Stray cats and dogs carrying zoonotic *Enterocytozoon bieneusi* genotype D in China: a public health concern

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Abstract

Enterocytozoon bieneusi is reported to be a common microsporidian of humans and animals in various countries. However, scarce information on *E. bieneusi* has been recorded in cats (*Felis catus*) and dogs (*Canis familiaris*) in China. As such, we undertook molecular epidemiological investigation of *E. bieneusi* in cats and dogs in Shanghai, China. A total of 359 genomic DNAs were extracted from individual faecal samples from cats (n = 59) and dogs (n = 300), and then tested using a nested PCR-based sequencing approach employing internal transcribed spacer (ITS) of nuclear ribosomal DNA as the genetic marker. *Enterocytozoon bieneusi* was detected in 34 of all 359 (9.5%) faecal samples from cats (32.2%; 19/59) and dogs (5.0%; 15/300), including 24 stray cats and dogs (22.6%; 24/106), as well as ten household/raised cats and dogs (4.0%; 10/253). Correlation analyses revealed that *E. bieneusi* positive rates were significantly associated with stray cats and dogs ($P < 0.05$). The analysis of ITS sequence data revealed the presentation of five known genotypes CD7, CHN-HD2, D, PtEb IX and Type IV and two novel genotypes D-like1 and PtEb IX-like1. Zoonotic genotype D was the predominant type with percentage of 61.8 (21/34). Phylogenetic analysis of ITS sequence data sets showed that genotypes D, D-like1 and Type IV clustered within Group 1, showing zoonotic potential. The others were assigned into Group 10 with host specificity. These findings suggested that cats and dogs in Shanghai harbor zoonotic genotype D of *E. bieneusi* and may have a significant risk for zoonotic transmission. Further insight into the epidemiology of *E. bieneusi* in animals, water and the environment from other areas in China will be important to have an informed position on the public health significance of microsporidiosis caused by this microbe.

Keywords: *Enterocytozoon bieneusi*, Genotypes, Prevalence, Risk factors, Cats, Dogs, Shanghai

Introduction

Enterocytozoon bieneusi is the commonest pathogen responsible to most of human microsporidiosis, causing chronic or severe diarrhea, malabsorption or wasting [1, 2]. This microbe can transmit through faecal-oral route, via spores contaminated water, food or direct contact with infected individuals or their droppings [3]. Typically, molecular method PCR-based sequencing of internal transcribed spacer (ITS) of ribosomal DNA has been widely used to identify *E. bieneusi* [2]. Using this approach, more than 600 genotypes have been identified in a broad host range [4] (review). Some of these genotypes can be only found in animals (e.g., genotypes SCC-2 [Common chipmunk]), however, many other genotypes have been recorded in both humans and animals showing zoonotic potential (e.g., genotypes EbpC, D and Type IV). Thus, the National Institute of Allergy and Infectious Diseases (NIAID) classifies *E. bieneusi* as a Category B Priority Pathogen [5].

Numerous studies have been investigated *E. bieneusi* from humans and a large group of animal species, including various orders of mammals (Artiodactyla, Carnivora, Diprotodontia, Lagomorpha, Perissodactyla, Primates and Rodentia), birds (Anseriformes, Columbiformes, Falconiformes, Galliformes, Passeriformes, Psittaciformes and Struthioniformes) and reptiles (Squamata) as well as insects (Diptera) in more than 40 countries [4]. Although, there have been > 30 studies investigating this microbe in cats and dogs worldwide [6-8], only ten investigations of *E. bieneusi* was conducted in China, leading the systematic epidemiological studies and risk factors (e.g., temperature and humidity) of *E. bieneusi* in cats and dogs are scarce.

Shanghai is a developed metropolitan city with nearly 25,000 thousand people, and lots of residents in Shanghai have pets (e.g., cats and dogs). Xu et al. studied *E. bieneusi* in cats and dogs in Shanghai with the prevalence of 5.9% [9]. Also, Liu et al. investigated this pathogen

from stray dogs (8.8%) and found stray dogs have higher risk to infect humans than pets [10]. Previously, we carried out epidemiological studies of *E. bieneusi* from alpacas [11], cats and dogs [12], farmed cattle [13], farmed goats and sheep [14], wild deer [15], wild marsupials [16], zoo animals [17] and humans [18], in Australia and China. The prevalence and risk factors such as host species, age, sex, location, temperature and season were analysed and *E. bieneusi* genotypes were identified. The results show potential zoonotic transmission and a strong significant association between some risk factors and *E. bieneusi* prevalence. Here, in this study, we investigated *E. bieneusi* in cats and dogs in Shanghai. The aims of this study are to investigate the prevalence of *E. bieneusi* and its risk factors, characterise genotypes and analyze their zoonotic potential.

Materials and Methods

Samples and DNA isolation

In total, 359 faecal samples were collected from cats (*Felis catus*) (n = 59) and dogs (*Canis familiaris*) (n = 300), including household/raised cats (n = 9) and dogs (n = 244) from pet clinics (n = 193) and breeding centers (n = 60), as well as stray cats (n = 50) and dogs (n = 56) in Minhang (n = 309) and Jingan (n = 50) districts in Shanghai from October 2019 to July 2020, corresponding to three seasons: autumn (n = 121), spring (n = 128) and summer (n = 110) (Table 1). All cats and dogs from pet clinics were maintained in individual cages, while others from breeding centers were raised together. Most of them were apparently healthy. Faecal samples were collected from cats and dogs rectum and most of them were firm and solid, except for a few soft and watery cases. Genomic DNA was extracted directly from 0.1 g to 0.4 g of each of the

359 faecal samples (i.e., right after the sample collection) using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA).

Nested PCR-based sequencing of *E. bieneusi* ITS

Individual genomic DNA samples were subjected to nested PCR-coupled sequencing of the ITS region using an established technique [15]. Briefly, in the first PCR round, primers MSP-1 (forward: 5′-TGA ATG KGT CCC TGT-3′) and MSP-2B (reverse: 5′-GTT CAT TCG CAC TAC T-3′) were used to amplify 601 bp of ITS plus flanking gene sequences. In the second round, primers MSP-3 (forward: 5′-GGA ATT CAC ACC GCC CGT CRY TAT-3′) and MSP-4B (reverse: 5′-CCA AGC TTA TGC TTA AGT CCA GGG AG-3′) were employed to amplify a product of 535 bp containing 130 bp of the 3′-end of the small subunit (*SSU*) of the nuclear rRNA gene, 243 bp of the ITS and 162 bp of the 5′-region of the large subunit (*LSU*) rRNA gene.

Nested PCR for amplification of ITS was conducted in a reaction volume of 50 µl in a standard buffer containing 4.0 µM $MgCl_2$, 0.4 mM dNTPs, 50 pmol of each primer, 1.25 U of Ex Taq DNA Polymerase (TaKaRa Bio Inc., Beijing, China) and DNA template - except for the negative (no-template) controls. Known test-positive, test-negative and no template controls were included in each PCR run. The cycling conditions for both primary and secondary (nested) PCRs were: 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 45 s (denaturation), 54 °C for 45 s (annealing) and 72 °C for 1 min (extension), followed by 72 °C for 10 min (final extension).

The secondary PCR products were examined by gel electrophoresis on a 1.5% agarose gel containing 4S Green Plus Nucleic Acid Stain (Sangon Biotech, Shanghai, China) using TBE (65 mM Tris-HCl, 27 mM boric acid, 1 mM EDTA, pH 9; Bio-Rad, Hercules, CA, USA) as the

114 buffer, and their size estimated using a 2000 bp-DNA ladder (TaKaRa Bio Inc., Beijing, China)
115 as a reference and directly sequenced using primers MSP-3 and MSP-4B in separate reactions.
116 ITS sequences obtained (GenBank accession nos. OQ597705-OQ597711) were inspected for
117 quality using the program Geneious v.10 [19], and compared with reference sequences acquired
118 from the GenBank database (S1 Table). Genotypes of *E. bieneusi* were named according to the
119 recommendations by Santín and Fayer [3, 20].
120

121 ## Phylogenetic analysis

122 ITS sequences from this and previous studies were aligned over a consensus length of 301
123 positions using the methods from Zhang et al. [18], and then subjected to phylogenetic analyses
124 using the Bayesian inference (BI) and Monte Carlo Markov Chain (MCMC) methods in
125 MrBayes v.3.2.3 [21]. The Akaike Information Criteria (AIC) test in jModeltest v.2.1.7 [22] was
126 used to evaluate the likelihood parameters set for BI analysis. Posterior probability (pp) values
127 were calculated by running 2,000,000 generations with four simultaneous tree-building chains,
128 with trees saved every one hundredth generation. A 50% majority rule consensus tree for each
129 analysis was constructed based on the final 75% of trees generated by BI. *Enterocytozoon*
130 *bieneusi* clades and subclades were assigned using an established classification system [23-27].
131

132 ## Statistical analysis

133 The multivariate logistic linear regression were utilised to compare *E. bieneusi* test-positives
134 (faecal samples) with risk factors, and to test the association between the prevalence of *E.*
135 *bieneusi* DNA and season. The strength of association between *E. bieneusi* prevalence and a

univariate risk factor was measured using the odds ratio (OR) calculated with 95% confidence intervals (95% CI). A *P*-value of < 0.05 was considered statistically significant. IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses [18].

Results

Prevalence of *E. bieneusi* and risk factors

Enterocytozoon DNA was detected in 34 of the 359 (9.5%) faecal samples from cats (32.2%; 19/59) and dogs (5.0%; 15/300), including 24 stray cats and dogs (19 stray cats and five stray dogs) (22.6%; 24/106), as well as ten household/raised cats and dogs (4.0%; 10/253) (Table 1). None of *E. bieneusi* test-positivity was found in household cats. The prevalences in each season are 20.0% (22/110) in summer, 5.5% (7/128) in spring and 4.1% (5/121) in autumn (Table 2). The association analyses showed that *E. bieneusi* contamination in cats and dogs in summer was higher than that in autumn (OR = 2.841; 95% CI [0.967-8.343]) and spring (OR = 2.117; 95% CI [0.803-5.580]) without significance ($P > 0.05$). There were significant associations of *E. bieneusi*-positivity with living status of cats and dogs (Table 2), stray cats and dogs had 7.112 times higher risk of *E. bieneusi* infection than household/raised cats and dogs (OR = 7.112; 95% CI [3.263-15.500]) ($P < 0.05$).

Genotypes and phylogeny

The sequencing of the 301 ITS amplicons (241 - 243 bp) and their subsequent comparisons with reference sequences from the GenBank database revealed that five known genotypes CD7 (1), CHN-HD2 (1), D (21), PtEb IX (7) and Type IV (1) representing 31 amplicons and two

158 novel genotypes D-like1 (2) and PtEb IX-like1 (1) (S2 Table). Genotype D was the most
159 frequent type with the percentage of 61.8% (21/34), followed by genotypes PtEb IX 20.6% (7/34)
160 and D-like1 5.9% (2/34). The rest of genotypes had the same percentage of 2.9% (1/34).

161 The ITS sequences for all seven genotypes defined herein were aligned with sequences
162 representing all eleven established Groups of *E. bieneusi* [23-27] and then subjected to
163 phylogenetic analysis (Fig 1). In this analysis, All groups were each strongly supported (pp =
164 0.92 to 1.00) except for Groups 2, 6 and 7 (i.e., pp < 0.85 are not shown). Based on this analysis,
165 genotypes D, D-like1 and Type IV were assigned to Group 1 with strong statistical support (pp =
166 0.92), others were fall into Group 10 (pp = 0.99).

167

168 # Discussion

169 Here, we investigated the distribution and genetic identity of *E. bieneusi* in cats and dogs
170 faecal DNA samples by PCR-based sequencing of ITS in Shanghai, China. In total, 34 of 359
171 (9.5%) faecal DNA samples were test-positive for *E. bieneusi*, including 19 in cats (32.2%;
172 19/59) and 15 in dogs (5.0%; 15/300). This is the first time that the highest prevalence of *E.*
173 *bieneusi* was found in cats around the world, and genotype D - the most frequently identified in
174 humans, was widely recorded in both cats and dogs.

175 This study revealed a prevalence of *E. bieneusi* of 32.2% (19/59) in cats and 5.0% (15/300)
176 in dogs from pet clinics, breeding centers and shelters in Shanghai, China. The total prevalences
177 of *E. bieneusi* in cats and dogs worldwide are reported to range from 1.4% (2/143) [28] to 31.3%
178 (25/80) [29] and 0.8% (2/237) [6] to 22.9% (149/651) [30], respectively (Table 3). The
179 prevalence of *E. bieneusi* in cats herein is the highest record globally and the *E. bieneusi* test-
180 positives in cats were all stray cats. Whereas, *E. bieneusi* prevalence in dogs is only higher than

that recorded in a few *E. bieneusi* studies in dogs (e.g., 0.8% (2/237) [6]; 2.5% (2/79) [31]; 3.23% (2/62) [32]; 4.36% (26/597) [33] and 4.88% (4/82) [34]). These results indicate a higher *E. bieneusi* infection in stray cats in shanghai in this study, posing a public health concern, although it can not be entirely excluded that *E. bieneusi* spores may only pass through the gastrointestinal tract (pseudoparasitism), as identification of *E. bieneusi* DNA from faecal samples is not direct evidence of infection.

Here, we took the first step to carry out the association analysis between the risk factor of season and *E. bieneusi* prevalence in cats and dogs. Higher prevalence of *E. bieneusi* was observed in summer, but there was no significant support ($P > 0.05$) (Table 2). Association analysis revealed that stray cats and dogs were significantly associated with higher *E. bieneusi* prevalence than household cats and dogs ($P < 0.05$). Stray cats and dogs had 7.112 times higher risk to infect *E. bieneusi* than that in pet clinics and breeding centers (OR = 7.112; 95% CI [3.263-15.500]) (Table 2). Wang et al. studied *E. bieneusi* from pets and stray cats and dogs in Yunnan in China, and found that stray dogs had higher contaminations of *E. bieneusi* ($P < 0.05$) [7], same as the study of Liu et al. [10]. Similarly, Kváč, et al. conducted *E. bieneusi* investigations from pets and stray cats from three countries (Czech Republic, Poland and Slovakia), and they found that stray cats had higher *E. bieneusi* detection rates than pets [35]. This indicates that stray cats and dogs may have higher risk of *E. bieneusi* infections than that in pets, showing a public health threat. Thus, more studies are needed to monitor this pathogen in stray cats and dogs to prevent the outbreaks of human infections of *E. bieneusi*.

The analysis of ITS sequences data revealed seven *E. bieneusi* genotypes, i.e., CD7, CHN-HD2, D, D-like1, PtEb IX, PtEb IX-like1 and Type IV. Zoonotic genotype D (synonyms: CEbC, Peru9, PigEBITS9, PtEb VI, Peru2, WL8, NCF7, SHW1, MJ10, MJ11, MJ12, isolate 20, ZJR7

and FJS) was the commonest genotype found in humans worldwide, and it was also recorded in 68 animal species in more than 38 countries [23]. Similarly, genotype D was the predominant type in stray cats and dogs in the present study (61.8%; 21/34) (i.e., none of genotype D was found in household cats and dogs in pet clinics and breeding centers in this study), similar to most of other studies (S3 Table). This indicates that stray cats and dogs carrying zoonotic genotype D represent the host reservoirs transmitting *E. bieneusi* from them to humans. Obviously, more studies are needed for further verification.

Genotypes CD7 and PtEb IX found in the present study were commonly found in cats and dogs, also, they were sporadically reported in Bactrian camel, sika deer and white-lipped deer in China [36], whooper swan in China [37] and European badger in Spain [38] (S4 Table). Furthermore, none of these two genotypes had been found in humans yet. The result revealed that these genotypes mainly spread among cats and dogs with occasional dispersal in other animal hosts. Interestingly, genotypes PtEb IX was commonly found in drinking source water, sewer water and wastewater in China [39-41], showing that PtEb IX might be transmissible to susceptible hosts (e.g., cats and dogs) via spore-contaminated water or the environment. However, the exact source and transmission pattern of genotype PtEb IX in cats and dogs are difficult to track. As stated, it is clear that more studies of *E. bieneusi* from humans, other animals and the environment are necessary.

To assess the zoonotic potential of *E. bieneusi* genotypes in the present study, our phylogenetic analysis included ITS sequences of seven genotypes and representatives from ten established *E. bieneusi* Groups (Fig 1). The analysis of these sequence data sets revealed that genotypes CD7, CHN-HD2, PtEb IX and PtEb IX-like1 fall into Group 10, which was mainly reported in cats and dogs, and none of them has been identified in humans yet, showing host

specificity. However, genotypes D, D-like1 and Type IV were inferred to be in Group 1 with zoonotic potential (Fig 1). The identification of potentially zoonotic genotypes in cats and dogs in the present study suggested that they might act as host reservoirs transmitting *E. bieneusi* from them to humans, *vice versa*.

Conclusions

This study recorded *E. bieneusi* in cats and dogs in Shanghai in China. The prevalence of *E. bieneusi* in stray cats and dogs was higher than that in housing cats and dogs, showing that stray cats and dogs have a higher potential to transmit *E. bieneusi* from them to humans, showing a public health threat. The predominant genotype D of *E. bieneusi* identified here in stray cats and dogs have been detected commonly in humans and water samples in other countries, suggesting that stray cats and dogs might act as a reservoir for genotype D that are transmissible to humans. Future studies should elucidate the epidemiology of *E. bieneusi* in humans, animals, water and the environment, in order to provide an informed position on its public health importance in this country. Other studies could be conducted to establish whether some of the genotypes recognised to be potentially zoonotic actually occur in humans in China.

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Authors' contributions

Sample collection: RSM, YDZ, LMX, HZH, HYG, YH and TM. Designed the study and performed the experiments: YZ and YDZ. Analysis and interpretation: YZ and YDZ. Write the manuscript: YZ. Review the draft: ZGC. All authors read and approved the final version of the manuscript.

References

1. Didier ES, Weiss LM. Microsporidiosis: not just in AIDS patients. Curr Opin Infect Dis. 2011;24(5):491-5. doi: https://doi.org/10.1097/QCO.0b013e32834aa152 PMID: 21844802
2. Santín-Durán M. *Enterocytozoon bieneusi*. In: Xiao LH, Ryan UM, Feng YY, editors. Biology of Foodborne Parasites. First Edition. Boca Raton, FL, USA: CRC Press; 2015. p. 149-74.
3. Santín M, Fayer R. Microsporidiosis: *Enterocytozoon bieneusi* in domesticated and wild animals. Res Vet Sci. 2011;90(3):363-71. doi: https://doi.org/10.1016/j.rvsc.2010.07.014 PMID: 20699192

4. Zhang Y. Molecular epidemiological investigations of *Enterocytozoon bieneusi* [PhD thesis]. Melbourne, Australia: The University of Melbourne; 2019.

5. Didier ES, Weiss LM. Microsporidiosis: current status. Curr Opin Infect Dis. 2006;19(5):485-92. doi: https://doi.org/10.1097/01.qco.0000244055.46382.23 PMID: 16940873

6. Pekmezci D, Pekmezci GZ, Yildirim A, Duzlu O, Inci A. Molecular detection of zoonotic microsporidia in domestic cats in Turkey: a preliminary study. Acta Parasitol. 2019;64(1):13-8. doi: https://doi.org/10.2478/s11686-018-00003-x PMID: 30645737

7. Wang YG, Zou Y, Yu ZZ, Chen D, Gui BZ, Yang JF, et al. Molecular investigation of zoonotic intestinal protozoa in pet dogs and cats in Yunnan province, southwestern China. Pathogens. 2021;10(9):1107. doi: https://doi.org/10.3390/pathogens10091107 PMID: 34578141

8. Zhou K, Liu M, Wu Y, Zhang R, Wang R, Xu H, et al. *Enterocytozoon bieneusi* in patients with diarrhea and in animals in the northeastern Chinese city of Yichun: genotyping and assessment of potential zoonotic transmission. Parasite. 2022;29:40. doi: https://doi.org/10.1051/parasite/2022041 PMID: 36047999

9. Xu H, Jin Y, Wu W, Li P, Wang L, Li N, et al. Genotypes of *Cryptosporidium* spp., *Enterocytozoon bieneusi* and *Giardia duodenalis* in dogs and cats in Shanghai, China. Parasit Vectors. 2016;9(1):121. doi: https://doi.org/10.1186/s13071-016-1409-5 PMID: 26932267

10. Liu H, Xu J, Shen Y, Cao J, Yin J. Genotyping and zoonotic potential of *Enterocytozoon bieneusi* in stray dogs sheltered from Shanghai, China. Animals (Basel). 2021;11(12):3571. doi: https://doi.org/10.3390/ani11123571 PMID: 34944346

294 11. Koehler AV, Rashid MH, Zhang Y, Vaughan JL, Gasser RB, Jabbar A. First cross-sectional,
295 molecular epidemiological survey of *Cryptosporidium*, *Giardia* and *Enterocytozoon* in
296 alpaca (*Vicugna pacos*) in Australia. Parasit Vectors. 2018;11(1):498. doi:
297 https://doi.org/10.1186/s13071-018-3055-6 PMID: 30185227
298 12. Zhang Y, Koehler AV, Wang T, Cunliffe D, Gasser RB. *Enterocytozoon bieneusi* genotypes
299 in cats and dogs in Victoria, Australia. BMC Microbiol. 2019;19(1):183. doi:
300 https://doi.org/10.1186/s12866-019-1563-y PMID: 31395004
301 13. Zhang Y, Koehler AV, Wang T, Haydon SR, Gasser RB. *Enterocytozoon bieneusi*
302 genotypes in cattle on farms located within a water catchment area. J Eukaryot Microbiol.
303 2018;66(4):553-9. doi: https://doi.org/10.1111/jeu.12696 PMID: 30358006
304 14. Zhang Y, Mi RS, Yang JB, Wang JX, Gong HY, Huang Y, et al. *Enterocytozoon bieneusi*
305 genotypes in farmed goats and sheep in Ningxia, China. Infect Genet Evol. 2020;85:104559.
306 doi: https://doi.org/10.1016/j.meegid.2020.104559 PMID: 32961363
307 15. Zhang Y, Koehler AV, Wang T, Haydon SR, Gasser RB. First detection and genetic
308 characterisation of *Enterocytozoon bieneusi* in wild deer in Melbourne's water catchments
309 in Australia. Parasit Vectors. 2018;11(1):2. doi: https://doi.org/10.1186/s13071-017-2577-7
310 PMID: 29295716
311 16. Zhang Y, Koehler AV, Wang T, Haydon SR, Gasser RB. New operational taxonomic units
312 of *Enterocytozoon* in three marsupial species. Parasit Vectors. 2018;11(1):371. doi:
313 https://doi.org/10.1186/s13071-018-2954-x PMID: 29954462
314 17. Zhang Y, Mi R, Yang L, Gong H, Xu C, Feng Y, et al. Wildlife is a potential source of
315 human infections of *Enterocytozoon bieneusi* and *Giardia duodenalis* in southeastern China.

316 Front Microbiol. 2021;12:692837. doi: https://doi.org/10.3389/fmicb.2021.692837 PMID:
317 34447356

318 18. Zhang Y, Koehler AV, Wang T, Robertson GJ, Bradbury RS, Gasser RB. *Enterocytozoon*
319 *bieneusi* genotypes in people with gastrointestinal disorders in Queensland and Western
320 Australia. Infect Genet Evol. 2018;65:293-9. doi:
321 https://doi.org/10.1016/j.meegid.2018.08.006 PMID: 30125732

322 19. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious basic:
323 an integrated and extendable desktop software platform for the organization and analysis of
324 sequence data. Bioinformatics. 2012;28(12):1647-9. doi:
325 https://doi.org/10.1093/bioinformatics/bts199 PMID: 22543367

326 20. Santín M, Fayer R. *Enterocytozoon bieneusi* genotype nomenclature based on the internal
327 transcribed spacer sequence: a consensus. J Eukaryot Microbiol. 2009;56(1):34-8. doi:
328 https://doi.org/10.1111/j.1550-7408.2008.00380.x PMID: 19335772

329 21. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees.
330 Bioinformatics. 2001;17(8):754-5. doi: https://doi.org/10.1093/bioinformatics/17.8.754
331 PMID: 11524383.

332 22. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics
333 and parallel computing. Nat Methods. 2012;9(8):772. doi:
334 https://doi.org/10.1038/nmeth.2109 PMID: 22847109

335 23. Zhang Y, Koehler AV, Wang T, Gasser RB. *Enterocytozoon bieneusi* of animals—with an
336 'Australian twist'. Adv Parasitol. 2021;111:1-73. doi:
337 https://doi.org/10.1016/bs.apar.2020.10.001 PMID: 33482973

24. Karim MR, Dong H, Li T, Yu F, Li D, Zhang L, et al. Predomination and new genotypes of *Enterocytozoon bieneusi* in captive nonhuman primates in zoos in China: high genetic diversity and zoonotic significance. PLoS One. 2015;10(2):e0117991. doi: https://doi.org/10.1371/journal.pone.0117991 PMID: 25705879

25. Li W, Li Y, Song M, Lu Y, Yang J, Tao W, et al. Prevalence and genetic characteristics of *Cryptosporidium*, *Enterocytozoon bieneusi* and *Giardia duodenalis* in cats and dogs in Heilongjiang province, China. Vet Parasitol. 2015;208(3):125-34. doi: https://doi.org/10.1016/j.vetpar.2015.01.014 PMID: 25665462

26. Li W, Feng YY, Zhang LX, Xiao LH. Potential impacts of host specificity on zoonotic or interspecies transmission of *Enterocytozoon bieneusi*. Infect Genet Evol. 2019;75:104033. doi: https://doi.org/10.1016/j.meegid.2019.104033 PMID: 31494271

27. Li W, Feng YY, Santín M. Host specificity of *Enterocytozoon bieneusi* and public health implications. Trends Parasitol. 2019;35(6):436-51. doi: https://doi.org/10.1016/j.pt.2019.04.004 PMID: 31076351

28. Li WC, Qin J, Wang K, Gu YF. Genotypes of *Enterocytozoon bieneusi* in dogs and cats in eastern China. Iran J Parasitol. 2018;13(3):457-65. PMID: 30483338

29. Mori H, Mahittikorn A, Thammasonthijarern N, Chaisiri K, Rojekittikhun W, Sukthana Y. Presence of zoonotic *Enterocytozoon bieneusi* in cats in a temple in central Thailand. Vet Parasitol. 2013;197(3-4):696-701. doi: https://doi.org/10.1016/j.vetpar.2013.07.025 PMID: 23932454

30. Wang HY, Lin XH, Sun YX, Qi NS, Lv MN, Xiao WW, et al. Occurrence, risk factors and genotypes of *Enterocytozoon bieneusi* in dogs and cats in Guangzhou, southern China: high

genotype diversity and zoonotic concern. BMC Vet Res. 2020;16(1):8. doi: https://doi.org/10.1186/s12917-020-02421-4 PMID: 32552737

31. Abe N, Kimata I, Iseki M. Molecular evidence of *Enterocytozoon bieneus*i in Japan. J Vet Med Sci. 2009;71(2):217-9. doi: https://doi.org/10.1292/jvms.71.217 PMID: 19262036

32. Karim MR, Rume FI, Rahman ANMA, Zhang Z, Li J, Zhang L. Evidence for zoonotic potential of *Enterocytozoon bieneusi* in its first molecular characterization in captive mammals at Bangladesh national zoo. J Eukaryot Microbiol. 2020;67(4):427-35. doi: https://doi.org/10.1111/jeu.12792 PMID: 32115792

33. Phrompraphai T, Itoh N, Iijima Y, Ito Y, Kimura Y. Molecular detection and genotyping of *Enterocytozoon bieneusi* in family pet dogs obtained from different routes in Japan. Parasitol Int. 2019;70:86-8. doi: https://doi.org/10.1016/j.parint.2019.02.010 PMID: 30825524

34. Piekarska J, Kicia M, Wesołowska M, Kopacz Ż, Gorczykowski M, Szczepankiewicz B, et al. Zoonotic microsporidia in dogs and cats in Poland. Vet Parasitol. 2017;246:108-11. doi: https://doi.org/10.1016/j.vetpar.2017.09.011 PMID: 28969771

35. Kváč M, Hofmannová L, Ortega Y, Holubová N, Horčičková M, Kicia M, et al. Stray cats are more frequently infected with zoonotic protists than pet cats. Folia Parasitol. 2017;64:034. doi: https://doi.org/10.14411/fp.2017.034 PMID: 29214976

36. Zhang K, Zheng S, Wang Y, Wang K, Wang Y, Gazizova A, et al. Occurrence and molecular characterization of *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi*, and *Blastocystis* sp. in captive wild animals in zoos in Henan, China. BMC Vet Res. 2021;17(1):332. doi: https://doi.org/10.1186/s12917-021-03035-0 PMID: 34663327

382 37. Wang Y, Zhang K, Zhang Y, Wang K, Gazizova A, Wang L, et al. First detection of
383 *Enterocytozoon bieneusi* in whooper swans (*Cygnus cygnus*) in China. Parasit Vectors.
384 2020;13(1):5. doi: https://doi.org/10.1186/s13071-020-3884-y PMID: 31910900
385 38. Santín M, Calero‐Bernal R, Carmena D, Mateo M, Balseiro A, Barral M, et al. Molecular
386 characterization of *Enterocytozoon bieneusi* in wild carnivores in Spain. J Eukaryot
387 Microbiol. 2018;65(4):468-74. doi: https://doi.org/10.1111/jeu.12492 PMID: 29230898
388 39. Ye J, Yan J, Xu J, Ma K, Yang X. Zoonotic *Enterocytozoon bieneusi* in raw wastewater in
389 Zhengzhou, China. Folia Parasitol. 2017;64:1. doi: https://doi.org/10.14411/fp.2017.002
390 PMID: 28148905
391 40. Fan Y, Wang X, Yang R, Zhao W, Li N, Guo Y, et al. Molecular characterization of the
392 waterborne pathogens *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi*,
393 *Cyclospora cayetanensis* and *Eimeria* spp. in wastewater and sewage in Guangzhou, China.
394 Parasit Vectors. 2021;14(1):66. doi: https://doi.org/10.1186/s13071-020-04566-5 PMID:
395 33472683
396 41. Li N, Xiao LH, Wang L, Zhao S, Zhao X, Duan L, et al. Molecular surveillance of
397 *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi* by genotyping and
398 subtyping parasites in wastewater. PLoS Negl Trop Dis. 2012;6(9):e1809. doi:
399 https://doi.org/10.1371/journal.pntd.0001809 PMID: 22970334
400 42. Dashti A, Santín M, Cano L, de Lucio A, Bailo B, de Mingo MH, et al. Occurrence and
401 genetic diversity of *Enterocytozoon bieneusi* (Microsporidia) in owned and sheltered dogs
402 and cats in northern Spain. Parasitol Res. 2019;118(10):2979-87. doi:
403 https://doi.org/10.1007/s00436-019-06428-1 PMID: 31435764

404 43. Prado JBF, do Nascimento Ramos CA, da Silva Fiuza VR, Terra VJB. Occurrence of
405 zoonotic *Enterocytozoon bieneusi* in cats in Brazil. Rev Bras Parasitol Vet. 2019;28(1):80-
406 90. doi: https://doi.org/10.1590/S1984-296120180096 PMID: 30785555
407 44. Kwak D, Seo MG. Genetic analysis of zoonotic gastrointestinal protozoa and microsporidia
408 in shelter cats in South Korea. Pathogens. 2020;9(11):894. doi:
409 https://doi.org/10.3390/pathogens9110894 PMID: 33121067
410 45. Dengjel B, Zahler M, Hermanns W, Heinritzi K, Spillmann T, Thomschke A, et al.
411 Zoonotic potential of *Enterocytozoon bieneusi*. J Clin Microbiol. 2001;39(12):4495-9. doi:
412 https://doi.org/10.1128/JCM.39.12.4495-4499.2001 PMID: 11724868
413 46. Mathis A, Breitenmoser AC, Deplazes P. Detection of new *Enterocytozoon* genotypes in
414 faecal samples of farm dogs and a cat. Parasite. 1999;6(2):189-93. doi:
415 https://doi.org/10.1051/parasite/1999062189 PMID: 10416194
416 47. Karim MR, Dong H, Yu F, Jian F, Zhang LX, Wang R, et al. Genetic diversity in
417 *Enterocytozoon bieneusi* isolates from dogs and cats in China: host specificity and public
418 health implications. J Clin Microbiol. 2014;52(9):3297-302. doi:
419 https://doi.org/10.1128/JCM.01352-14 PMID: 24989604
420 48. Zhong Y, Zhou Z, Deng L, Liu H, Zhong Z, Ma X, et al. Prevalence and new genotypes of
421 *Enterocytozoon bieneusi* in sheltered dogs and cats in Sichuan province, southwestern
422 China. Parasite. 2021;28:31. doi: https://doi.org/10.1051/parasite/2021029 PMID: 33812463
423 49. Santín M, Trout JM, Cortés Vecino JA, Dubey JP, Fayer R. *Cryptosporidium*, *Giardia* and
424 *Enterocytozoon bieneusi* in cats from Bogota (Colombia) and genotyping of isolates. Vet
425 Parasitol. 2006;141(3):334-9. doi: https://doi.org/10.1016/j.vetpar.2006.06.004 PMID:
426 16860480

50. Delrobaei M, Jamshidi S, Shayan P, Ebrahimzade E, Tamai IA, Rezaeian M, et al. Molecular detection and genotyping of intestinal microsporidia from stray dogs in Iran. Iran J Parasitol. 2019;14(1):159-66. doi: https://doi.org/10.18502/ijpa.v14i1.731 PMID: 31123481

51. Cao Y, Tong Q, Zhao C, Maimaiti A, Chuai L, Wang J, et al. Molecular detection and genotyping of *Enterocytozoon bieneusi* in pet dogs in Xinjiang, northwestern China. Parasite. 2021;28:57. doi: https://doi.org/10.1051/parasite/2021057 PMID: 34283021

52. Zhang X, Wang Z, Su Y, Liang X, Sun X, Peng S, et al. Identification and genotyping of *Enterocytozoon bieneusi* in China. J Clin Microbiol. 2011;49(5):2006-8. doi: https://doi.org/10.1128/JCM.00372-11 PMID: 21389159

53. Phrompraphai T, Itoh N, Iijima Y, Ito Y, Kimura Y, Kameshima S. Molecular determination of *Enterocytozoon bieneusi* in pet shop puppies and breeding kennel dogs. Trends Med. 2018;18(6):1-4. doi: https://doi.org/10.1016/j.parint.2019.02.010

54. Galván-Díaz AL, Magnet A, Fenoy S, Henriques-Gil N, Haro M, Gordo FP, et al. Microsporidia detection and genotyping study of human pathogenic *E. bieneusi* in animals from Spain. PLoS One. 2014;9(3):e92289. doi: https://doi.org/10.1371/journal.pone.0092289 PMID: 24651457

55. Santín M, Cortés Vecino JA, Fayer R. *Enterocytozoon bieneusi* genotypes in dogs in Bogota, Colombia. Am J Trop Med Hyg. 2008;79(2):215-7. doi: https://doi.org/10.4269/ajtmh.2008.79.215 PMID: 18689627

Abbreviations

AIC: Akaike information criteria

BI: Bayesian inference

ITS: Internal transcribed spacer of nuclear ribosomal DNA

LSU: Large subunit of nuclear ribosomal DNA gene

MCMC: Monte Carlo Markov Chain

pp: Posterior probability

SSU: Small subunit of nuclear ribosomal DNA gene

Declarations

Ethics approval and consent to participate

All faecal samples were donated from Shanghai/Minhang Center(s) for Animal Disease Control and Prevention with the consent of their owners or staff. During the whole experimental process, all laboratory work on the study specimens was covered under the Animal Experimental Protocol of Shanghai Veterinary Research Institute (201008): "Use of animal samples for the determination of zoonotic pathogen".

Data Availability Statement

All relevant data are within the manuscript and its Supporting Information files.

Competing interests

The authors declare that they have no competing interests.

470 **Table 1. The information regarding faecal samples collected from household cats and dogs**
471 **from pet clinics (*n* = 193) and breeding centers (*n* = 60), as well as stray cats and dogs (*n* =**
472 **106) located in Minhang and Jingan districts in Shanghai, China (2019 - 2020).**

473

Host **Season**	**Tested sample nos.**	**Sample source** **Pet clinic/Breeding center/Stray**	**Location** **Minhang/ Jingan**	***E. bieneusi* prevalence %** **(test-positive sample nos./total tested sample nos.)**
Cat	***59***	***9/0/50***	***9/50***	***32.2 (19/59)***
Spring	-	-	-	-
Summer	50	0/0/50	0/50	38.0 (19/50)
Autumn	9	9/0/0	9/0	-
Dog	***300***	***184/60/56***	***300/0***	***5.0 (15/300)***
Spring	128	128/0/0	128/0	5.5 (7/128)
Summer	60	0/60/0	60/0	5.0 (3/60)
Autumn	112	56/0/56	112/0	4.5 (5/112)
Total	**359**	**193/60/106**	**309/50**	**9.5 (34/359)**

474

475 **Table 2. Association analysis of the risk factors (seasons and living status) with**
476 ***Enterocytozoon bieneusi* test-positivity assessed using the multivariate logistic linear**
477 **regression.**

478

Factors	Test positive samples nos.	Total tested sample nos.	Prevalence (%)	Odds ratio (95% CI)	*P*-value
Seasons					
Spring	7	128	5.5	2.117 (0.803-5.580)	0.152
Summer[1]	22	110	20.0	-	-
Autumn	5	121	4.1	2.841 (0.967-8.343)	0.075
Living status					
Stray	24	106	22.6	7.112 (3.263-15.500)	0.000*
Household/raised	10	253	4.0	-	-

479 The strength of association was measured using an odds ratio calculated with 95% confidence
480 intervals (95% CI), and statistical significance was given as a *P*-value. * = Statistically
481 significant ($P < 0.05$). - = Not available. [1] = Value were used as references when odds ratio was
482 calculated.

483 **Table 3. Prevalences of *Enterocytozoon bieneusi* recorded previously in cats and dogs**
484 **worldwide.**

485

Host species	Prevalence of *E. bieneusi* % (*E. bieneusi* test-positive sample nos./total tested sample nos.)	Country	Reference
Cat	1.40 (2/143)	China	[28]
Cat	2.34 (4/171)	China	[7]
Cat	2.54 (3/118)	Czech Republic	[35]
Cat	3.03 (3/99)	Spain	[42]
Cat	3.33 (2/60)	Brazil	[43]
Cat	3.80 (6/158)	South Korea	[44]
Cat	5.00 (3/60)	Germany	[45]
Cat	5.56 (4/72)	Turkey	[6]
Cat	5.63 (9/160)	China	[9]
Cat	5.77 (3/52)	China	[25]
Cat	6.25 (4/64)	Slovakia	[35]
Cat	6.85 (5/73)	Poland	[35]
Cat	8.33 (1/12)	Switzerland	[46]
Cat	9.09 (4/44)	Poland	[34]
Cat	11.46 (11/96)	China	[47]
Cat	11.63 (20/172)	Australia	[12]
Cat	14.10 (22/156)	China	[48]

Cat	14.29 (1/7)	Japan	[31]
Cat	17.39 (8/46)	Colombia	[49]
Cat	20.31 (79/389)	China	[30]
Cat	31.25 (25/80)	Thailand	[29]
Dog	0.84 (2/237)	Spain	[6]
Dog	2.53 (2/79)	Japan	[31]
Dog	3.23 (2/62)	China	[8]
Dog	4.36 (26/597)	Japan	[33]
Dog	4.88 (4/82)	Poland	[34]
Dog	5.33 (4/75)	Iran	[50]
Dog	5.85 (20/342)	Australia	[12]
Dog	5.98 (29/485)	China	[9]
Dog	6.29 (38/604)	China	[51]
Dog	6.74 (18/267)	China	[25]
Dog	7.69 (2/26)	China	[52]
Dog	8.02 (21/262)	China	[7]
Dog	8.02 (75/935)	Japan	[53]
Dog	8.33 (3/36)	Switzerland	[46]
Dog	8.57 (27/315)	China	[28]
Dog	8.82 (24/272)	China	[10]
Dog	9.59 (7/73)	Spain	[54]
Dog	15.00 (18/120)	Colombia	[55]
Dog	15.52 (54/348)	China	[47]

Dog	18.78 (136/724)	China	[48]
Dog	22.89 (149/651)	China	[30]

Genotyping studies using previously confirmed *E. bieneusi* samples were excluded.

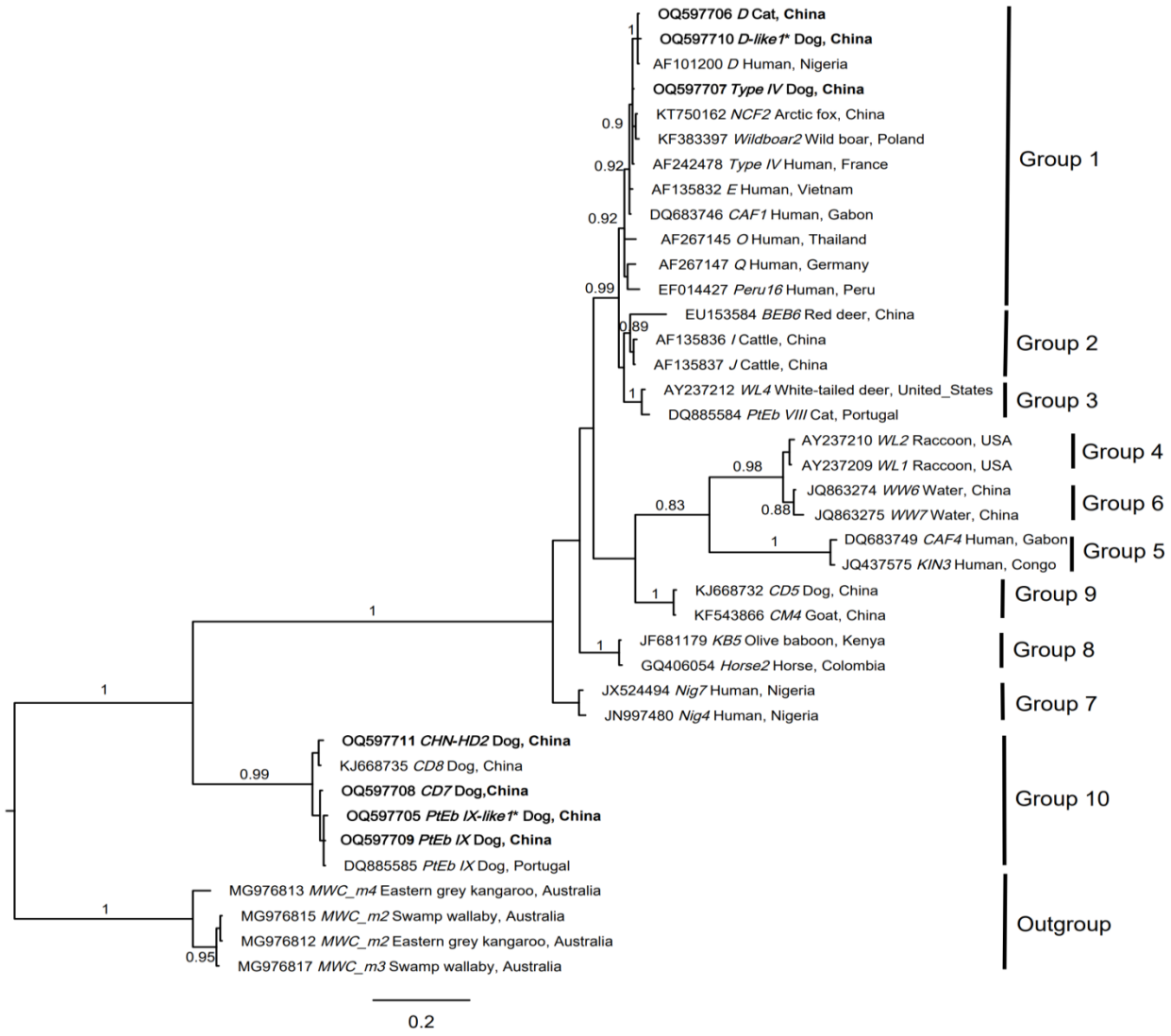


Figure 1. Relationships among genotypes of *Enterocytozoon bieneusi* recorded in cats and dogs in this study inferred from phylogenetic analysis of sequence data for the internal transcribed spacer (ITS) of nuclear ribosomal DNA by Bayesian inference (BI). Sequences from a range of distinct *E. bieneusi* genotypes from published papers were included for comparison in the analysis (S1 Table) [23-27]. Statistically significant posterior probabilities (pp) are indicated on branches. Individual GenBank accession numbers precede genotype designation (in italics), followed by sample and locality descriptions. *Enterocytozoon bieneusi* genotypes identified and characterised from faecal DNA samples in the present study are indicated in bold-type. Clades were assigned group names based on the classification system [23-27]. The scale-

497 bar represents the number of substitutions per site. The *E. bieneusi* genotypes MWC_m2-m4

498 from marsupials were used as outgroups.

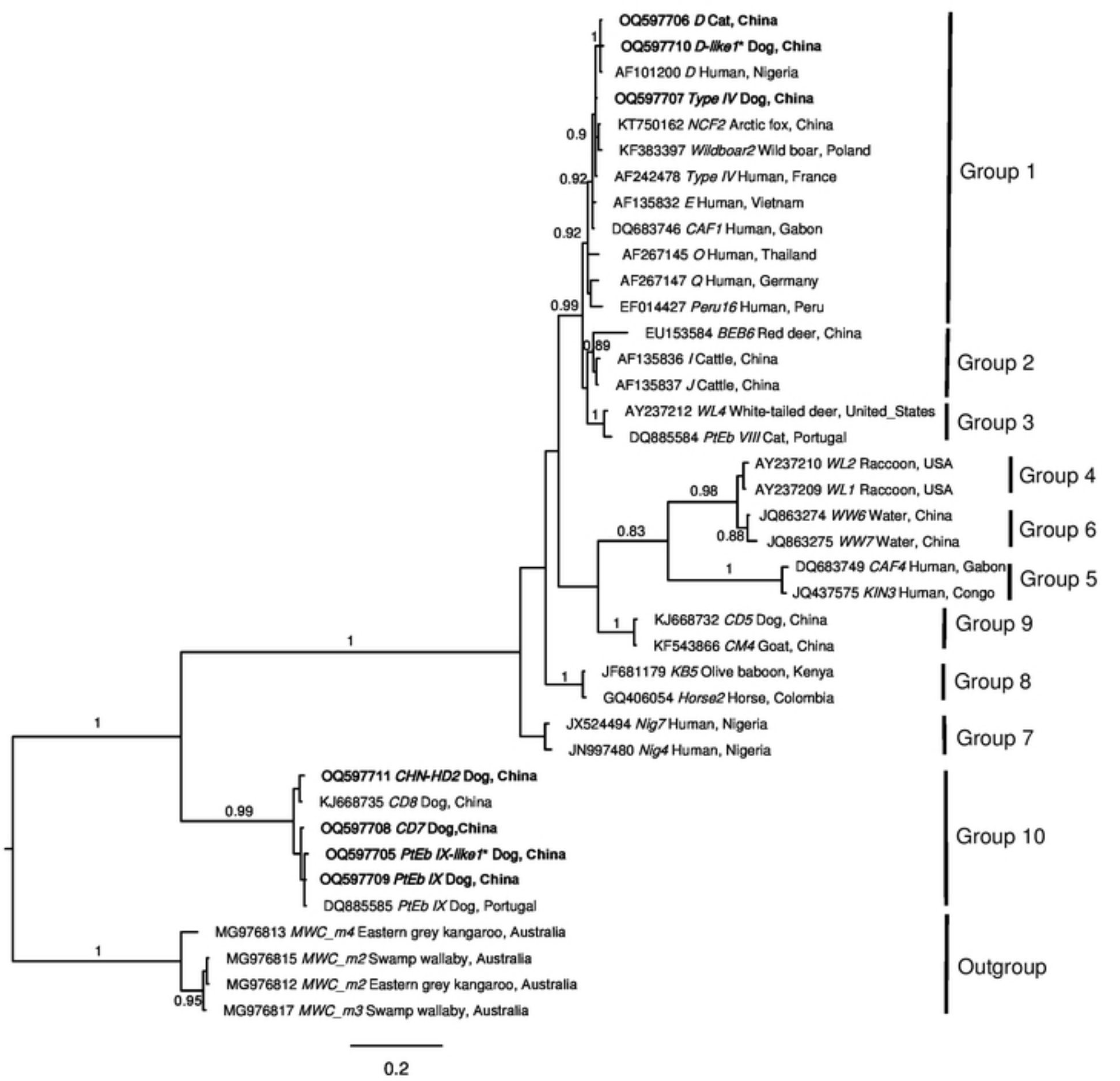
Supporting information

S1 Table. GenBank accession numbers of all internal transcribed spacer (ITS) of nuclear ribosomal DNA sequences used for phylogenetic analysis (Fig 1), and associated information.

S2 Table. Genotypes of *Enterocytozoon bieneusi* characterised from 359 individual faecal samples (sample codes given) from cats and dogs in this study.

S3 Table. All *Enterocytozoon bieneusi* genotypes recorded previously in cats (*Felis catus*) and dogs (*Canis familiaris*) worldwide.

S4 Table. Genotypes PtEb IX, Type IV, D, CD7 and CHN-HD2 of *Enterocytozoon bieneusi* recorded in different host species and water samples in previous studies. These genotypes were also recorded in the present study.


OQ597706 D Cat, China
OQ597710 D-like1* Dog, China
AF101200 D Human, Nigeria
OQ597707 Type IV Dog, China
KT750162 NCF2 Arctic fox, China
KF383397 Wildboar2 Wild boar, Poland
AF242478 Type IV Human, France
AF135832 E Human, Vietnam
DQ683746 CAF1 Human, Gabon
AF267145 O Human, Thailand
AF267147 Q Human, Germany
EF014427 Peru16 Human, Peru
EU153584 BEB6 Red deer, China
AF135836 I Cattle, China
AF135837 J Cattle, China
AY237212 WL4 White-tailed deer, United_States
DQ885584 PtEb VIII Cat, Portugal
AY237210 WL2 Raccoon, USA
AY237209 WL1 Raccoon, USA
JQ863274 WW6 Water, China
JQ863275 WW7 Water, China
DQ683749 CAF4 Human, Gabon
JQ437575 KIN3 Human, Congo
KJ668732 CD5 Dog, China
KF543866 CM4 Goat, China
JF681179 KB5 Olive baboon, Kenya
GQ406054 Horse2 Horse, Colombia
JX524494 Nig7 Human, Nigeria
JN997480 Nig4 Human, Nigeria
OQ597711 CHN-HD2 Dog, China
KJ668735 CD8 Dog, China
OQ597708 CD7 Dog,China
OQ597705 PtEb IX-like1* Dog, China
OQ597709 PtEb IX Dog, China
DQ885585 PtEb IX Dog, Portugal
MG976813 MWC_m4 Eastern grey kangaroo, Australia
MG976815 MWC_m2 Swamp wallaby, Australia
MG976812 MWC_m2 Eastern grey kangaroo, Australia
MG976817 MWC_m3 Swamp wallaby, Australia
1
0.9
0.92
0.92
0.99
0.89
1
0.98
0.88
0.83
1
1
1
1
1
0.99
1
0.95
0.2
Group 1
Group 2
Group 3
Group 4
Group 6
Group 5
Group 9
Group 8
Group 7
Group 10
Outgroup

Figure