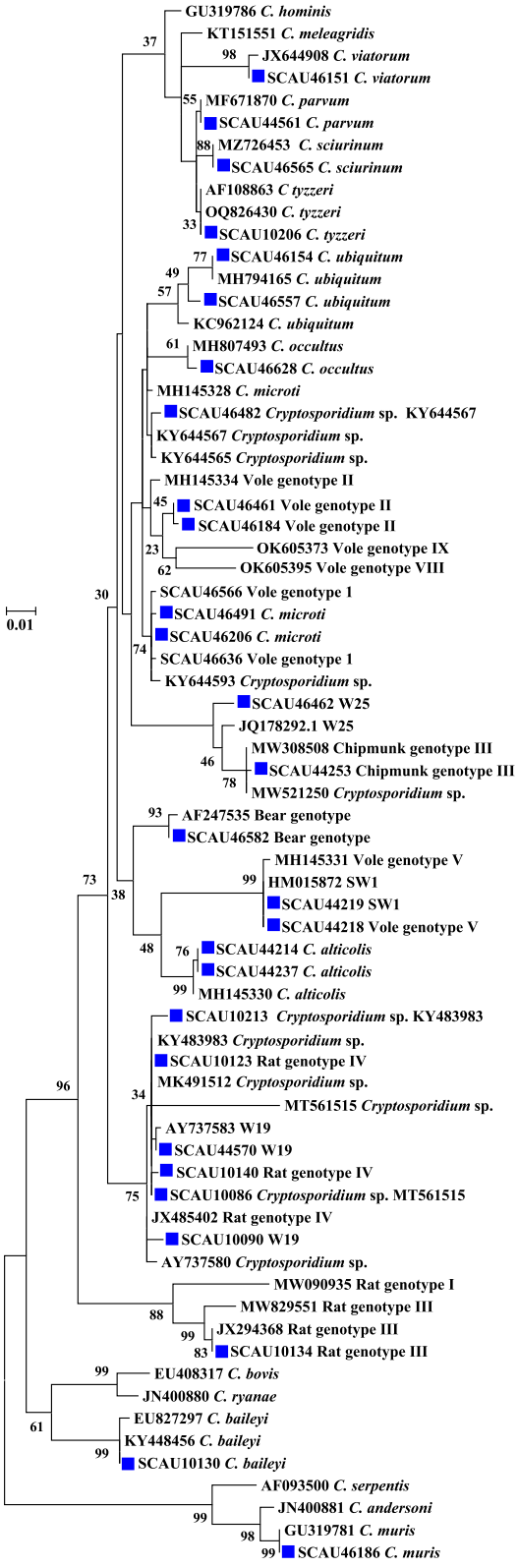

Fig. 2. Phylogenetic relationship of *Cryptosporidium* spp. based on the maximum likelihood analyses of the SSU rRNA gene. Bootstrap values >50% from 1000 replicates are displayed. The blue squares indicate known *Cryptosporidium* species and genotypes identified in the present study. The scale bar indicates 0.01 nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sichuan; chipmunk genotype III and SW1 were dominant in Xinjiang. For the three zoonotic species, *C. parvum* was detected only in Guangdong, while *C. viatorum* and *C. ubiquitum* were found exclusively in Sichuan. By rodent species, the most diverse *Cryptosporidium* species and genotypes were identified in brown rats. *Cryptosporidium parvum* was found only in a brown rat, *C. viatorum* in South China field mice and Coxing's white-bellied rats, and *C. ubiquitum* in a China field mouse and a Pallas's squirrel.

3.3. Subtypes of zoonotic Cryptosporidium spp

The common zoonotic species of *C. parvum*, *C. viatorum* and *C. ubiquitum* identified were further characterized at the *gp60* gene. The *C. parvum* isolate was identified as subtype IIdA20G1. Altogether, 11 of 14*C. viatorum* samples were successfully amplified, leading to the identification of two new subtype families that were named as XVe and XVf in accordance with the established nomenclature [25]. In phylogenetic analysis, XVe and XVf clustered together and genetically related to the known subtype family XVa (Fig. 3a). Both of XVe and XVf had 96–98% nucleotide sequence similarity to XVa. Within the two new subtype families, five subtypes XVeA4, XVeA5, XVeA6, XVfA4 and XVfA5 were identified based on the number of contiguous TCA trinucleotides in the serine repeat region. In addition, a known subtype family XIIh and a new subtype family XIIj were identified in two *C. ubiquitum* isolates. The new subtype family XIIj was named in accordance with established subtype nomenclature [24]. Phylogenetically, XIIj clustered with the known subtype family XIIf but with 55 nucleotide substitutions (89% sequence identity) (Fig. 3b).

3.4. Infection of Giardia spp

Due to the early collection time in 2018–2019, 64 fecal samples from 42 brown rats and 22 Lesser rice-field rats in Guangzhou and their DNA extracts were fully used up before the detection of *Giardia* spp. Therefore, only 378 samples were tested for *Giardia* spp. The overall infection rate of *Giardia* spp. in wild rodents was 19.8% (75/378) based on the sequence analysis of the *bg* locus (Table 2). The highest prevalence of *Giardia* spp. was found in Xinjiang (43.2%, 57/132), followed by Sichuan (8.1%, 15/185) and Guangdong (4.9%, 3/61). Among various wild rodents, voles had the significantly higher prevalence of *Giardia* spp. (86.4%, 57/66; $P < 0.001$) than Coxing's white-bellied rats (16.7%, 7/42), South China field mice (8.1%, 8/99) and brown rats (5.5%, 3/55). No *Giardia* infection was found in the remaining rodent species.

3.5. Giardia species, genotypes and subtypes

Among the 75 *Giardia*-positive samples at the *bg* locus, three *Giardia* species were identified, including *G. microti* ($n = 57$), *G. muris* ($n = 15$), and *G. duodenalis* ($n = 3$) (Table 2, Fig. 4a). All 57 sequences of *G. microti* had 100% identity to the GenBank sequence MG676943. Four sequence types of *G. muris* were identified, including one sequence type identical to GenBank sequence MG677059, one sequence type having 1 SNP compared with MG677059, and two sequence types having 98% similarity to the GenBank sequence MT713338. For *G. duodenalis*, assemblage G ($n = 2$) and assemblage A ($n = 1$) were identical to the GenBank sequences MT114176 and LC437420, respectively. The assemblage A isolate was identified as subtype A5, which belonged to the sub-assemblage AI. The *bg*-positive samples were further characterized at the *gdh* and *tpi* loci. Sequence analysis of the *gdh* and *tpi* PCR products revealed the presence of *G. microti* and *G. duodenalis* assemblage G, but failed to identify *G. muris* and *G. duodenalis* assemblage A (Fig. 4b and c).

By sampling locations, *G. duodenalis*, *G. muris*, and *G. microti* were found only in Guangdong, Sichuan, and Xinjiang, respectively. By rodent species, *G. muris* was found in South China field mice and Coxing's white-bellied rats, while *G. microti* and *G. duodenalis* were found only in

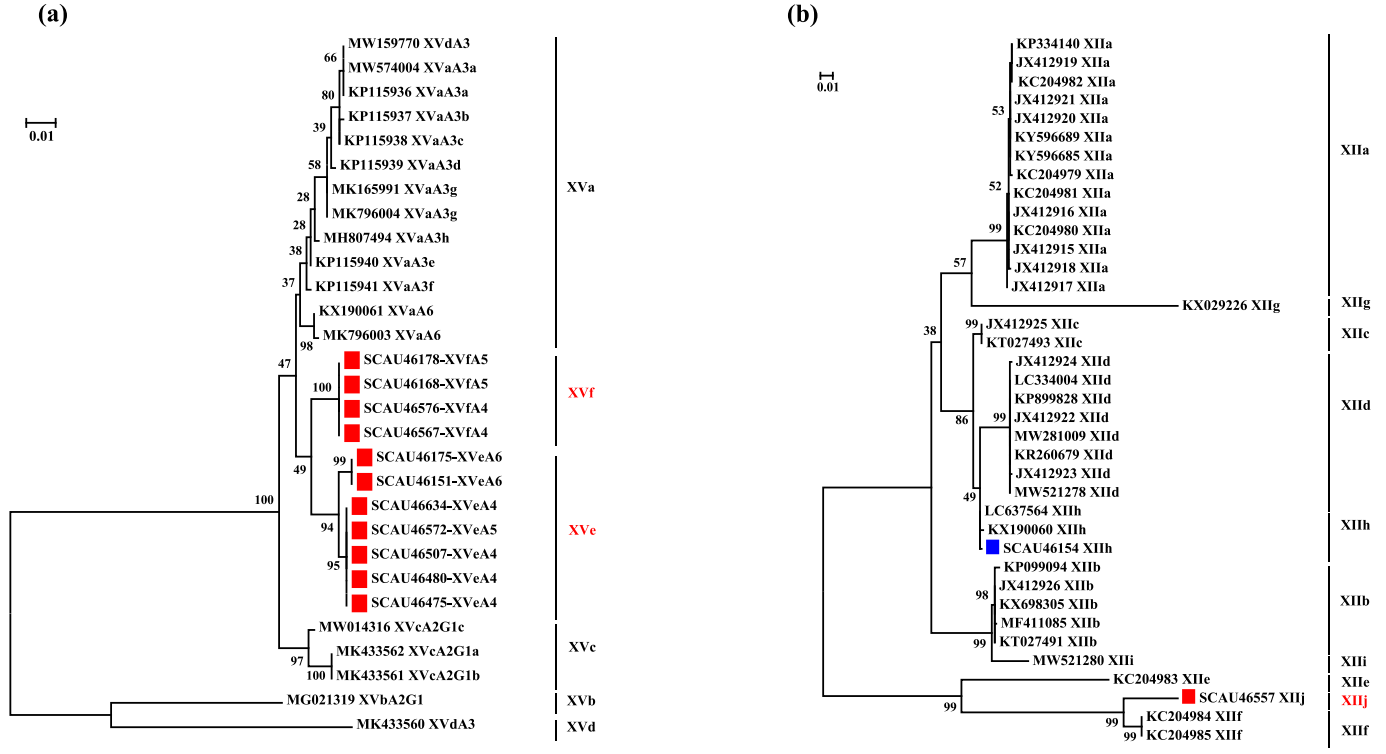

Fig. 3. Phylogenetic relationships among various subtypes of *C. viatorum* (a) and *C. ubiquitum* (b). Phylogenetic trees based on the *gp60* gene sequences of *C. viatorum* and *C. ubiquitum* are constructed by the maximum likelihood method with 1000 bootstrap replicates. Known and novel subtypes are indicated by bule and red squares, respectively. The scale bar indicates 0.01 nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Infection and distribution of *Giardia* spp. and *Enterocytozoon bieneusi* in wild rodents in China.

Samples	*Giardia* spp.			*E. bieneusi*	
	No. positive/no. examined (%)	Species (n)	*G. duodenalis* Assemblages (n)	No. positive/no. examined (%)	Genotypes (n)
By sampling locations					
Guangdong	3/61 (4.9)	*G. duodenalis* **(3)**	G (2), A (1)	40/125 (32.0)	D (20), Type IV (12), PigEBITS7 (5), Peru 8 (2), J (1)
Sichuan	15/185 (8.1)	*G. muris* (15)	/	9/185 (4.9)	SCAR01 (3), Korea-WL6 (1), SCAR02 (1), SCAR03 (1), SCR05 (1), HNFS01 (1), SCMR (1)
Xinjiang	57/132 (43.2)	*G. microti* (57)	/	5/132 (3.8)	XJUR (4), J (1)
By rodent species (Common Name)					
Rattus norvegicus (Brown rat)	3/55 (5.5)	*G. duodenalis* (3)	G (2), A (1)	29/97 (29.9)	D (13), Type IV (9), PigEBITS7 (5), Peru 8 (2)
Rattus losea **(Lesser Rice-field rat)**	0/4 (0)	/		11/26 (42.3)	D (7), Type IV (3), J (1)
Niviventer coninga (Coxing's White-bellied rat)	7/42 (16.7)	*G. muris* (7)	/	2/42 (4.8)	HNFS01 (1), SCAR02 (1)
Bandicota indica (Greater Bandicoot Rat)	0/2 (0)	/	/	0/2 (0)	/
Niviventer fulvescens (Chestnut White-bellied Rat)	0/1 (0)	/	/	1/1 (100.0)	SCMR (1)
***Apodemus draco* (South China field mouse)**	8/99 (8.1)	*G. muris* (8)	/	4/99 (4.0)	SCR05 (1), Korea-WL6 (1), SCAR01 (1), SCAR03 (1)
Myospalax fontanieri (Common Chinese zokor)	0/1 (0)	/	/	1/1 (100.0)	SCAR01 (1)
***Microtus arvalis* (Vole)**	57/66 (86.4)	*G. microti* (57)	/	1/66 (1.5)	J (1)
***Eothenomys miletus* (Yunnan Red-backed vole)**	0/41 (0)	/	/	1/41 (2.4)	SCAR01 (1)
Callosciurus erythraeus (Pallas's squirrel)	0/1 (0)	/	/	0/1 (0)	/
***Spermophilus undulatus* (Long-tailed Ground squirrel)**	0/66 (0)	/	/	4/66 (6.1)	XJUR (4)
Total	75/378 (19.8)	*G.microti* (57), *G. muris* (15), *G. duodenalis* (3)	G (2), A (1)	54/442 (12.2)	D (20), Type IV (12), PigEBITS7 (5), XJUR (4), SCAR01 (3), J (2), Peru 8 (2), SCR05 (1), SCMR (1), Korea-WL6 (1), SCAR02 (1), HNFS01 (1), SCAR03 (1)

voles and brown rats, respectively (Table 2).

3.6. Infection of *E. bieneusi*

The overall prevalence of *E. bieneusi* was 12.2% (54/442) in wild rodents (Table 2). The *E. bieneusi* infection rate was highest in Guangdong (32.0%, 40/125), followed by Sichuan (4.9%, 9/185) and Xinjiang (3.8%, 5/132). By rodent species, the highest infection rate of *E. bieneusi* was found in lesser rice-field rats (42.3%, 11/26), followed by brown rats (29.9%, 29/97), long-tailed ground squirrels (6.1%, 4/66), Coxing's white-bellied rats (4.8%, 2/42), South China field mice (4.0%, 4/99), Yunnan red-backed voles (2.4%, 1/41), and voles (1.5%, 1/66). The chestnut white-bellied rat and common Chinese zokor sampled were both positive for *E. bieneusi*, while the greater bandicoot rats and one Pallas's squirrel sampled were negative for *E. bieneusi*.

3.7. *Enterocytozoon bieneusi* genotypes

Based on sequence analysis of the ITS locus, we detected 13 genotypes of *E. bieneusi*, including eight known genotypes (D, Type IV, PigEBITS7, J, Peru 8, SCR05, Korea-WL6, and HNFS01) and five novel genotypes (XJUR, SCAR01, SCAR02, SCAR03, and SCMR) (Table 2). Among them, the zoonotic genotypes D ($n = 20$) and Type IV ($n = 12$) were dominant. Phylogenetically, the 13 genotypes belonged to Groups 1, 2, 10, 14 and 15 (Fig. 5). The novel genotypes SCAR01 and SCAR02 were placed in Group 1, SCAR03 in Group 10, SCMR in Group 14, and XJUR in Group 15.

By sampling locations, the common human-pathogenic genotypes D, Type IV, PigEbITS7 and Peru8 were found only in Guangdong, while the remaining host-adapted genotypes were found in Sichuan and Xinjiang. By rodent species, the common human-pathogenic genotypes D, Type IV, PigEbITS7 and Peru8 were only detected in brown rats and Lesser rice-field rats, while the host-adapted genotypes were identified in the remaining rodent species (Table 2).

3.8. Co-infection of the enteric pathogens

Among the 442 rodents examined in this study, 11 rodents had co-infections of *Cryptosporidium* spp. and *E. bieneusi*, mainly brown rats and Lesser rice-field rats from Guangdong ($n = 7$). Eighteen rodents had co-infections of *Cryptosporidium* spp. and *Giardia* spp., mainly voles from Xinjiang ($n = 12$) and Coxing's white-bellied rats from Sichuan ($n = 4$). A vole from Xinjiang and a brown rat from Guangdong were found to be co-infected with *Giardia* spp. and *E. bieneusi*. Nevertheless, co-infection

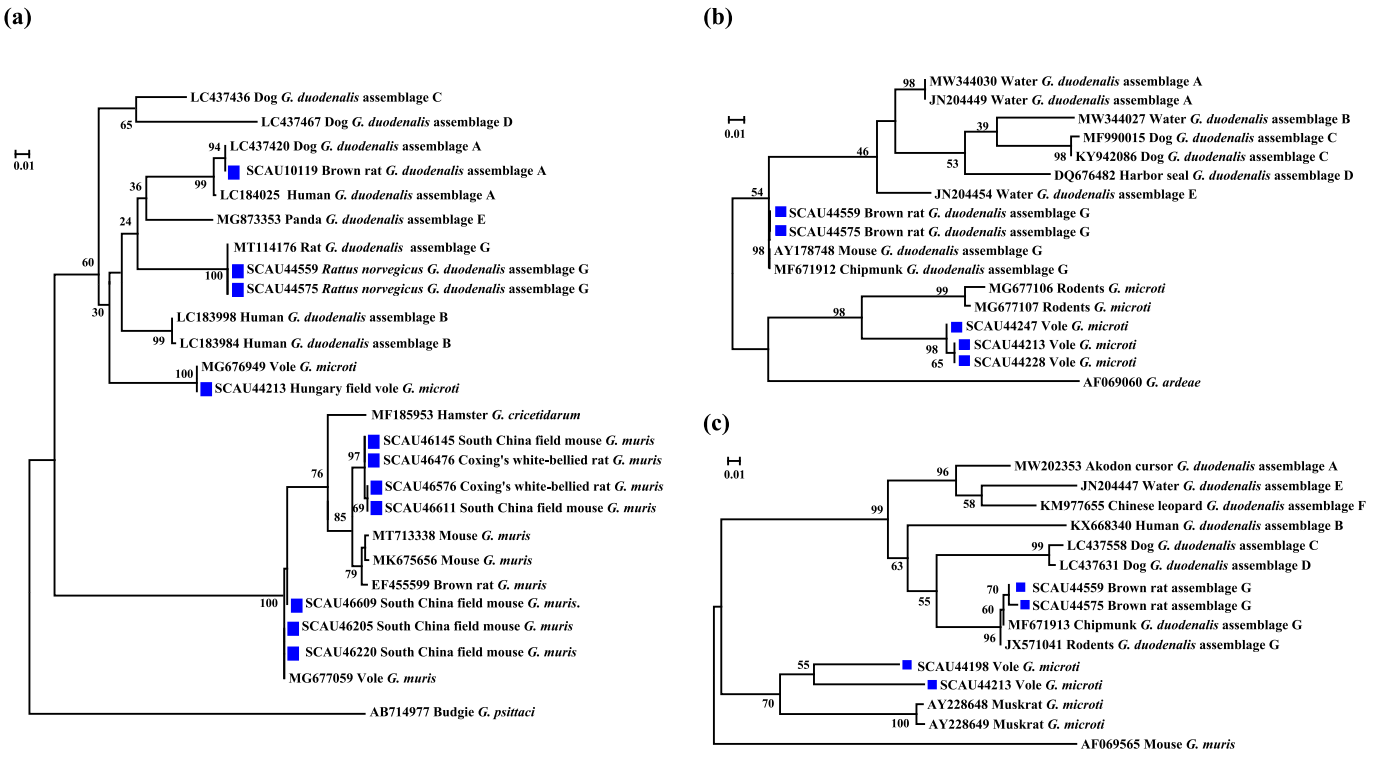

Fig. 4. Phylogenetic relationships of *Giardia* spp. based on the maximum likelihood analyses of the *bg* gene (a), *gdh* gene (b) and *tpi* gene (c). Bootstrap values >50% from 1000 replicates are displayed. The blue triangles indicate known species identified in the present study. The scale bar indicates 0.01 nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the three enteric pathogens was not detected.

4. Discussion

A high prevalence of *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi* was found in wild rodents from three provinces in China, with infection rates of 19.9%, 19.8% and 12.2%, respectively. Overall, the infection rates of these pathogens are consistent with previous reports worldwide showing the prevalence in rodents ranged from 0.7% to 100.0% for *Cryptosporidium* spp. [9], 1.6% to 66.3% for *Giardia* spp. [12,30,31], and 2.0% to 38.9% for *E. bieneusi* [5,18,32]. The prevalence of these pathogens in wild rodents is affected by many factors, including animal species, animal gender, animal age, sample size, sampling time, sampling locations and ecological conditions [9]. In the present study, the prevalence of these pathogens varies among different sampling locations or different rodent species. Co-infections of these pathogens were also found to be common here. As only a small number of samples were collected from a few rodent species, including 2 greater bandicoot rats, 1 Chestnut white-bellied rat, 1 common Chinese zokor and 1 Pallas's squirrel, further studies involving extensive sampling of these rodent species are needed to better understand the prevalence of enteric pathogens in wild rodents.

A high genetic diversity of *Cryptosporidium* spp. was observed in wild rodents in this study, with 23 known *Cryptosporidium* species and genotypes identified. To date, 28 known *Cryptosporidium* species and 41 genotypes have been identified in rodents worldwide [9]. The majority of *Cryptosporidium* species and genotypes detected in this study have been found in rodents previously, with the exception of six species and genotypes being found elsewhere, including *C. baileyi* in poultry, bear genotype in black bear, and sw1, w19, w25and *Cryptosporidium* sp. KY483983 in water. The distribution of these *Cryptosporidium* species varies among different sampling locations or rodent species, which is in agreement with previous findings in rodents worldwide [9].

Cryptosporidium parvum, *C. viatorum* and *C. ubiquitum* found in wild rodents in this study are common zoonotic species frequently found in humans. They have been reported previously in various species of rodents, such as rats, mice, voles, and squirrels [33,34,35]. In this study, *C. parvum* was identified in a brown rat in Guangdong and belonged to the IIdA20G1 subtype. This subtype has been found in farm animals and humans worldwide [36,37], and recently caused two outbreaks of cryptosporidiosis in pre-weaned calves in China [38]. *Cryptosporidium viatorum* is another common zoonotic *Cryptosporidium* species, which was identified here in South China field mice and Coxing's white-bellied rats in Sichuan. The XVa subtype family of *C. viatorum* was known to infect humans [25], however, the *C. viatorum* isolates identified in rodents here belonged to two novel subtype families XVe and XVf with unknown human-infective potential. Additionally, *C. ubiquitum* was identified in a Pallas's squirrel and a China field mouse in Sichuan, belonging to a known subtype family XIIh and a new subtype family XIIj, respectively. XIIh has been reported from both environmental and rodent sources [39], but further studies are needed to understand the host range of the new subtype family XIIj. In addition to the three zoonotic *Cryptosporidium* species, some other species like *C. muris*, *C. occultus* and *C. tyzzeri* were also identified in wild rodents here, which have been found in humans occasionally [40,41,42].

Apparent host adaptation and geographical differences were observed among *Giardia* spp. in wild rodents in the present study. *Giardia microti* was found only in voles in Xinjiang, *G. muris* only in South China field mice and Coxing's white-bellied rats in Sichuan, *G. duodenalis* only in brown rats in Guangdong. Geographical differences of the distribution of *Giardia* spp. might be due to different rodent species sampled in each region. Thus, the existence of host adaptation is apparent here, which is supported by previous finding of *Giardia* distribution in wild rodents in Germany [43]. *Giardia microti* and *G. muris* are considered as rodent-adapted species without infectivity to humans [44]. Within the only zoonotic species *G. duodenalis*, assemblages G and A were identified. Assemblage G has been commonly found in mice and rats but not in humans [45]. The assemblage A isolate belonged to the

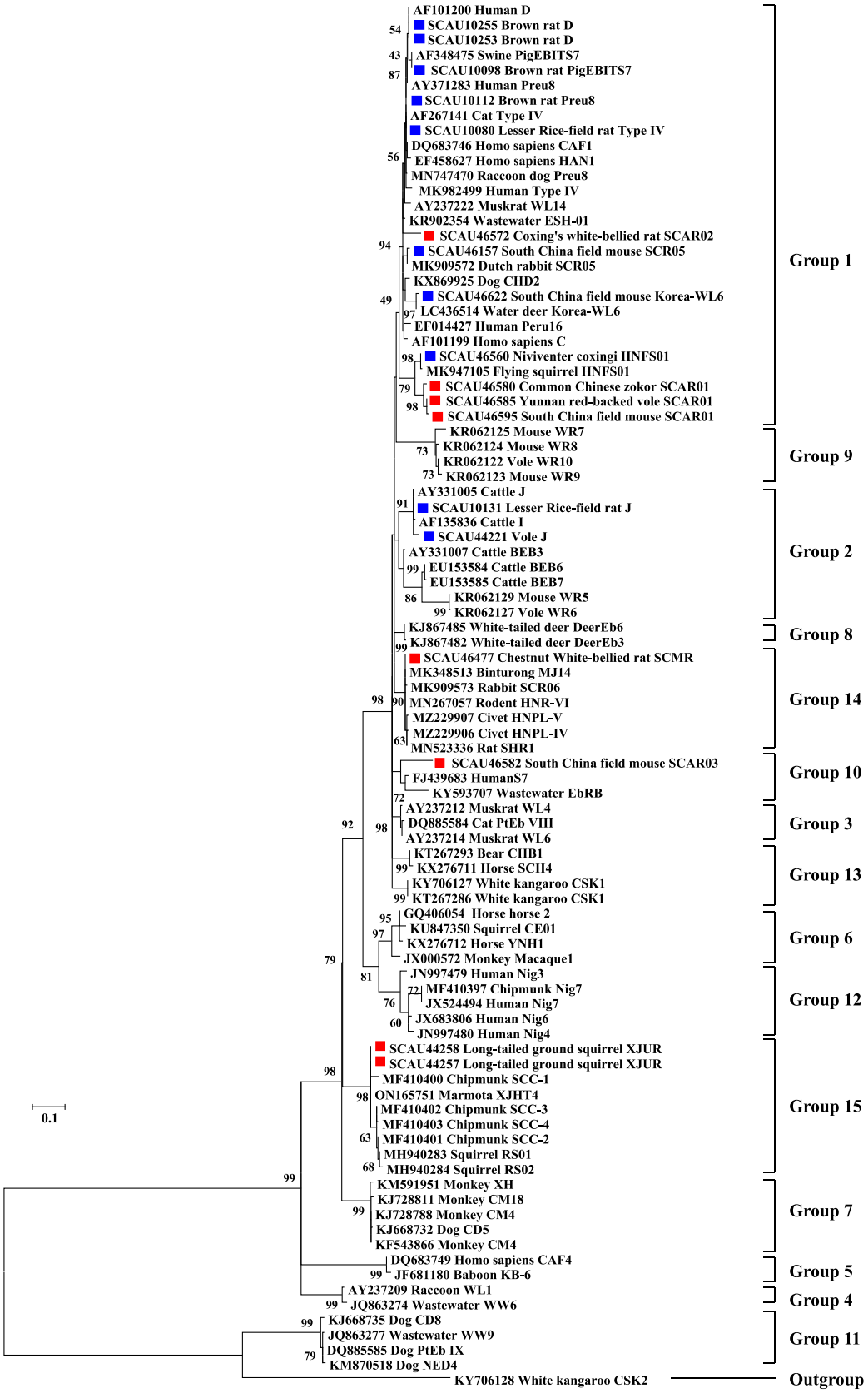

Fig. 5. Phylogenetic relationship among *Enterocytozoon bieneusi* genotypes based on the maximum likelihood analyses of the ITS locus. Bootstrap values >50% from 1000 replicates are displayed. Known and novel genotypes are indicated by bule and red squares, respectively. The scale bar indicates 0.1 nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sub-assemblage AI, which has been detected in humans in many studies [46]. Nevertheless, because of only one assemblage A isolate identified, the zoonotic potential of *Giardia* spp. in wild rodents in this study is limited.

High genetic diversity and zoonotic potential of *E. bieneusi* were observed in wild rodents. Altogether, 13 genotypes of *E. bieneusi* were identified and belonged to Groups 1, 2, 10, 14 and 15, with genotypes in Groups 10, 14 and 15 being firstly reported in wild rodents. Nine genotypes were found belonging to the zoonotic Group 1, including D, Type IV, PigEBITS7, Peru 8, SCR05, Korea-WL6, HNFS01, SCAR01, and SCAR02. Among them, genotypes D, Type IV, PigEbITS7 and Peru8 were detected in brown rats and Lesser rice-field rats in Guangdong, which have been found in numerous human cases [47]. These four common human-pathogenic genotypes were also reported in rodents in China previously [16,20]. In addition, two novel genotypes SCAR01 and SCAR02 were phylogenetically placed in the zoonotic Group 1. Therefore, the data indicated that natural transmission of *E. bieneusi* among rodents and humans might occur.

Some species and genotypes of *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi* identified in wild rodents in this study have been previously found in humans and domestic animals in China. Although there is no direct evidence of overlap with human and rodent infections in the geographical areas studied, some species and genotypes of these pathogens have been found in humans in other regions of China, such as *C. parvum*, *C. viatorum*, *G. duodenalis* assemblage A, and *E. bieneusi* genotype D and Type IV [48,49,50]. In addition, we have noticed that some domestic animals in the study areas were infected with these rodent-related pathogens, including dogs and cats in Guangdong infected with *C. muris*, rat genotype IV [51], and *E. bieneusi* genotype D and Type IV [52], sheep in Sichuan infected with *C. ubiquitum* [53], and dairy cattle in Guangdong and Xinjiang infected with *E. bieneusi* genotype J [54,55]. It suggests that wild rodents may pose a potential risk for the transmission of zoonotic diseases. Hygiene and One Health measures should be implemented in urban environments and rural farms to reduce the possible direct and indirect transmission of these rodent-related pathogens.

Brown rats frequently occurring in urban areas could carry human-pathogenic *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi*. In the trapped brown rats in streets and food stores in Guangzhou city in Guangdong, some zoonotic species and genotypes of these pathogens were identified, including *C. parvum* subtype IIdA20G1, *G. duodenalis* assemblage A, and *E. bieneusi* genotypes D, Type IV, PigEbITS7 and Peru8. This is supported by previous findings of zoonotic *C. parvum* and *E. bieneusi* distribution in brown rats in China and elsewhere [5,9]. In previous studies in China, the IIdA15G1 subtype of *C. parvum* was detected in Fujian, and *E. bieneusi* genotype D was detected in Hainan [16,56]. The rodent-specific assemblage G of *G. duodenalis* was commonly found in brown rats worldwide [45,57], however, in addition to assemblage G, the zoonotic assemblage A was also identified in brown rats in this study. Feces of brown rats could contaminate urban environment, food stores, and human habitations, thus brown rats may play an important role in the zoonotic transmission of these enteric pathogens in urban areas.

5. Conclusions

In conclusion, this study revealed the high prevalence and genetic diversity of *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi* in wild rodents in three provinces of China. Apparent host adaptation and geographical differences were observed among *Cryptosporidium* spp., *Giardia* spp. and *E. bieneusi* genotypes in eleven wild rodent species. Moreover, the presence of zoonotic *Cryptosporidium* spp. and *E. bieneusi* genotypes indicates that wild rodents could be potential reservoirs for human and domestic animal infections with these pathogens due to their large numbers and wide distributions, especially brown rats in urban areas. Therefore, hygiene and One Health measures should be implemented in urban streets and food stores to prevent brown rats from transmitting these zoonotic pathogens. An increase in the number of wild rodents from broader geographical locations and diversity of rodent species surveyed for these pathogens may help gain a much-improved understanding of the role of wild rodents in the epidemiology of cryptosporidiosis, giardiasis and microsporidiosis.

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CRediT authorship contribution statement

Kangli Feng: Data curation, Formal analysis, Investigation, Writing – original draft. **Shenghua Yang:** Data curation, Investigation, Writing – original draft. **Yanhua Xu:** Data curation, Investigation, Writing – original draft. **Luxing Wen:** Investigation, Resources, Validation. **Jia Chen:** Formal analysis, Validation. **Wenbao Zhang:** Investigation, Resources. **Shouyi Chen:** Investigation, Resources. **Yongyi Shen:** Investigation, Resources. **Lihua Xiao:** Conceptualization, Supervision, Writing – review & editing. **Yaqiong Guo:** Formal analysis, Methodology. **Yaoyu Feng:** Methodology, Project administration, Conceptualization, Funding acquisition. **Na Li:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors have no conflicting interest to declare.

Data availability

Data will be made available on request.

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