Occurrence and genetic characteristics of Cryptosporidium spp. and Enterocytozoon bieneusi in pet red squirrels (Sciurus vulgaris) in China

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Cryptosporidium spp. and Enterocytozoon bieneusi are two well-known protist pathogens which can result in diarrhea in humans and animals. To examine the occurrence and genetic characteristics of Cryptosporidium spp. and E. bieneusi in pet red squirrels (Sciurus vulgaris), 314 fecal specimens were collected from red squirrels from four pet shops and owners in Sichuan province, China. Cryptosporidium spp. and E. bieneusi were examined by nested PCR targeting the partial small subunit rRNA (SSU rRNA) gene and the ribosomal internal transcribed spacer (ITS) gene respectively. The infection rates were 8.6% (27/314) for Cryptosporidium spp. and 19.4% (61/314) for E. bieneusi. Five Cryptosporidium species/genotypes were identified by DNA sequence analysis: Cryptosporidium rat genotype II (n = 8), Cryptosporidium ferret genotype (n = 8), Cryptosporidium chipmunk genotype III (n = 5), Cryptosporidium rat genotype I (n = 4), and Cryptosporidium parvum (n = 2). Additionally, a total of five E. bieneusi genotypes were revealed, including three known genotypes (D, SCC-2, and SCC-3) and two novel genotypes (RS01 and RS02). Phylogenetic analysis revealed that genotype D fell into group 1, whereas the remaining genotypes clustered into group 10. To our knowledge, this is the first study to report Cryptosporidium spp. and E. bieneusi in pet red squirrels in China. Moreover, C. parvum and genotype D of E. bieneusi, previously identified in humans, were also found in red squirrels, suggesting that red squirrels may give rise to cryptosporidiosis and microsporidiosis in humans through zoonotic transmissions. These results provide preliminary reference data for monitoring Cryptosporidium spp. and E. bieneusi infections in pet red squirrels and humans.

Cryptosporidium spp. and Enterocytozoon bieneusi, causative agents of cryptosporidiosis and microsporidiosis, are two important opportunistic intestinal pathogens that can infect vertebrate and invertebrate, posing a significant threat to public health1. Humans are infected with these pathogens mainly via the fecal-oral route with anthropogenic and zoonotic transmission or via food-borne and water-borne transmission2,3. Clinical manifestations of infection with these pathogens are often inconsistent due to the variabilities in the health condition of infected hosts4,5. In healthy individuals, these pathogens usually cause asymptomatic infection or self-limiting diarrhea. However, infection may also result in chronic or life-threatening diarrhea in immunocompromised individuals, such as patients with acquired immunodeficiency syndrome and patients who had undergone organ transplantation7. In addition to humans, there are a variety of animals that can act as hosts for these two pathogens, including various mammals, reptiles, birds, amphibians and insects8. Therefore, Cryptosporidium spp. and

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**Table 1.** Occurrence of Cryptosporidium spp. and *Enterocytozoon bieneusi* in pet red squirrels from different sources in Southwestern China.

| Sources          | No. of examined | Cryptosporidium spp. | E. bieneusi |
|------------------|-----------------|-----------------------|-------------|
|                  | No. of positive | Prevalence (%) (95% CI) | OR (95% CI) | Species/Genotype (n) | No. of positive | Prevalence (%) (95% CI) | OR (95% CI) | Genotype (n) |
| Pet shop 1       | 58              | 2                     | 3.4% (0.014–0.083) | reference rat genotype I (2) | 5              | 8.6% (0.012–0.161) | reference D (3), RS01 (2) |
| Pet shop 2       | 74              | 12                    | 16.2% (0.076–0.248) | rat genotype II (8), chipmunk genotype III (3), *C. parvum* (1) | 16             | 21.6% (0.12–0.312) | 2.9 (1.0–8.5) | D (6), SCC-2 (8), SCC-3 (2) |
| Pet shop 3       | 76              | 8                     | 10.5% (0.035–0.176) | ferret genotype (6), chipmunk genotype III (2) | 21             | 27.6% (0.173–0.379) | 4.0 (1.4–11.5) | D (13), SCC-2 (6), RS02 (2) |
| Pet shop 4       | 61              | 4                     | 6.6% (0.002–0.129) | rat genotype II (2), ferret genotype (2) | 14             | 23% (0.121–0.338) | 3.2 (1.1–9.4) | D (4), SCC-3 (10) |
| owners           | 45              | 1                     | 2.2% (0.023–0.067) | *C. parvum* (1) | 5              | 11.1% (0.016–0.207) | 1.3 (0.4–4.9) | D (1), SCC-2 (4) |
| Total            | 314             | 27                    | 8.6% (0.055–0.117) | rat genotype II (8), ferret genotype (8), chipmunk genotype III (5), rat genotype I (4), *C. parvum* (2) | 61             | 19.4% (0.150–0.238) | D (27), SCC-2 (18), SCC-3 (12), RS01 (2), RS02 (2) |

*E. bieneusi* have been recognized as category B pathogens by the National Institutes of Health due to their ease of transmission in spite of low mortality. To detect and evaluate potential zoonotic transmissions, it is necessary to accurately distinguish *Cryptosporidium* spp. and *E. bieneusi* on the molecular level. To date, at least 37 species and over 70 genotypes of *Cryptosporidium* spp. have been described. Among them, 11 *Cryptosporidium* species have been identified in rodents, *Cryptosporidium parvum* and *C. muris* are the most common. For *E. bieneusi*, more than 474 genotypes have been identified based on the internal transcribed spacer (ITS) region of the rRNA gene, and more than 35 genotypes have been determined in rodents. These genotypes can be classified into eleven groups (groups 2–11) as the host-adapted groups, which are mostly found in specific hosts or water. In China, *Cryptosporidium* spp. and *E. bieneusi* have been detected in a wide range of hosts, including carnivores, lagomorphs, primates, birds, and rodents. Pet rodents, in particular (e.g. chinchillas, red-bellied tree squirrels, guinea pigs, and chipmunks), are considered potential sources of *Cryptosporidium* spp. and *E. bieneusi* infections in humans. The red squirrel (*Sciurus vulgaris*) is a popular pet in China, which is widely bred in pet shops and homes for its appearance and mild-mannered nature. However, there is no published data regarding the prevalence of *Cryptosporidium* spp. and *E. bieneusi* in pet red squirrels, and the role of the red squirrels in the transmission of the two pathogens remains poorly investigated. Thus, we examined the occurrence of *Cryptosporidium* spp. and *E. bieneusi* in red squirrels, and evaluated their potential role in the zoonotic transmission of human cryptosporidiosis and microsporidiosis.

**Results**

**Occurrence of Cryptosporidium spp. and *E. bieneusi*.** The overall prevalence of *Cryptosporidium* spp. in pet red squirrels was 8.6% (27/314, 95% CI: 5.5–11.7%). All pet shops were positive for *Cryptosporidium*, and the prevalence ranged from 2.2% to 16.2%; significant differences were observed ($\chi^2 = 0.028$, df = 4, $P < 0.05$; Table 1). The prevalence of *Cryptosporidium* spp. among males and females were 8.1% and 9%, respectively, but the difference was not statistically significant ($\chi^2 = 0.886$, df = 1, $P > 0.05$). The differences in prevalence of *Cryptosporidium* spp. among squirrels of different ages were not statistically significant ($\chi^2 = 0.093$, df = 1, $P > 0.05$) (Table 2). Moreover, a significant correlation between the different sources and *Cryptosporidium* spp. infection ($P = 0.01$) was observed by logistic regression analysis.

The overall prevalence of *E. bieneusi* in pet red squirrels was 19.4% (61/314, 95% CI: 15–23.8%). *E. bieneusi* was found in all four pet shops investigated, with infection rates ranging between 8.6% and 27.6%. The difference was statistically significant ($\chi^2 = 0.036$, df = 4, $P < 0.05$; Table 1). The prevalence of *E. bieneusi* in female red squirrels (20.5%) was higher than male (18.2%), but the difference was not statistically significant ($\chi^2 = 0.251$, df = 1, $P > 0.05$). The differences in prevalence of *E. bieneusi* among squirrels of different ages were not statistically significant ($\chi^2 = 0.353$, df = 1, $P > 0.05$) (Table 2). No mixed infections of the two pathogens were found in red squirrels in our study. Similarly, a significant correlation between the different sources and *E. bieneusi* infection ($P = 0.01$) was observed by logistic regression analysis.

**Cryptosporidium** species/genotypes. Twenty-seven *Cryptosporidium*-positive samples were genotyped by sequence analysis of the SSU rRNA gene, and five *Cryptosporidium* species/genotypes were identified: *Cryptosporidium* rat genotype II (8/27, 30%), *Cryptosporidium* ferret genotype (8/27, 30%), *Cryptosporidium* chipmunk genotype III (5/27, 18.5%), *Cryptosporidium* rat genotype I (4/27, 14.8%), and *C. parvum* (2/27, 7.4%). Phylogenetic relationship analysis confirmed the identity of *Cryptosporidium* species/genotypes (Fig. 1). *Cryptosporidium* rat genotype II and *Cryptosporidium* ferret genotype were the two most predominant genotypes (Table 1).

At the SSU rRNA locus, eight *Cryptosporidium* rat genotype II isolates had 100% homology between each other and were identical to that (GQ121025) from *Rattus tanezumi* in China and those from *R. rattus* in Southwestern China.
C. parvum, chipmunk genotype III, and Cryptosporidium identified, including in humans in Henan province, China. Moreover, Siberian chipmunks, chinchillas, and Bamboo rats in China, highlighting the prevalence of C. parvum in Japan, mice and red-backed voles in the USA, brown rats in Iran, striped field mice in Slovak Republic, and hamsters, Siberian chipmunks, chinchillas, and Bamboo rats in China, highlighting the prevalence of C. parvum in various rodents in China (Table 3). In this study, five different genotypes were identified, including two novel genotypes, which were named RS01 and RS02. Genotype D was the most prevalent (44.3%, 27/61) and showed 100% homology with the sequences JF927954 (from humans in China) and AY371284 (from humans in Peru). Genotypes SCC-2 and SCC-4 had 100% homology with the two sequences MF410401 and MF410403, respectively.

With regard to the two novel genotypes, RS01 displayed two single nucleotide polymorphisms (SNPs) within the 243 bp of the ITS gene sequence of E. bieneusi, when compared with the genotype SCC-2 (MF410401), which showed 99% homology. RS02 had three SNPs (G/A at positions 178 and 324) respectively.

A phylogenetic analysis of the ITS gene sequences of all the genotypes of E. bieneusi obtained here and reference genotypes published previously revealed that genotype D clustered in group 1 and further clustered into 1a, whereas genotypes SCC-2, SCC-4, and two novel genotypes (RS01 and RS02) clustered in group 10 (Fig. 2).

**Discussion**

In 314 fecal samples of red squirrel, we first demonstrated the presence of Cryptosporidium spp. (8.6%, 27/314) was lower than the average prevalence previously reported in multiple rodent species, with an infection rate of 1.5% in laboratory rats to 85.0% in guinea pigs. The relatively low occurrence of Cryptosporidium spp. in this study may be explained by the fact that the pet Cryptosporidium infections have been observed in brown rats in Iran (6.6%), interpreted in a separate cage. The prevalence of E. bieneusi was 19.4%, which was similar to that in two recent studies in Sichuan province for E. bieneusi infection rates in red-bellied tree squirrels (16.7%, 24/144) and chipmunks (17.6%, 49/279). The prevalence of E. bieneusi in rodents ranged from 1.1% to 100% (Table 4). As proposed in other studies, factors contributing to the prevalence of these pathogens may include the examination method, age, sex, season, host health status, feeding density, sample size, geo-ecological conditions, and living conditions.

Previous studies have indicated that five Cryptosporidium species and nine Cryptosporidium genotypes exist in various rodents in China (Table 3). In this study, five different Cryptosporidium species/genotypes were identified, including Cryptosporidium rat genotypes I and II, Cryptosporidium ferret genotype, Cryptosporidium chipmunk genotype III, and C. parvum. Cryptosporidium rat genotypes I and II have been found in brown rats in the Philippines, Nigeria, Australia, and China, even in South Nation River watershed, raw wastewater, and environmental samples in the United Kingdom, Canada, and China. Cryptosporidium ferret genotype has been found in ferrets and red squirrels in Italy. The Cryptosporidium chipmunk genotype III was previously reported in red squirrels, eastern squirrels, eastern chipmunks, and deer mice in the USA. To date, little is known regarding the disease-causing potential of the four genotypes in humans and livestock; thus bringing attention to the need for epidemiological molecular surveillance of Cryptosporidium spp. for the assessment of infectivity across different hosts.

C. parvum is one of the two predominant Cryptosporidium species in humans. C. parvum has been identified in humans in Henan province, China. Moreover, C. parvum infections have been observed in brown rats in Japan, mice and red-backed voles in the USA, brown rats in Iran, striped field mice in Slovak Republic, and hamsters, Siberian chipmunks, chinchillas, and Bamboo rats in China, highlighting the prevalence of C. parvum in various rodents in China.

### Table 2. Occurrence of Cryptosporidium spp. and Enterocytozoon bieneusi in pet red squirrels according to sex and age.

| Factor | Characteristics | No. of examined | No. of positive | Prevalence (%) (95% CI) | OR (95% CI) | No. of positive | Prevalence (%) (95% CI) | OR (95% CI) |
|--------|-----------------|----------------|----------------|-------------------------|-------------|-----------------|-------------------------|-------------|
| Sex    | Male            | 148            | 12             | 8.1% (0.037–0.126)      | reference   | 27              | 18.2% (0.119–0.245)     | reference   |
|        | Female          | 166            | 15             | 9% (0.046–0.134)        | 1.1 (0.5–2.5)| 34              | 20.5% (0.143–0.267)     | 1.2 (0.7–2.0) |
| Age    | ≤3 months       | 154            | 14             | 9.1% (0.045–0.137)      | reference   | 32              | 20.8% (0.143–0.273)     | reference   |
|        | >3 months       | 160            | 13             | 8.1% (0.038–0.124)      | 0.9 (0.4–1.9)| 29              | 18.1% (0.121–0.242)     | 0.8 (0.5–1.5) |
rodents (Table 3)\textsuperscript{12,25,27}. In addition, \textit{C. parvum} has also been found in other animals, such as cattle, sheep, goats, deer, alpacas, horses, dogs, gray wolves, raccoon dogs, cats, and pigs\textsuperscript{43,44}. In this study, only two \textit{C. parvum} isolates were identified in investigated red squirrels; however, these isolates may result in emerging zoonotic infections through the oral-fecal route.

Three known genotypes (D, SCC-2, and SCC-3) and two novel genotypes (RS01 and RS02) were identified in this study. Genotype D was the predominant genotype (44.3\%, 27/61). This finding was similar to previous reports in mice in Czech Republic and Germany\textsuperscript{45} (32.3\%, 10/31), mice in Poland\textsuperscript{46} (33.3\%, 10/30), and brown

![Phylogenetic tree](image)

**Figure 1.** Phylogenetic relationships between the partial \textit{Cryptosporidium} SSU rRNA gene from red squirrels and the \textit{Cryptosporidium} spp. or genotypes deposited in GenBank. The GenBank accession number of each \textit{Cryptosporidium} species or genotype is shown in parentheses. Bootstrap values above 50\% from 1,000 replicates are shown at the nodes. The newly generated sequences are indicated in bold.
rats (89.5%, 17/19) and red-bellied tree squirrels (75%, 18/24) in China. In China, genotype D has been identified in various animals, such as nonhuman primates, cattle, sheep, horses, pigs, dogs, cats, and in wastewater. This study demonstrated the presence of genotype D in red squirrels for the first time, suggesting that red squirrels could play a potential role in the disease dissemination of *E. bieneusi* to humans.

Figure 2. Phylogenetic relationships of the *E. bieneusi* genotypes identified in this study and other reported genotypes. The phylogeny was inferred with a neighbor-joining analysis of the internal transcribed spacer (ITS) sequences based on distances calculated with the Kimura two-parameter model. Bootstrap values greater than 50% from 1,000 replicates are shown at the nodes. Genotypes with open circles and solid circles are known and novel genotypes identified in this study, respectively.
Table 3. Occurrence of Cryptosporidium species/genotypes in rodents from different countries. *-represents unknown.

| Country       | Host (common name) | Scientific name | No. of samples | No. of positive (%) | Species/Genotype (n) | References |
|---------------|--------------------|-----------------|----------------|---------------------|----------------------|------------|
| Japan         | Brown rats         | Rattus norvegicus | 50             | 19 (38)             | C. meleagris (1), C. parvum (1), New genotypes (11) | Kimura et al., 2007 |
| USA           | Opossum            | Didelphis virginiana | 2              | Marsupial genotype (2) | Feng et al., 2007 |
| Philippines   | Chipmunk           | Tamias striatus | —              | 1                   | C. baylei (1)       |            |
|               | Gray squirrel      | Sciurus carolinensis | —              | 1                   | Skunk genotype (1)  |            |
|               | White-footed mouse| Peromyscus leucopus | 3              | C. parvum (3)       |            |
|               | Deer mouse         | Peromyscus maniculatus | 3            | C. parvum (2), Muskrat II genotype (1) |            |
|               | Red-backed vole    | Clethrionomys gapperi | 2              | C. parvum (1), Muskrat II genotype (1) |            |
|               | Meadow vole        | Micrurus pennsylvanicus | 5              | Muskrat II genotype (5) |            |
|               | House mouse        | Mus musculus | —              | 1                   | C. parvum (1)       |            |
| Australia     | Black rats         | Rattus rattus | 85             | 7 (8.2)             | rat genotype III (4), rat genotype II (3) | Koehler et al., 2018 |
|               | Swamp rats         | Rattus lutreolus | 21             | 3 (14.3)            | C. viatorum (3)     |            |
| Philippines   | Asian house rat    | Rattus tanezumi | 83             | 37 (44.6)           | rat genotype III (18), rat genotype IV (5), suis-like genotype (5), C. scrofarum (3), rat genotype I (1), rat genotype II (1), C. muris (1) | Nghiublin et al., 2013 |
|               | Brown rat          | Rattus norvegicus | 70             | 12 (18.6)           | rat genotype II (5), rat genotype IV (1), C. muris (2), rat genotype I (2), C. scrofarum (1), rat genotype III (1) |            |
| Iran          | Brown rat          | Rattus norvegicus | 91             | 6 (6.6)             | C. parvum + C. muris (6) | Gholiapoury et al., 2016 |
| Nigeria       | Laboratory rats    | Rattus norvegicus | 134            | 2 (1.5)             | C. andersoni (1), rat genotype II (1) | Ayinnmode et al., 2017 |
| Slovak Republic | Striped field mouse | Apodemus agrarius | 103            | 34 (33)             | C. scrofarum (18), C. parvum (9), Muskrat genotype II (3), C. environment isolate (3), C. hominis (1) | Danišová et al., 2017 |
|               | Bank vole          | Myodes glareolus | 72             | 16 (22.2)           | C. scrofarum (4), C. parvum (3), Muskrat genotype I (3), C. environment isolate (6) |            |
|               | Yellow-necked mouse| Apodemus flavicollis | 73          | 15 (20.5)           | C. scrofarum (5), C. suis (4), C. parvum (3), C. environment isolate (3) |            |
| Italy         | Red squirrels      | Sciurus vulgaris | 70             | 17 (24.3)           | ferret genotype (15), chipmunk genotype I (2) | Krček et al., 2008 |
| China         | Brown rat          | Rattus norvegicus | 64             | 4 (6.2)             | C. tyzzeri (3), rat genotype III (1) | Lv et al., 2009 |
|               | Asian house rat    | Rattus tanezumi | 33             | 5 (15.2)            | C. tyzzeri (1), rat genotype II (2), rat genotype III (2) |            |
|               | Laboratory mouse   | Mus musculus | 229            | 4 (1.7)             | C. tyzzeri (4)      |            |
|               | Laboratory rat     | Rattus norvegicus | 25             | 1 (4)               | C. tyzzeri (1)      |            |
|               | Golden hamster     | Mesocricetus auratus | 50            | 16 (32)             | C. muris (7), C. andersoni (5), C. parvum (4) |            |
|               | Siberian hamster   | Phodopus sungorus | 51            | 4 (7.8)             | C. parvum (2), C. muris (1), hamster genotype (1) |            |
|               | Campbell hamster   | Phodopus campbelli | 30            | 3 (10)              | C. parvum (2), C. andersoni (1) |            |
|               | Red squirrel       | Sciurus vulgaris | 19             | 5 (26.3)            | ferret genotype (5) |            |
|               | Siberian chipmunk  | Tamias sibiricus | 20             | 6 (30)              | ferret genotype (4), C. parvum (1), C. muris (1), chipmunk genotype III (1) |            |
|               | Guinea pig         | Cavia porcellus | 40             | 34 (85)             | C. wrairi (30)      |            |
|               | Chipmunk           | Chinchilla laniger | 140           | 14 (10)             | C. ubiquitum (13), C. parvum (1), | Qi et al., 2015 |
|               | Brown rats         | Rattus norvegicus | 242            | 22 (9.1)            | rat genotype I (14), rat genotype IV (6), suis-like genotype (1), C. ubiquitum (1) | Zhao et al., 2018 |
|               | Squirrel monkey    | Saimiri sciureus | 24             | 1 (4.2)             | C. hominis monkey genotype II (1) | Liu et al., 2015a |
|               | Bamboo rats        | Rhizomys sinensis | 92             | 3 (3.3)             | C. parvum (3)       | Liu et al., 2015b |

Conclusions

This is the first report on the incidence of Cryptosporidium spp. and E. bieneusi in pet red squirrels in China. The infection rates of Cryptosporidium spp. and E. bieneusi were 8.6% and 19.4%, respectively. The detection of zoonotic C. parvum and genotype D of E. bieneusi suggests that red squirrels are a potential source of crypto- sporidiosis and microsporidiosis in humans. However, the infection sources and transmission dynamics between red squirrels and humans remain unknown, thus emphasizing on the importance of further follow-up studies of the transmission dynamics of these pathogens.
Table 4. Occurrence and genotypes of *Enteroctytozoon bieneusi* in rodents from different countries. aRepresents positive samples in feces and spleen.

| Country          | Host (common name) | Scientific name                  | No. of samples | No. of positive (%) | Genotypes (no.) | References                  |
|------------------|--------------------|----------------------------------|----------------|---------------------|------------------|-----------------------------|
| Czech Republic and Germany | East-European house mice | *Mus musculus musculus* | 127            | 14 (11)             | D (6), PgiEBITSS (4), EphA (2), C (1), H (1) | Sak et al., 2011           |
|                  | West-European house mice | *Mus musculus domesticus* | 162            | 17 (10.5)           | D (4), Peru 8 (4), CZ3 (4), PgiEBITSS (3), S6 (1), C (1) |                         |
| United States    | Eastern gray squirrel | *Sciurus carolinensis*          | 34             | 11 (32.4)           | WL4 (5), Type IV (3), WW6 (2), PpiEV + WL21 (1) | Guo et al., 2014           |
|                  | Eastern chipmunk   | *Tamias striatus*                | 7              | 5 (71.4)            | WL4 (3), Type IV (1), WL23 (1) |                             |
|                  | Woodchuck          | *Marmota monax*                 | 5              | 5 (100)             | WL4 (2), Type IV + WL20 (1), WL22 (1), WW6 (1) |                             |
|                  | Deer mouse         | *Peromyscus sp.*                | 55             | 13 (23.6)           | WL4 (10), WL23 (2), WL25 (1) |                             |
|                  | Boreal red-backed vole | *Myodes gapperi*               | 3              | 1 (20)              | WL20 + WL21 (1) |                             |
|                  | Meadow vole        | *Microtus pennsylvanicus*       | 10             | 3 (33)              | Peru11 (1), Peru11 + type IV (1), WL21 + unknown (1) |                         |
|                  | Guinea pigs        | *Cavia porcellus*               | 60             | 4 (6.7)             | Peru 16 (4) | Cama et al., 2007           |
|                  | Black-tailed prairie dogs | *Cynomys ludovicianus*      | 153            | 14 (9.2)            | Row (14) |                             |
| Poland           | Pallas             | *Apodemus agrarius*             | 184            | 79*                 | D (6), WR8 (2), WR5 (1), WR7 (1), gorilla 1 (1) | Perek-Matsyaik et al., 2015 |
|                  | Yellow-necked mouse | *Apodemus flavicollis*         | 60             | 18*                 | D (2), WR6 (6), WR4 (1), WR1 (1), WR9 (1) |                             |
| Slovakia         | House mouse        | *Mus musculus musculus*        | 280            | 3 (1.1)             | Unknown | Daniova et al., 2015        |
| China            | Chinchillas        | *Chinchilla lanigera*           | 140            | 5 (3.6)             | D (2), BEB6 (3) | Qi et al., 2015           |
|                  | Brown rats         | *Rattus norvegicus*             | 242            | 19 (7.9)            | D (17), Peru6 (2) | Zhao et al., 2018          |
|                  | Red-bellied tree squirrels | *Callosciurus erythraeus*    | 144            | 24 (16.7)           | D (18), EphC (3), SCC02 (1), CE01 (1), CE02 (1), CE03 (1), CE04 (1) | Deng et al., 2016         |
|                  | Chipmunks          | *Eutamias asiatica*            | 279            | 49 (17.6)           | D (6), SCC-1 (17), SCC-2 (9), SCC-3 (5), CHY1 (5), Nig 7 (4), CHG9 (2), SCC-4 (1) | Deng et al., 2018a         |

Materials and Methods

Ethics statement. The present study protocol was reviewed and approved by the Research Ethics Committee and the Animal Ethical Committee of Sichuan Agricultural University, and all methods were performed in accordance with the relevant guidelines and regulations. Permission was obtained from the owners or shop managers before the fecal specimens were collected.

Collection of specimens. A total of 314 fecal specimens were collected from red squirrels from four pet shops (n = 269) and owners (n = 45) in the Sichuan province, southwestern China between September 2016 and December 2017 (Table 1). All tested pet shops only raised red squirrels and served as suppliers of red squirrels to other pet shops. Sample size was approximately 20% of the squirrels from each shop, and small-scale shops (population less than 50) were not included. The four pet shops are distributed in Jianyang (104°32′E, 30°24′N), Pengzhou (103°57′E, 30°51′N), Wenjiang (103°51′E, 30°40′N), and Jintang (104°24′E, 30°59′N–30°51′N). In both pet shops and homes, red squirrels were housed in separate cages. Approximately 30–50 g fresh fecal samples were collected from the bottom of each cage after defecation using a sterile disposal latex glove and then immediately placed into individual disposable plastic bags. No obvious clinical signs were observed at the time of sampling, and the age, sex, and source were recorded at the same time. All fecal specimens were stored in 2.5% potassium dichromate solution at 4 °C until processing.

DNA extraction. The fecal specimens were washed three times in distilled water with centrifugation at 3,000 × g for 10 min to remove the potassium dichromate. Genomic DNA was extracted from approximately 200 mg of each processed fecal specimen using an E.Z. N. A. R Stool DNA kit (Omega Biotek Inc., Norcross, GA, USA) according to the manufacturer’s recommended instructions. The extracted DNA was stored at −20 °C until molecular analysis.

Genotyping of *Cryptosporidium* spp. and *E. bieneusi*. *Cryptosporidium* spp. were identified by nested polymerase chain reaction (PCR) amplification of an SSU rRNA gene fragment of ~850 bp designed by Xiao et al., 2011. *E. bieneusi* genotypes were determined by nested PCR amplification of a 392-bp fragment containing the entire ITS (243 bp) and portions of the flanking large and small subunits of the rRNA gene (Supplementary Table 1). TaKaRa Taq DNA Polymerase (TaKaRa Bio, Otsu, Japan) was used for PCR amplification. Positive controls (camel-derived *C. andersoni* DNA for *Cryptosporidium* spp. and horse-derived genotype D DNA for...
E. bieneusi) and negative control with no DNA added were included in all PCR assays. The secondary PCR products were examined by agarose gel electrophoresis and visualized after ethidium bromide staining.

**Sequence analysis.** All nested PCR positive-products were sequenced using the same PCR primers as those used for the secondary PCRs on an ABI 3730 instrument (Applied Biosystems, Foster City, CA, USA) at the BioSune Biotechnology Company (Shanghai, China). The nucleotide sequences of each obtained gene were aligned and analyzed using the Basic Local Alignment Search Tool and Clustal X (http://www.clustal.org/) with reference sequences retrieved from GenBank to identify Cryptosporidium spp. and E. bieneusi genotypes.

**Phylogenetic analyses.** To support the Cryptosporidium species/genotypes and assess the genetic relationships between the E. bieneusi genotypes in the present study and reference sequences previously published in GenBank, phylogenetic analysis was performed using Phylip version 3.69 package and by constructing a neighboring-joining tree using Mega 6 software (http://www.megasoftware.net/), which is based on evolutionary distances calculated using a Kimura 2-parameter model. The MegaAlign program in the DNA Star software package (version 5.0) was used to determine the degree of sequence identity. The reliability of these trees was assessed using bootstrap analysis with 1,000 replicates.

**Statistical analysis.** Variations in the occurrence of Cryptosporidium spp. (y1) and E. bieneusi (y2) in red squirrels according to age (x1), sex (x2), and geographical location (x3) were analyzed by χ² test using SPSS V20.0 (IBM, Chicago, IL, USA). Each of these variables was included in the binary logit model as an independent variable by multivariable regression analysis. When the P value was less than 0.05, the results were considered statistically significant. The adjusted odds ratio (OR) and 95% confidence interval (CI) for each variable were calculated with binary logistic regression, and all risk factors were entered simultaneously.

**GenBank accession numbers.** Representative nucleotide sequences were deposited in GenBank with the following accession numbers: MH940281-MH940290.

**Data availability**

All data generated or analysed during this study are included in this published article and its Supplementary Information Files.

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Author contributions
L.D. designed the project, performed experiments and discussed the data. Y.C. performed experiments and analyzed the data. R.L. analyzed and discussed the data. L.Y. collected the fecal samples. J.Y. collected the fecal samples. Z.Z. designed the project and analyzed the data. W.W. collected the fecal samples. L.X. performed experiments. H.F. analyzed and discussed the data. H.L. analyzed the data. Z.Z. designed the project. C.Y. collected the fecal samples. W.C. designed the project. G.P. designed the project and analyzed and discussed the data. All authors prepared final manuscript.

Competing interests
The authors declare no competing interests.

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