Identification of *Cryptosporidium* species, *Enterocytozoon bieneusi* genotypes, and *Giardia duodenalis* assemblages in birds in Henan, China

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Research

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Abstract

Background

Domesticated, wild, and migratory birds have been known to transmit diseases such as diarrhea in humans and other animals, but studies specifically on the zoonotic pathogens *Cryptosporidium* spp., *Enterocytozoon bieneusi*, and *Giardia duodenalis* in birds in Henan Province, China are lacking. Hence, this study sought to characterize the prevalence of these pathogens, and to identify the different species of *Cryptosporidium* and their phylogenetic relationships, the genotypes of *E. bieneusi*, and the assemblages of *G. duodenalis*, in birds in the province.

Methods

Fresh fecal samples were collected from birds in parks and pet shops in Henan, China and were screened for the presence of the pathogens using nest-PCR amplification of the small subunit ribosomal RNA (SSU rRNA) gene and the internal transcribed spacer (ITS) gene.

Results

A total of 1,005 fecal samples were collected from 32 species of birds. 21 fecal samples (2.09%) were found positive for *Cryptosporidium* spp., 45 (4.48%) for *E. bieneusi*, and 33 (3.28%) for *G. duodenalis.* This study identified five *Cryptosporidium* species: *C. baileyi* (10 out of 21 fecal samples, 47.62%) in crested myna (*Acridotheres cristatellus*), Java sparrow (*Lonchura oryzivora*), Chinese hwamei (*Garrulax canorus*), common quail (*Coturnix coturnix*), and Chinese grosbeak (*Eophona migratoria*); *C. galli* (5/21, 23.81%) in Chinese blackbird (*Turdus mandarinus*), zebra finch (*Taeniopygia guttata*), and white-eyes (*Zosterops* sp.); *C. andersoni* (1/21, 4.76%) in a white-eye for the first time; *C. meleagridis* (4/21, 19.05%) in parrots and crested myna; and *C. parvum* (1/21, 4.76%) in a pigeon. Two *E. bieneusi* genotypes: Peru6 and PtEb I were found in pigeons and European turtle dove (*Streptopelia turtur*). The *G. duodenalis* assemblage E was detected in parrots, common hill myna, crested myna, Java sparrow, white-eyes, black-throated laughingthrush, and other birds.

Conclusions

Our findings indicate that the aforementioned species of birds in Henan, China could be a source of zoonotic pathogens, such as *C. meleagridis*, *C. andersoni*, *C. parvum*, *E. bieneusi* genotype Peru6, and *G. duodenalis* assemblage E, that cause diseases in humans.

Background

Cryptosporidium spp., *Enterocytozoon bieneusi*, and *Giardia duodenalis* are zoonotic pathogens that cause acute or chronic diarrhea, vomiting, and respiratory ailments in both humans and animals via fecal-oral transmission [1].

Cryptosporidiosis is a parasitic disease in caged, domesticated, and wild birds [2]. Currently, four *Cryptosporidium* species, including *C. meleagridis*, *C. baileyi*, *C. galli*, and *C. avium*, and several genotypes including the avian genotypes I-V, Eurasian woodcock genotype, black duck genotype and goose genotypes I-V have been detected in birds through sequence analysis of the SSU rRNA gene [2-4]. Moreover, birds infected with *C. hominis*, *C. serpentis*, *C. andersoni*, and the muskrat genotype I and with the zoonotic pathogens *C. parvum, C. muris*, *C. andersoni* and *C. meleagridis* have also been reported [5].

The pervasive obligate intracellular eukaryotic fungi *E. bieneusi* infects humans and various animals, including birds, fish, insects, amphibians, and mammals, specifically dogs, cats, pigs, rabbits, and sheep. More than 300 genotypes of *E.*

bieneusi have been reported based on differences in ITS gene sequences [6]. Based on phylogenetic analysis, *E. bieneusi* could be clustered into nine groups, one of which is zoonotic group 1 that can infect humans and animals. The genotypes of *E. bieneusi* that have been detected in birds include J, D, Peru6, A, and EbpA, which belong to group 1, as well as co101, co102, PtEb, and Peru6-var [7-9].

Giardiasis is another parasitic disease caused by six *Giardia* species, which are zoonotic pathogens that have been differentiated from others based on morphological, host-source, and electron microscopic nuances. As far as public health is concerned, the most zoonotic among these species is *G. duodenalis*. Based on genetic analyses, *G. duodenalis* consists of eight distinct assemblages from A to H, of which only the zoonotic assemblages A and B and nonzoonotic assemblages D and F have been found in birds [10-11].

Birds that are in close contact with humans and those that migrate have been known to play a consequential role in the transmission and spread of zoonotic parasites [1]. Nevertheless, there have only been a few studies on the epidemiological and genetic characteristics of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis* in birds in Henan Province, China. For this reason, this study aimed to primarily evaluate the prevalence of the pathogens and specifically identify, through nucleotide sequencing, the different species of *Cryptosporidium* and their phylogenetic relationships, the genotypes of *E. bieneusi*, and the assemblages of *G. duodenalis* present in birds in Henan, China.

Material And Methods

Fecal sample collection

A total of 1,005 fresh fecal samples were collected from 32 species of birds (Table 1) in parks and pet shops in seven areas in Henan Province, China from July 2018 to May 2019 (Figure 1). Approximately 1-10 birds of the same species were caged in groups, and only one specimen was collected from each cage. Using sterile gloves, the specimens were briefly collected from the ground or cages. The time of collection, location, taxonomic identification, deworming conditions, feeding habits, and the shape of the feces of the birds were recorded. About 200-300 mg of each fecal sample was dispensed into a 2.5-ml centrifuge tube and was stored at 4°C before DNA extraction. No mixed infection was observed among the samples.

DNA extraction and PCR amplification

Whole genomic DNA was extracted from each of the fecal samples using an EZNA Stool DNA Kit (Omega Bio-tek Inc., Norcross, GA), and according to the manufacturer's instructions it was eluted in 200 µl of elution buffer and was stored at -20°C until PCR amplification. All the extracted DNA was screened for the presence of *Cryptosporidium* spp. and *G. duodenalis* through the amplification of the small subunit ribosomal RNA (SSU rRNA) gene [12,13] and the presence of *E. bieneusi* was established through the amplification of the internal transcribed spacer (ITS) region of the rRNA gene [14]. Negative and positive control samples were included in every PCR reaction. Secondary PCR products were subjected to electrophoresis in 1% agarose gel, they were stained with DNA Green (TIAND, Beijing, China) and were visualized with a UV transilluminator.

Sequence analysis and phylogenetic analysis of *Cryptosporidium* spp.

All the PCR-positive products were submitted to the Beijing Nuosai Biological Engineering Company for bidirectional sequencing using an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences obtained in this study were compared with the reference sequences downloaded from the GenBank database and were aligned using ClustalX 2.0 (http://www.clustal.org/). The phylogenetic analysis of *Cryptosporidium* spp. based on the SSU rRNA gene was performed using software MEGA 7.0 (https://www.megasoftware.net/). Neighbor-joining

phylogenetic trees were constructed based on the evolutionary distances calculated using Kimura's two-parameter model, and the reliability of the trees was assessed through bootstrap analysis with 1,000 replicates.

Statistical analysis and nucleotide sequence accessioning

All statistical analyses were performed using SPSS 22.0. Statistical differences among the sampling areas, deworming conditions, and fecal shapes were evaluated using a Chi-square test. Values with $p < 0.05$ were considered significant, and a 95% confidence interval was determined. Representative nucleotide sequences generated in this study were deposited under accession numbers MN410718 to MN410725 to the GenBank database.

Results

Overall prevalence of *Cryptosporidium* spp., *E. bieneusi* and *G. duodenalis*

A total of 1,005 fecal samples were collected from 32 species of birds in Henan, China. The zoonotic parasite *E. bieneusi* showed the highest prevalence in these birds, being PCR-positive in 45 fecal samples (4.48%), followed by *G. duodenalis* that was detected in 33 samples (3.28%) and *Cryptosporidium* spp. that were found in 21 samples (2.09%) (Table 1). The highest infection rate (16.56%) was observed in Luohe ($\chi^2 = 30.414$, $p < 0.001$), whereas the lowest infection rate (0) was recorded in a Zhumadian pet shop. Fecal shapes ($\chi^2 = 30.35$, $p < 0.001$) and deworming conditions ($\chi^2 = 8.24$, $p < 0.05$) were found to be significantly different between birds (Table 2).

Cryptosporidium species and sequence analysis

Among the fecal samples that were found to be positive for *Cryptosporidium* spp., five species were further identified (Figure 2). The most predominant among them was *C. baileyi* (10 out of 21 fecal samples, 47.62%), whose nucleotide sequences were detected in three crested mynas (*Acridotheres cristatellus*), two Java sparrows (*Lonchura oryzivora*), one Chinese hwamei (*Garrulax canorus*), two common quails (*Coturnix coturnix*), and two Chinese grosbeaks (*Eophona migratoria*) and were found to be 100% homologous with a sequence (KT151550) identified in quail in Iraq. Another species, *C. meleagridis* (4/21, 19.05%), was identified in three parrots and in one crested myna. Its sequences were 100% homologous and were identical to that of an immunocompetent patient (MK311181) from Poland. The sequences of *C. galli* (5/21, 23.81%) were detected in three white-eyes (*Zosterops* sp.), one zebra finch (*Taeniopygia guttata*) and one Chinese blackbird (*Turdus mandarinus*), and they were 99% homologous with a sequence (MG516766) identified in an ibis in Australia. The pathogen *C. andersoni* (1/21, 4.76%) was detected in one white-eye for the first time, and its sequence showed 100% homology with that of a human patient (KF826301) in China. The sequence of *C. parvum* (1/21, 4.76%) was found in a pigeon of family Columbidae and was found to be 100% homologous with a sequence (MK241967) detected in cattle from India.

E. bieneusi genotypes and sequence analysis

The sequences of *E. bieneusi* were found in ten European turtle doves (*Streptopelia turtur*) as well as in a number of Columbidae pigeons in different areas: 13 pigeons in Luohe (15.23%), 6 pigeons in Puyang (5.88%), 1 pigeon in Nanyang (1.25%), and 15 pigeons in Zhengzhou (3.11%). Based on the results of the nucleotide sequencing analysis of the ITS gene locus, the genotype Peru 6 of *E. bieneusi* was found in 34 pigeons and 10 European turtle doves, whereas the genotype PtEb I was identified in a pigeon for the first time. Peru 6 and PtEb I had 100% homology with two isolates: KY012356 and DQ425107.

G. duodenalis assemblages and sequence analysis

The pathogen *G. duodenalis* was identified in 12 parrots, three common hill mynas, 13 crested mynas, one Java sparrow, one white-eye, one black-throated laughingthrush and two other birds in Zhengzhou (6.85%). All the samples positive for *G. duodenalis* were identified to be from assemblage E and were 100% homologous with the isolate MH794176 from a sheep in China (Table 2).

Phylogenetic analysis of *Cryptosporidium* spp.

Phylogenetic analyses based on SSU rRNA sequences identified five *Cryptosporidium* species: *C. baileyi, C. meleagridis, C. galli, C. andersoni* and *C. parvum* (Figure 2).

Discussion

This study principally demonstrated the varying prevalence and genetic characteristics of *Cryptosporidium* spp., *E. bieneusi*, and *G. duodenalis* in birds in Henan Province, China. As far as we know, our molecular data on *E. bieneusi* and *G. duodenalis* in birds are the first to have been generated in the region. *Cryptosporidium* spp. have been reported in many species of domesticated and wild birds. However, in this study, *Cryptosporidium* spp. showed a prevalence or infection rate of only 2.09%. This is higher than that reported in Poland (1.1%) [15] and Japan (0%) [16] but lower than that recorded in domesticated and wild birds in Italy (5.7%) [17], Malaysia (10%), Brazil (4.86%) [4], northwestern Spain (8.3%), Hungary (8.8%), Australia (6.3%), the United States (7.2%), Greece (13.0%), and Japan (9.1%), and particularly in China, it is lower than that documented for central China (8.1%) [18] and northeast China (11.1%) [19]. Molecular techniques were employed in this study to record the infection rate of *Cryptosporidium* spp., but through microscopic examination, Gu [20] found that the infection rate of *Cryptosporidium* in birds in Anhui Zoo was only 0.97%. Thus, using molecular techniques to detect *Cryptosporidium* may be more efficient and accurate than performing microscopic examination. The differences in infection rates between the current study and previous ones could be attributed to the differences in study area, methods, bird species, and bird feeding habits.

A total of five *Cryptosporidium* species were detected in birds in this study. The species *C. baileyi*, *C. meleagridis*, and *C. galli* are considered the most common pathogens in birds worldwide. This study was the first to identify *C. baileyi* in Chinese grosbeak and Chinese hwamei, *C. meleagridis* in crested myna, *C. galli* in zebra finch, Chinese blackbird, and a species of white-eye, and *C. andersoni* in a white-eye. Reboredo-Fernandez reported the detection of *C. parvum* in sparrowhawk and black-billed magpie (*Pica hudsonia*) in northwest Spain, but in the current study, *C. parvum* was found in a pigeon. Importantly, *C. meleagridis, C. andersoni* and *C. parvum* can infect humans. The results of this study indicate that the range of bird species hosting *Cryptosporidium* spp. could be wider, and the diversity of *Cryptosporidium* spp. acting as zoonotic pathogens could be greater than what was previously known.

The average infection rate (4.48%) of *E. bieneusi* documented in this study is lower than that recorded in Heilongjiang Province (22.2% in pet birds), Portugal (12.8% in pet birds), and Czech Republic (21.3% in exotic birds), but it is higher than that reported in Poland (1.4% in pigeons) [21] and Brazil (3.8% in exotic birds). Furthermore, *E. bieneusi* was detected with an infection rate of 8.18% in a total of 35 pigeons. Different infection rates of the pathogen have been reported among pigeons in many countries: Brazil (7.8%), Portugal (43.2%) [22], Spain (15.3%), Iran (8.8%) [23] and the Netherlands (5.4%). The pervasiveness of *E. bieneusi* in pigeons, as demonstrated in this study, suggests that these birds play a critical role in the spread of the pathogen. The infection rate of 27.78%, as well as the pioneering detection of *E. bieneusi* in European turtle doves, further substantiates the expanded range of bird species harboring the pathogen.

Only nine genotypes, including J, D, Peru6, A, EbpA, co101, co102, PtEb, and Peru6-var, of *E. bieneusi* have been identified in birds worldwide, and five of them (J, D, Peru6, A and EbpA) have also been detected in humans. In this

study, Peru6 was identified in 44 samples, suggesting that the genotype is dominant. Peru6 belongs to group 1 which is of notable zoonotic importance because it largely affects humans. The genotype has been isolated from HIV-infected patients in Peru, but not yet in China. However, Peru6 has already been found in pigeons, cranes, geese, and ducks in China. In this study, PtEb I was found for the first time in one pigeon in Luohe. The genotype has never been attributed to human infections.

Although *G. duodenalis* has been commonly detected in mammals, it has been scarcely reported in birds especially in China. This study is the first to identify the pathogen in birds in China. The infection rate (3.28%) recorded for *G. duodenalis* in this study is lower than that documented in Italy (4.3% in pet birds) [17], Spain (8.3% in aquatic birds) [24] and Japan (16.1% in pet birds). On the contrary, the prevalence of *G. duodenalis* that this study found in pet birds is higher than that in Brazil (1.1% in captive birds), northwest Spain (2.1% in wild birds) [25], Poland (2.2% in captive birds), and Japan (1.7% in zoo birds).

In this study, all the samples positive for *G. duodenalis* assemblage E were collected in Zhengzhou. It was previously reported that *G. duodenalis* assemblage E mainly infects artiodactyls, but in this study, *G. duodenalis* assemblage E was found in birds, probably because their food or water was contaminated with the pathogen. There have been occasional reports of *G. duodenalis* assemblage E in the human body [26], indicating that *G. duodenalis* assemblage E is a zoonotic risk.

Conclusions

Overall, this study reported the occurrence and molecular diversity of *Cryptosporidium* spp., *E. bieneusi*, and *G. duodenalis* in birds in Henan Province, China. Our findings suggest that these pathogens infect a diversity of avian species, notably including those that were found to be positive for these pathogens for the first time. The detection of *C. meleagridis*, *C. andersoni*, *C. parvum*, *E. bieneusi* genotype Peru 6 and *G. duodenum* assemblage E in birds suggests that exposure to these birds pose a threat to human health. Most of the birds in which the pathogens were detected were ostensibly healthy, and they appeared to be asymptomatic carriers of the parasites. Pet owners and animal keepers who are responsible for the regular maintenance and feeding of birds are the most vulnerable to zoonotic infection. In addition, birds can travel long distances and could be important for spreading the pathogens. Hence, the potential mechanisms underlying the zoonotic transmission of diseases between birds and humans should be further investigated.

Abbreviations

ITS:Internal transcribed spacer; SSU: Small subunit

Declarations

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Availability of date and material

All date generated or analysed during this study are included in this published article. Sequence were submitted to the GenBank database under the accession number MN410718-MN410725.

Author contribution

fL and LZ designed the study. ML and RC collected and analyzed the specimens. RC, JL, and CB analyzed the date. RC, JD and LZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study protocol was reviewed and approved by the research ethics committee of Henan Agricultural University (Zhengzhou city, China). Before beginning this study, we contacted the owners or managers of the animals and acquired their permission. No birds were hurt during sample collection.

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Tables

Table 1. Evaluation of prevalence and genotyping of pathogens in birds in Henan Province, China

Scientific name / taxon (common name)	Total number of fecal samples	*Cryptosporidium* spp. (Number of fecal samples)	1. *bieneusi* genotype (Number of fecal samples)	*G. duodenalis* assemblage (Number of fecal samples)
Family Columbidae (pigeons)	428	*C. parvum* (1)	Peru6 (34) PtEbI (1)	0
Serinus canaria (island canary)	47	0	0	0
Order Psittaciformes (parrots)	100	1. *meleagridis* (3)	0	Assemblage E (12)
Gracula religiosa (common hill myna)	86	0	0	Assemblage E (3)
Acridotheres cristatellus (crested myna)	132	*C. baileyi* (3) *C. meleagridis* (1)	0	Assemblage E (13)
Family Picidae (woodpeckers)	3	0	0	0
Taeniopygia guttata (zebra finch)	23	*C. galli* (1)	0	0
Corvus splendens (house crow)	2	0	0	0
Coturnix coturnix (common quail)	12	*C. baileyi* (2)	0	0
Eophona migratoria (Chinese grosbeak)	11	*C. baileyi* (2)	0	0
Lonchura oryzivora (Java sparrow)	9	*C. baileyi* (2)	0	Assemblage E (1)
Garrulax canorus (Chinese hwamei)	13	*C. baileyi* (1)	0	0
Poecile palustris (marsh tit)	2	0	0	0
Lanius schach (long-tailed shrike)	3	0	0	0
Zosterops sp. (white-eyes)	22	*C. galli* (3) *C. andersoni* (1)	0	Assemblage E (1)
Pyrrhocorax pyrrhocorax (red-billed chough)	1	0	0	0
Pycnonotus sinensis (light-vented bulbul)	4	0	0	0
Turdus mandarinus (Chinese blackbird)	8	*C. galli* (1)	0	0
Leiothrix argentauris (silver-eared mesia)	4	0	0	0
Garrulax chinensis (black-throated laughingthrush)	2	0	0	Assemblage E (1)
Periparus ater (coal tit)	1	0	0	0
Phoenicurus auroreus (daurian redstart)	1	0	0	0
Machlolophus spilonotus (yellow-cheeked tit)	22	0	0	0
Aethopyga sp. (sunbirds)	2	0	0	0
Streptopelia turtur (European turtle dove)	36	0	Peru 6 (10)	0
Family Trochilidae (hummingbirds)	1	0	0	0
Other birds	30	0	0	Assemblage E (2)
Total	1005	*C. meleagridis* (4) *C. galli* (5) *C. baileyi* (10) *C. andersoni* (1) *C. parvum* (1)	Peru 6(44) PtEbI (1)	Assemblage E (33)

Table 2. Factors associated with the prevalence of *Cryptosporidium* spp., *Enterocytozoon bieneusi* and *Giardia duodenalis* in birds in Henan Province, China.

Factors	Categories	No. of positive samples / total no. of samples (%)	Prevalence			*Cryptosporidium* species (Number of fecal samples)	*E. bieneusi* Genotype (Number of fecal samples)	*G. duodenalis* Assemblage (Number of fecal samples)
			Cryptosporidium spp.	*E. bieneusi*	*G. duodenalis*			
Areas	Zhengzhou	63/509 (12.38)	2.95% (15)	2.95% (15)	6.48% (33)	*C. meleagridis* (3); *C. Baileyi* (9); *C. galli* (2); *C. andersoni* (1)	Peru 6 (15)	E (33)
	Puyang	6/102 (5.8)	0	5.88 % (6)	0	--	Peru 6 (6)	--
	Kaifeng	3/79 (3.80)	3.80% (3)	0	0	*C. Baileyi* (1); *C. galli* (2)	--	--
	Nanyang	1/80 (1.25)	0	1.25% (1)	0	--	Peru 6 (1)	--
	Zhumadian	0/57 (0)	0	0	0	--	--	--
	Luoyang	1/27 (3.70)	3.70% (1)	0	0	*C. meleagridis* (1)	--	--
	Luohe	25/151 (16.56)	1.32% (2)	15.23% (23)	0	*C. galli* (1); *C. Parvum* (1);	Peru 6 (22); PtEb I (1)	--
Fecal shapes	Soft feces	22/63 (34.92)	1.59% (1)	25.40% (16)	7.94% (5)	*C. andersoni* (1)	Peru 6 (28); PtEb I (1)	E (5)
	Normal feces	77/868 (8.87)	2.30% (20)	3.34% (29)	3.23% (28)	*C. meleagridis* (4); *C. galli* (5); *C. Baileyi* (10); *C. Parvum* (1)	Peru 6 (16)	E (28)
	Dry, hard stool	0/74 (0)	0	0	0	--	--	--
Deworming conditions	Dewormed	15/275 (5.45)	0	5.45% (15)	0	--	Peru 6 (15)	--
	Undewormed	84/730 (11.51)	2.88% (21)	4.11% (30)	4.52% (33)	*C. meleagridis* (4); *C. galli* (5); *C. baileyi* (10); *C. anderson* (1); *C. parvum* (1)	Peru 6 (29); PtEb I (1)	E (33)
Total	--	99/1005	2.09% (21)	4.48% (45)	3.28% (33)	*C. meleagridis* (4); *C. galli* (5); *C. Baileyi* (10); *C. Anderson* (1); *C. Parvum* (1)	Peru 6 (44); PtEb I (1)	E (33)

Figures

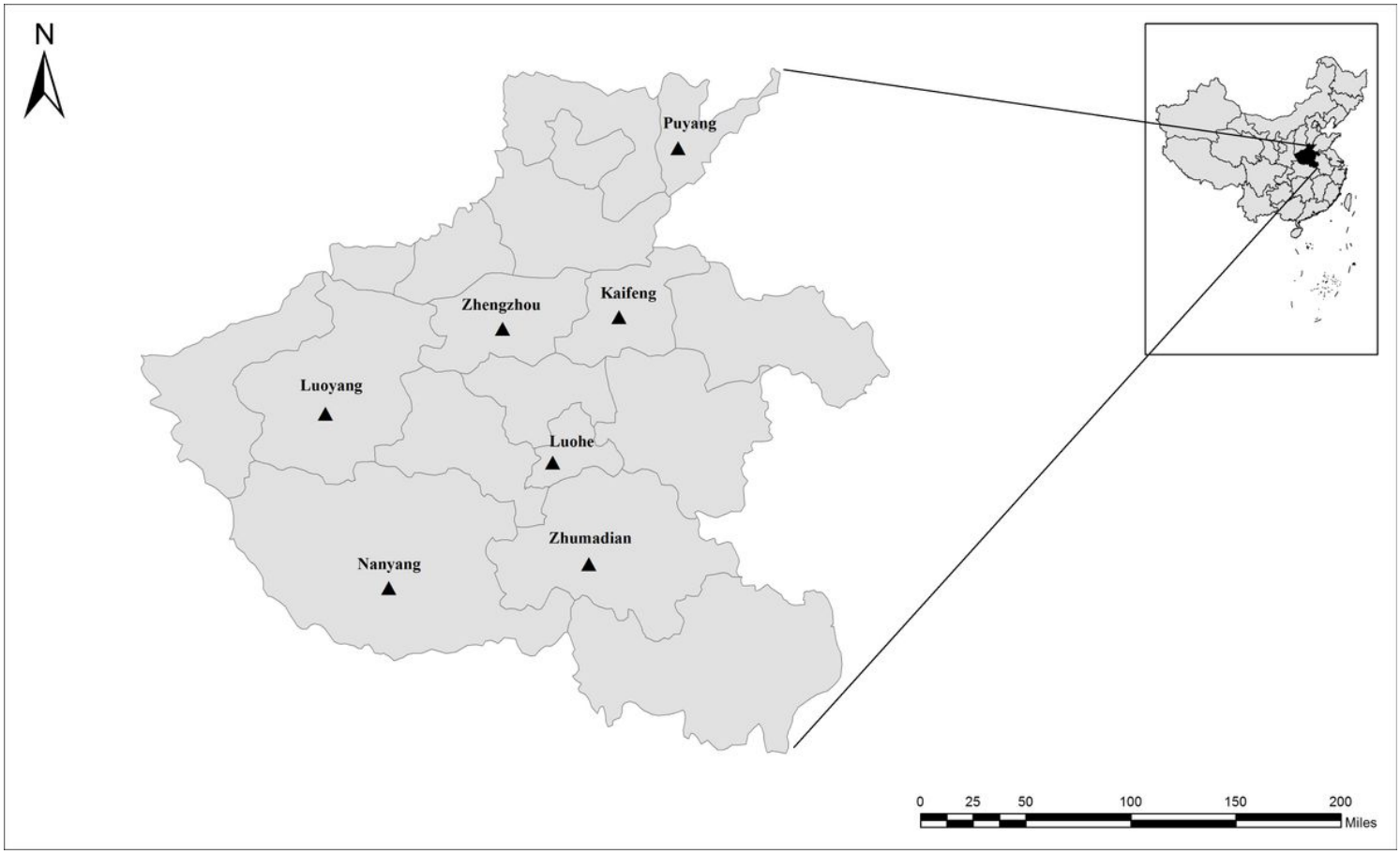

Figure 1

Sampling sites of birds in Henan province of China. Solid triangles indicate sampling sites. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

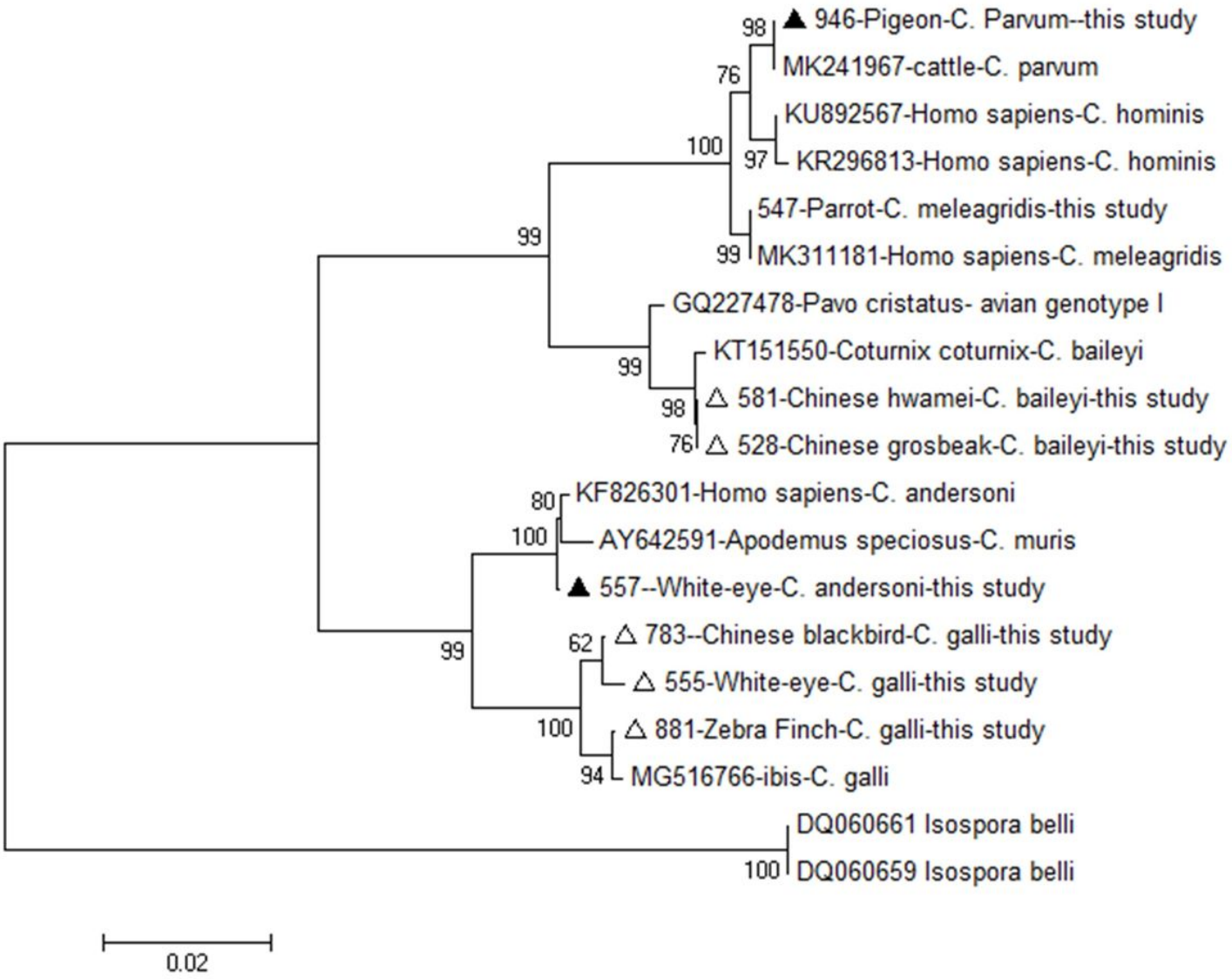

Figure 2

Phylogenetic relationships of Cryptosporidium species identified in this study and other known species in GenBank. Phylogenetic relationships were inferred using neighbor-joining analysis of the SSU rRNA based on Kimura's two-parameter model. Bootstrap values greater than 50% from 1,000 replicates are shown at the nodes. Solid and open triangles indicate zoonotic and nonzoonotic species identified in the present study.