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ORIGINAL ARTICLE

Multiyear Survey of Coccidia, Cryptosporidia, Microsporidia, Histomona, and Hematozoa in Wild Quail in the Rolling Plains Ecoregion of Texas and Oklahoma, USA

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ABSTRACT

We developed nested PCR protocols and performed a multiyear survey on the prevalence of several protozoan parasites in wild northern bobwhite (*Colinus virginianus*) and scaled quail (*Callipepla squamata*) in the Rolling Plains ecoregion of Texas and Oklahoma (i.e. fecal pellets, bird intestines and blood smears collected between 2010 and 2013). Coccidia, cryptosporidia, and microsporidia were detected in 46.2%, 11.7%, and 44.0% of the samples (n = 687), whereas histomona and hematozoa were undetected. Coccidia consisted of one major and two minor *Eimeria* species. Cryptosporidia were represented by a major unknown *Cryptosporidium* species and *Cryptosporidium baileyi*. Detected microsporidia species were highly diverse, in which only 11% were native avian parasites including *Encephalitozoon hellem* and *Encephalitozoon cuniculi*, whereas 33% were closely related to species from insects (e.g. *Antonospora*, *Liebermannia*, and *Sporanauta*). This survey suggests that coccidia infections are a significant risk factor in the health of wild quail while cryptosporidia and microsporidia may be much less significant than coccidiosis. In addition, the presence of *E. hellem* and *E. cuniculi* (known to cause opportunistic infections in humans) suggests that wild quail could serve as a reservoir for human microsporidian pathogens, and individuals with compromised or weakened immunity should probably take precautions while directly handling wild quail.

THE northern bobwhite (*Colinus virginianus*) and scaled quail (*Callipepla squamata*) are the most common quail species in Texas and adjacent regions. They are an important part of the ecosystem and a group of economically significant game birds. However, populations of both species are experiencing long-term declines in the wild in Texas and other regions of the USA, even in locations where suitable habitat conditions exist, and estimated declines of 75% and 66% in bobwhites and scaled quail have occurred in Texas since 2008 (also see descriptions at https://tpwd.texas.gov/publications/pwdpubs/media/pwd_rp_w7000_1025.pdf and http://www.quailresearch.org). The biotic and/or abiotic factors contributing to the wild quail population declines remain elusive. Parasites are one of the major biotic factors that can kill individuals (direct effect) or cause morbidity, making them more vulnerable to predators (indirect effect). However, little is known on the status and impact of parasites on wild quail populations in Texas (Peterson 2007). There are more surveys on parasitic arthropods and enteric nematodes, but only

few reports describing the prevalence of protozoan (protistan) parasites (e.g. coccidia and cryptosporidia) (Duszynski and Gutierrez 1981; Forrester et al. 2007; Peterson 2007).

To address the concern regarding the potential causes of the quail population decline, the Rolling Plain Quail Research Foundation (RPQRF) initiated a comprehensive and collaborative program named "Operation Idiopathic Decline" (OID) to investigate the status of infectious diseases and environmental factors in the Rolling Plains ecoregion of Texas and Oklahoma (http://www.quailresearch.org/projects1/). Under the OID program, a few recent studies have reported surveys on toxins, viral and bacterial agents, and helminth communities (Baxter et al. 2015; Bruno et al. 2015; Dunham et al. 2014; Moore et al. 2013; Su et al. 2014; Turaga et al. 2016; Urban et al. 2013; Xiang et al. 2013). The present study was part of the OID program aimed to gain knowledge on the prevalence of major protozoan parasites (protists) in northern bobwhites and scaled quail in the Rolling Plains ecoregion. In this project, we conducted a multiyear (2010–2013) molecular epidemiology survey on the prevalence of coccidia, cryptosporidia, microsporidia, histomonas, and hematozoa. We developed and optimized PCR-based molecular detection protocols that enabled sensitive detection of multiple parasites from limited amounts of intestinal contents and fecal samples collected from wild quail. In addition, we also microscopically examined quail blood smears for hematozoa.

MATERIALS AND METHODS

Sample collections

Three types of samples were collected: fecal samples, whole or partial intestines, and blood smears. Fecal samples were collected from the Rolling Plains Quail Research Ranch (RPQRR) in Fisher County, Texas (http://www.quailresearch.org) through a seasonal trap-and-release program in a separate conservation research project, and from 33 sites in the Rolling Plains ecoregion (Fig. 1) through the OID Central Receiving Laboratory at the Texas Institute of Environmental and Human Health at Texas Tech University, Lubbock, Texas. Fecal pellets were collected from cotton pillow cases where quail were placed overnight, placed into plastic bags, stored under refrigeration at RPQRR or Texas Tech University, and shipped with ice packs to Texas A&M University, College Station where samples were stored at −20 °C. Intestines were collected only via OID operation, in which quail captured from the 33 sites were euthanized and various tissues were collected after gross examination and autopsy for distribution to other OID team members. Intestines were frozen at the site of collection and shipped with dry ice to Texas A&M University, College Station where samples were stored at −20 °C. Blood samples were collected in the field from the jugular veins of selected quail for preparation of blood smears. Birds were trapped and handled under Texas Parks and Wildlife permit SRP-1098-984, USFWS permit MB014265-0, and the Animal Use Protocols approved by the Institutional Animal Care and Use Committees of Texas A&M University (#2011-193) and Texas Tech University (ACUC 11049-07 and ACUC 13027-03).

DNA isolation

Individual fecal samples were thawed and cleaned by removing feathers and other debris. Approximately the same volume of phosphate-buffered saline (PBS) was added into each fecal pellet sample that was thoroughly mixed by repeatedly squeezing the plastic bags. Each fecal sample was weighed and ~200 mg of solid wet sample was placed in a 2-ml microtube with screw cap and silicone seal, followed by three washes with 1.0 ml PBS by centrifugation. After final wash, 0.5 ml glass beads (∅ 0.5 mm) and 0.5 ml lysis buffer (i.e. ASL buffer included in the QIAamp DNA Stool Mini Kit) (Qiagen, Valencia, CA) were added into each sample. Microtubes were securely capped and subjected to one freeze/thaw cycle in liquid nitrogen, followed by homogenization in a Mini-Beadbeater-16 (BioSpec Products, Bartlesville, OK) at full-speed for 4 min. After a brief centrifugation to settle the pellets, an additional 0.9 ml of lysis buffer was added into each sample, mixed by vortex, and heated at 70 °C for 5 min. Intestinal DNA was also isolated using the QIAamp DNA Stool Mini Kit. Frozen intestines with lumen contents were thawed and individually minced and mixed with Stanley single edge razor blades. Approximately 200 mg of each sample was loaded into a 2-ml microtube preloaded with 0.2 ml ASL lysis buffer, and ground with a polypropylene pellet pestle. Additional 1.2 ml lysis buffer was added into each ground sample that were mixed by vortex and heated at 70 °C for 5 min. The remaining procedures for both fecal and intestinal samples followed the manufacturer's standard protocol (Qiagen). DNA was eluted into 200 μl elution buffer AE.

Design of PCR primers

Due to the lack of knowledge of molecular sequences for coccidia, cryptosporidia, and most other protozoan parasites from quail in the public databases, PCR primers could only be designed based on the available sequences from other relevant species. We selected small subunit ribosomal RNA (SSU rRNA) genes as the targets for detection because more reference sequences were available for comparison and designing primers with specificities at desired taxonomic levels. Eukaryotic SSU rRNA is commonly referred to as 18S rRNA, but 16S rRNA may be used for microsporidial SSU rRNA genes because they are much smaller than those from other eukaryotes. The general principles in designing primers were to choose regions that were relatively conserved within specified groups of parasites, but divergent from other parasites and from hosts for desired specificities, and each primer pair flanked at least one variable region of the SSU rRNA genes that allowed verification of the identity of PCR products by sequencing.

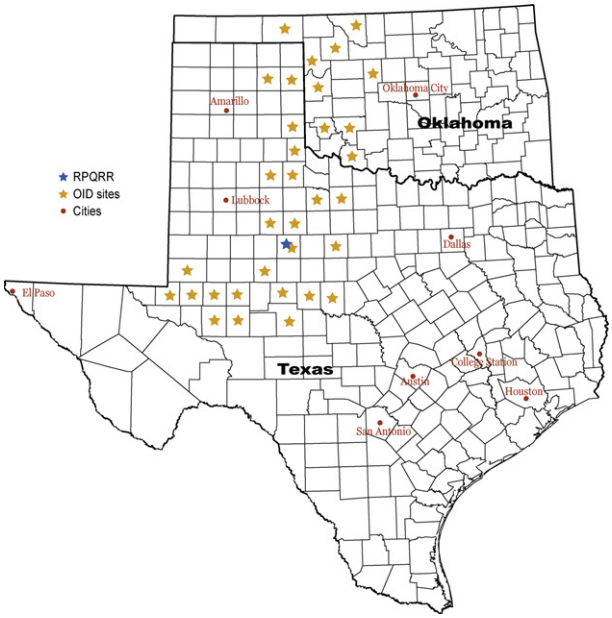

Figure 1 Illustration of the 33 sample collection sites during 2010–2013 in the Rolling Plains ecoregion of Texas and Oklahoma, USA. RPQRR, Rolling Plain Quail Research Ranch; OID, Operation Idiopathic Decline program.

In this study, we first retrieved SSU rRNA sequences from representative species available at the GenBank databases for each group of pathogens, other common intestinal protozoan pathogens and birds. Multiple DNA sequence alignments were performed using the MUSCLE algorithm implanted in the MacVector program (v14.0) (http://www.macvector.com) (MacVector, Inc., Apex, NC). Several possible primer pairs were manually selected at regions with desired conservativeness. For coccidia, primer sequences were conserved among major coccidian genera (e.g. *Eimeria*, *Isospora*, *Cyclospora*, and *Toxoplasma*), but relatively divergent from other apicomplexans (e.g. *Cryptosporidium*). For *Cryptosporidium* and *Histomonas*, primer sequences were only conserved within the specified genera. For microsporidia, primers were specific to all major microsporidian groups, but divergent from other common intestinal pathogens. All primers should contain one or more group-specific nucleotide bases at the 3′-end. Degenerate nucleotides might be included in the middle region of primers at positions divergent within each group of parasites. Candidate primers were tested with DNA samples from known species (e.g. *Eimeria tenella*, *Cryptosporidium parvum*, *Cryptosporidium baileyi*, *Histomonas meleagridis*, *Encephalitozoon hellem* [ATCC # 50451]) that were not necessarily the pathogens of bobwhites, but sufficient for evaluating the specificity of primers. Primers with satisfactory specificity and sensitivity were further evaluated using DNA isolated from negative fecal samples spiked with parasite oocysts (*E. tenella* and *C. parvum*) or pure DNA (*E. hellem* and *H. meleagridis*). Additionally, we also used previously reported primers for microsporidian species capable of infecting humans and for species-specific detection of *H. meleagridis*. The primers used are listed in Table 1.

Detection of parasites by nested PCR

We employed a unified nested PCR protocol that was verified to be suitable for detecting all four groups of parasites in fecal samples. The primary PCR reaction (20 μl/well) contained 2 μl fecal DNA, 1 μM each of the primers for coccidia or cryptosporidia as specified (Table 1), and RedTaq ReadyMix PCR reaction mix (Sigma-Aldrich, St. Louis, MO). Reactions started with a denaturation step at 95 °C for 5 min, followed by 10 thermal cycles at 95 °C for 45 s, 72 °C for 90 s and 72 °C for 90 s, and 30 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. All reactions included a final extension time at 72 °C for 5 min. The secondary reaction (20 μl/well) contained 2 μl of diluted primary reaction solution (5X dilution with pure water), 1 μM each of the nested primer pairs for coccidia or cryptosporidia (Table 1), and RedTaq ReadyMix PCR reaction mix. Reactions included an initial denaturation at 95 °C for 2 min, and 45 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, followed by a 5 min final extension at 72 °C. PCR amplifications were performed with a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). After amplification, PCR products were fractionated with 1.5% agarose gel that were stained with ethidium bromide and visualized in a Hitachi Genetic Miraibio CCDBIO Imaging System (MiraiBio, Alameda, CA). Each set of reactions contained negative and positive controls (i.e. DNA isolated from validated negative or positive fecal samples). In the case of *Histomonas* for which true positive fecal samples were unavailable, we used DNA isolated from spiked samples as described above. Reactions were repeated if controls failed to produce expected results for positive and negative controls. All detections were repeated at least twice or until consistent results were produced at least twice.

Molecular sequencing and analysis

PCR products were sequenced and analyzed to verify their identities. We employed a two-step approach to sequence a selected number of PCR products, because it was impractical to sequence all samples. The first step was to sequence a selected number of individual PCR products from the first few experiments to confirm that PCR detections yielded desired specificity (i.e. all sequences belonged to the expected groups of parasites). In this step, PCR products directly sequenced using one of the PCR primers after extraction from the agarose gel using Omega Gel Extraction Kit (Omega Bio-Tek, Norcross, GA). The second step was to sequence pooled PCR products by seasons, in which all PCR products for specified parasite groups from one particular season were pooled together and cloned into pCRII vector using TA Cloning Kit or pCRII-TOPO vector using TOPO TA Cloning Kit (Invitrogen/Life Technologies, Grand Island, NY).

When possible, primary PCR products were cloned and sequenced to give longer sequences than those derived from nested PCR. In each seasonal sample set, 10–20 clones were randomly selected for isolating plasmids and DNA sequencing. In all experiments, automated Sanger sequencing was performed at the Gene Technologies Lab, Texas A&M University (http://www.idmb.tamu.edu/gtl/). General sequence analysis was performed using MacVector program (v14.0) (http://www.macvector.com). Sequencing quality was assessed by manual inspecting chromatographic files. After removal of primer and vector sequences, all sequences were used as queries to search homologous genes in the nonredundant nucleotide databases at NCBI to confirm their identities.

Phylogenetic reconstructions

High-quality sequencing reads were assembled into consensus sequences (contigs) using the Phrap algorithm implanted in the MacVector. All contigs were used as queries to search the NCBI nucleotide databases and up to 500 top hits for each query contig in each parasite group were retrieved and combined. After moving duplicated sequences, multiple sequence alignments were performed using MUSCLE program (v3.8.31) (http://www.drive5.com/muscle/) (Edgar 2004a,b). The aligned datasets were subjected to neighbor-joining (NJ) phylogenetic constructions using Tamura-Nei nucleotide substitution model. Based on the NJ trees, redundant and over-represented taxa were removed. The procedures were repeated until a balanced selection of taxa was achieved for each dataset. The final datasets were trimmed to remove gaps and ambiguous positions, and then subjected to phylogenetic reconstructions by Bayesian inference (BI) method using MrBayes (version 3.2) (http://mrbayes.sourceforge.net). One million generations of searches were performed with four independent chain running in BI analysis. Parameters included the use of general time reversal (GTR) nucleotide substitution model and the consideration of fraction of invariance and 4-rate of discrete gamma (GTR + F_{inv} + Γ_4). Searches reached to convergence as determined by average standard deviation (SD) of split frequencies reaching to < 0.01, and potential scale reduction factor (PSRF) values for various approaching 1.0 (Gelman and Rubin 1992). Majority rule consensus trees were visualized using FigTree (v1.4.2) (http://tree.bio.ed.ac.uk/software/figtree/), followed by annotations using Adobe Illustrator CS6 (http://www.adobe.com).

Blood smear examination

Thin blood smears were prepared on microscope slides at the OID trapping sites, air-dried, and shipped to Texas A&M University laboratory that followed similar procedures as described (Zabransky et al. 2008). Slides were stained by Wright–Giemsa technique, and examined under a light microscope with 100X oil lens for blood protozoa.

Statistical analysis

Statistical significance between parasite groups were evaluated by a paired, two-tailed Student *t*-test. Effects of age and sex of quail in specified samples on infection rates were assessed by two-tailed Chi-square (χ^2) test.

RESULTS

Detection of coccidia and molecular analysis

Various coccidia are capable of infecting various vertebrates, in which at least some species of coccidia species are thought to be highly host-specific (see an excellent summary by "the Coccidia of the World" project at http://biology.unm.edu/coccidia/home.html). In the quail fecal and intestinal samples collected in this study, coccidia

Table 1. Primers used in detecting protozoan parasites in wild quail collected during 2010–2013 in the Rolling Plains ecoregion of Texas and Oklahoma, USA

Primer name	Primer sequence (5′ to 3′)	L (nt)	Specificity	Application
Cocci18S-175F	GGGGCTGTGTTTATTAGATACAA	23	Coccidia	Primary PCR
Cocci18S-874R	AATGCCCCCAACTGTCCCTATT	22	Coccidia	Primary PCR
Cocci18S-506F	GAATGTAAAACCCTTCCAGAGTAA	24	Coccidia	Nested PCR
Cocci18S-775R	GCGACAAGCCTGCTTGAAA	19	Coccidia	Nested PCR
Crypto18S-148F	TGGTAATTCTAGAGCTAATACA	22	Cryptosporidia	Primary PCR
Crypto18S-765R	CATGCTGGAGTATTCAAGGCA	21	Cryptosporidia	Primary PCR
Crypto18S-198F	TTGTATTTATTAGATAAAGAACCAAT	26	Cryptosporidia	Nested PCR
Crypto18S-505R	GTTTATACTTMACTCATTCCAATTA	25	Cryptosporidia	Nested PCR
Microsp16S-270F	GCCTGAGAGATGGCTMCYACG	21	Microsporidia	Primary PCR
Microsp16S-840R	GTTGAGTYAAATTAAGCMGCACA	23	Microsporidia	Primary PCR
Microsp16S-446F	GRATGCTGCAGTTAAARDGTC	21	Microsporidia	Nested PCR
Microsp16S-776R	TCACYCTTGCGAGCRTACTAT	21	Microsporidia	Nested PCR
Histo18S-77F	ACACTTATTATCTACTTGGAAA	22	*Histomonas*	Primary PCR
Histo18S-694R	GTTGATCTTATTTAACTTTACTG	23	*Histomonas*	Primary PCR
Histo18S-165F	TTTCATTGGAAATTAAATACAATG	24	*Histomonas*	Nested PCR
Histo18S-639R	TCATTCAAGTATATTTATCCTTAC	24	*Histomonas*	Nested PCR

were detected in 26.9–54.6% of the fecal samples (mean = 46.2%; total $N = 687$) (Table 2). Six of the seven seasonal sample sets had positive rates between 43.1% and 54.6%, while the spring 2013 fecal samples had the a lower positive rate at 26.9%. In the 2012 summer/fall intestinal samples for which age and/or sex of individual were available for individual specimens, the infection rates were not statistically significantly different between female and male or between adult and juvenile birds ($p > 0.05$ by χ^2 test) (Table 3).

The specificity of detection was confirmed by sequencing selected PCR products and subsequent BLAST analysis, in which top hits were all SSU rRNA gene sequences from *Eimeria* (98%), *Cyclospora* (1%) or *Isospora* (1%) species. Among them, 78 high-quality sequences were extracted and assembled into four consensus sequences (contigs) with sizes ranging from 270 to 700 bp (including primer sequences) (Table 4). The mostly represented two sequences (contig-01 and contig-02) were derived from 58 (74.4%) and 12 (15.4%) clones with top hits to *Eimeria dispersa* (GenBank accession number: HG793041) (Vrba and Pakandl 2014). However, contig-01 and contig-02 shares only 95–96% identities with HG793041, indicating they are not originated from *E. dispersa*. Contig-03 was derived from 7 (9.0%) clones with top hits to an uncultured *Eimeria* species from environmental samples (GenBank: EU044770) (Martynova-Vankley et al. 2008). Contig-04 was derived from a single clone from a sample collected in the spring 2013 with top hit to an *Isospora* species from a domestic canary *Serinus canaria* (GenBank: KR477877) (Yang et al. 2015).

Several *Eimeria* species may infect the northern bobwhite, and probably also scaled quail—*Eimeria lettyae*, *Eimeria colini*, *E. dispersa* and *Eimeria innocua*, in which *E. dispersa* and *E. innocua* were originally isolated from turkeys, but capable of infecting some other birds including bobwhites, pheasants and chickens (Doran 1978; Fisher and Kelley 1977; Gerhold et al. 2011; Ruff 1985; Ruff and Wilkins 1987). Some other coccidian species were found in related birds, such as *Eimeria crusti*, *Eimeria lophortygis*, *Eimeria okanaganensis* and *Eimeria oreortygis* in mountain quail (*Oreortyx pictus*) and *Eimeria uzura*, *Eimeria bateri* and *Eimeria tsunodai* in Japanese quail (*Coturnix japonica*) (Duszynski and Gutierrez 1981; Norton and Peirce 1971; Ruff et al. 1984; Tsutsumi and Tsunoda 1972). Among them, SSU rRNA sequences were available only for *E. dispersa* and *E. innocua* in the GenBank. However, our study did not detect any *E. dispersa* and *E. innocua* sequences, suggesting that these two parasites were not circulating in wild quail, at least recently in the Rolling Plains ecoregion. In fact, we were unable to determine the coccidian species detected in this study because of the lack of reference sequences in the databases.

To assess the molecular relationship of detected sequences, we reconstructed two phylogenetic trees by BI method using long and short sequences based on the sizes of contigs (Fig. 2). In the long sequence tree inferred from 106 sequences and 679 nucleotide positions, contig-01 and contig-02 were placed between turkey coccidia (*E. dispersa* and *E. innocua*) and other avian species including those from turkeys (e.g. *Eimeria adenoeides* and *Eimeria meleagrimitis*), peafowls (e.g. *Eimeria pavonina*), and chickens (e.g. *Eimeria necatrix* and *Eimeria tenella*) (Fig. 2A). The short sequence tree inferred from 108 sequences and 267 positions was not well resolved, but clustered the four contigs into three groups (Fig. 2B), in which contig-01 and contig-02 were monophyletic as in the long sequence tree, while contig-03 and contig-04 were grouped together one of the other *Eimeria* cluster and an *Isospora* species, respectively. These observations suggested the presence of three *Eimeria* parasites (one major and two minor species) and a minor, previously unreported *Isopora* species in wild quail in Texas and Oklahoma. Contig-04 was represented only by a single sequence, indicating that *Isospora* was likely a very minor species if truly present in quail, or a contaminant from other birds.

Detection of cryptosporidia and molecular analysis

Cryptosporidia were detected in 9.6–14.3% of the quail specimens (mean = 11.7%, total $N = 687$), which was ~ 4-fold less than the prevalence of coccidian parasites ($p < 0.001$ by Student *t*-test) (Table 2). Like coccidian infections, there were no statistically significant differences between female and male or between adult and juvenile birds in samples with identified ages and/or sexes ($p > 0.05$ by χ^2 test) (Tables 3).

Eleven percent of the sequenced clones belong to *C. baileyi*, which is a native avian parasite detected in a broad range of birds (Xiao et al. 2004). The remaining 89% clones were from undefined species with top hits to *Cryptosporidium* species or environmental sequences. We were able to assemble 69 high-quality sequences into three consensus sequences (contig-01 to contig-03) derived from 53 (76.8%), 15 (21.7%) and one (1.4%) clones, respectively. The contig-01 does not fully match any known sequences in the databases, indicating that wild quail carries a major, but yet unknown *Cryptosporidium* species (GenBank: FJ205700) (Feng et al. 2009). The contig-02 fully matches the sequences derived from *C. baileyi*, a parasite known to be avian-specific and previously reported in other domesticated quail species (Fig. 3A) (Current et al. 1986; Fayer 2004; Lindsay et al. 1991; Morgan et al. 2001; Wang et al. 2012; Xiao et al. 2004). It was also clustered within the *C. baileyi* clade with zero branch length in the phylogenetic tree (Fig. 4). Based on the number of clones represented by the contig-02 (21.7%) and the mean infection rate (11.7%), we estimated that ~ 2.5% of the birds were infected by *C. baileyi* (vs. 9.0% by the major species). Contig-03 was derived from only a single clone, for which the significance may be questionable. It may represent a very minor species infecting quail in the Rolling Plains ecoregion, or a contaminant from other animals. Phylogenetic analysis clustered contig-01 and contig-03 together with an environmental

Table 2. Positive rates of quail specimens collected during 2010–2013 in the Rolling Plains ecoregion of Texas and Oklahoma, USA as determined by nested PCR detection and statistical significance between pathogens

Sample			Positive rate		
Collection time	Type	*N*	Coccidia (%)	Cryptosporidia (%)	Microsporidia (%)
2010 Fall/2011 spring[a]	Fecal	124	46.9	10.5	33.1
2011 (Summer/fall)[b]	Fecal	109	54.6	12.5	34.4
2012 (Summer/fall)[b]	Fecal	95	49.5	11.5	36.8
2012 (Summer/fall)[b]	Intestinal	102	55.9	13.7	85.3
2013 Spring[a]	Fecal	54	43.1	14.3	27.3
2013 Fall[b]	Fecal	104	26.9	9.6	38.5
2013 Fall[b]	Intestinal	99	46.5	10.1	52.5
Total *N* or mean %		687	46.20	11.74	43.99

Student *t*-test (paired by seasons, two-tail)	*p*-Value
Cryptosporidia vs. Coccidia	< 0.001
Cryptosporidia vs. Microsporidia	< 0.001
Coccidia vs. Microsporidia	0.760

[a]Samples were collected solely from RPQRR.
[b]Samples were collected from the Operation Idiopathic Decline (OID) sites including RPQRR.

Table 3. Effect of age and sex of quail on positive detection rates in intestinal samples collected during 2010–2013 in the Rolling Plains ecoregion of Texas and Oklahoma, USA

Factor*	Sample type	Sample number	Number of positive samples and rate (%)		
			Coccidia	Cryptosporidia	Microsporidia
Sex	Female	41	23 (56.1)	6 (14.6)	36 (87.8)
	Male	51	31 (60.8)	7 (13.7)	43 (84.3)
	Total	92	54 (58.7)	13 (14.1)	79 (85.9)
Age	Adult	16	11 (68.8)	2 (12.5)	15 (93.7)
	Juvenile	83	45 (54.2)	12 (14.5)	69 (83.1)
	Total	99	56 (56.6)	14 (14.1)	84 (84.9)

**p*-Values > 0.05 by two-tailed Chi-square tests for all three parasites in both sex and age groups.

Cryptosporidium sequence (KF994570) (Prystajecky et al. 2014) (Fig. 4). However, their significant branch length variations indicated certain levels of nucleotide divergence between these sequences.

Detection of microsporidia and molecular analysis

Microsporidia were detected in 27.3–85.3% of the specimens (mean = 44.0%, total *N* = 687) (Table 2). Similar to what were observed for coccidia and cryptosporidia, the positive rates of microsporidia were not statistically different between female and male or between adult and juvenile birds ($p > 0.05$ by χ^2 test) (Table 3). However, unlike coccidia and cryptosporidian that only infect vertebrates, microsporidia also infect all types of invertebrates (Didier and Weiss 2011; Rodriguez-Tovar et al. 2011; Smith 2009; Stentiford et al. 2013; Szumowski and Troemel 2015; Troemel 2011; Vavra and Lukes 2013). Microsporidians (particularly spores) in the insects taken by birds could exist in the digestive tracts and some would be excreted with fecal pellets. Due to the lack of a full list of avian microsporidian species, our protocol was designed to detect any microsporidia. Therefore, the positive rates also included the detections of microsporidian species from insects (i.e. those carried by quail) together with those that truly infect birds (i.e. true infections).

For the reason described above, the positive rates reported here do not necessarily represent "true infection rates." This notion is supported by the sequence analysis, in which only 26% of the individually sequenced PCR products are derived from *E. hellem* (16.9%) or *Encephalitozoon cuniculi* (9.1%) that are known to truly infect birds (Kasickova et al. 2007, 2009; Lallo et al. 2012; Malcekova et al. 2011; Reetz et al. 2002; Sak et al. 2010; Slodkowicz-Kowalska et al. 2006), corresponding to roughly 11% (26% × 44.0% mean positive rate) of true microsporidian infection in wild quail. The remaining sequences corresponding to ~33% of the detected species (74% × 44.0%) belong to insect parasites including those related to *Antonospora*, *Liebermannia* and *Sporanauta* species (Slamovits et al. 2004; Sokolova et al. 2009, 2010; Vossbrinck and Andreadis 2007). The presence of larger portion of insect microsporidia could also explain the significantly higher positive rates in intestinal samples (i.e.

Table 4. Summary of the consensus sequences derived from high-quality reads of PCR products

Groups	Contigs	Length (nt)	Reads *N* (%)	Top hit accession numbers, % identities, and species (all SSU rRNA sequences)
Coccidia	Contig-01	697	58 (74.4)	HG793041 (95); *Eimeria dispersa*, strain KR
Coccidia	Contig-02	700	12 (15.4)	HG793041 (96); *Eimeria dispersa*, strain KR
Coccidia	Contig-03	270	7 (9.0)	EU044770 (100); Uncultured *Eimeria* clone 141
Coccidia	Contig-04	270	1 (1.3)	KR477877 (97); *Isospora* sp. RY-2015a
Cryptosporidia	Contig-01	313	53 (76.8)	FJ205700 (97); *Cryptosporidium* environmental sequence clone ECUST112
Cryptosporidia	Contig-02	308	15 (21.7)	JX548296 (99); *Cryptosporidium baileyi* strain D33
Cryptosporidia	Contig-03	313	1 (1.4)	FJ205700 (97); *Cryptosporidium* environmental sequence clone ECUST112
Microsporidia	Contig-01	570	19 (26.8)	EF016249 (92); *Liebermannia dichroplusae*
Microsporidia	Contig-02[a]	334	8 (11.3)	KM058744 (99); *Encephalitozoon hellem* isolate EhH2P_Ehell
Microsporidia	Contig-03	326	7 (9.9)	EU709818 (98); *Liebermannia covasacrae* isolate Laprida
Microsporidia	Contig-04	339	6 (8.5)	FJ756158 (82); *Microsporidium* sp. BSEI2 SE
Microsporidia	Contig-05[a]	336	5 (7.0)	DQ453123 (99); *Encephalitozoon cuniculi* strain 1268
Microsporidia	Contig-06	342	4 (5.6)	FJ865222 (96); *Antonospora psocopterae*
Microsporidia	Contig-07[a]	577	4 (5.6)	CP002718 (99); *Encephalitozoon hellem* ATCC 50504
Microsporidia	Contig-08	583	4 (5.6)	AY376351 (93); *Anostracospora rigaudi* isolate Ar_PR6
Microsporidia	Contig-09	316	3 (4.2)	JX915758 (99); *Anostracospora rigaudi* isolate Ar_PR6
Microsporidia	Contig-10	343	3 (4.2)	FJ865222 (98); *Antonospora psocopterae*
Microsporidia	Contig-11	343	2 (2.8)	AY376351 (99); *Antonospora locustae*
Microsporidia	Contig-12	343	1 (1.4)	AY305323 (98); *Paranosema whitei*
Microsporidia	Contig-13	348	1 (1.4)	FJ755988 (81); *Microsporidium* sp. BBRE3
Microsporidia	Contig-14	573	1 (1.4)	KC172651 (85); *Sporanauta perivermis*
Microsporidia	Contig-15	347	1 (1.4)	HM002483 (95); *Nosema oryzaephili*
Microsporidia	Contig-16	327	1 (1.4)	FJ755987 (93%); *Microsporidium* sp. BBRE2
Microsporidia	Contig-17	344	1 (1.4)	AY305323 (89); *Paranosema whitei*

[a]Native avian microsporidian species that are capable of causing opportunistic infections in humans.

85.3% in 2012 and 52.5% in 2013) than in feces (i.e. between 27.3% and 38.5%) (Table 2), because intestines would contain more insect microsporidia (i.e. partially digested developmental stages of microsporidia and spores in the insect diets), whereas fecal pellets might contain mostly nondigestible spores.

We assembled 71 high-quality sequences into 17 contigs. The first sequence (contig-01) contained the highest number of sequences (n = 19, 26.8%), while the second to fifth contigs (contig-02 to contig-05) contained 8 (11.3%), 7 (9.9%), 6 (8.5%), and 5 (7.0%) clones (Table 4). The next six contigs (contig-06 to contig-11) contained 4 to 2 clones (5.6–2.8%), and the remaining ones (contig-12 to contig-17) were each derived from single clones (1.4%). Among them, contig-02 (11.3%) and contig-07 (5.6%) fully matched the SSU rRNA gene sequence of *E. hellem*, while contig-05 (7.0%) matched that of *E. cuniculi* as indicated directly by sequence comparison and by phylogenetic analysis (Figs. 3, 5). These observations indicated that these two native avian microsporidian species were circulating in the quail populations, at least in the Rolling Plains ecoregion of Texas and Oklahoma, although their exact prevalence remained to be further determined.

None of the remaining clones fully match any known sequences, indicating that they are derived from potential new microsporidian species and/or known species lacking reference sequences in the databases (Fig. 5). They are mainly grouped with two clusters of microsporidia infecting land and aquatic invertebrates (e.g. bark lice, beetles, and grasshoppers), indicating that they were insects microsporidia carried by birds, rather than native avian parasites. However, a few species were known to be capable of causing opportunistic infections in immunocompromised human patients (e.g. *Nosema algerae*), but they were not considered as native human or avian pathogens (Didier and Weiss 2011).

Absence of blackhead disease pathogen (*Histomonas meleagridis*) in quail samples

Blackhead disease (histomoniasis) is caused by a *Histomonas* parasite and transmitted by cecal worms. Presently, *H. meleagridis* is the only described species in the genus. Blackhead disease is deadly in turkeys, but outbreaks in other avian species have also been reported, including chickens and farm-reared bobwhites (McDougald et al. 2012; Stokholm et al. 2010). However, in our survey, we were unable to detect the presence of *H. meleagridis* DNA in the fecal and intestinal samples. The molecular detection data agree with the gross examination results at the OID receiving laboratory at Texas Tech University and the visual inspection of intestinal samples at the Texas A&M University prior to DNA isolation, in which no apparent lesions related to histomoniasis were observed.

Absence of hematozoa in blood smears

Avian hematozoa including species of *Plasmodium, Haemoproteus*, and *Leucocytozoon* can cause morbidity and

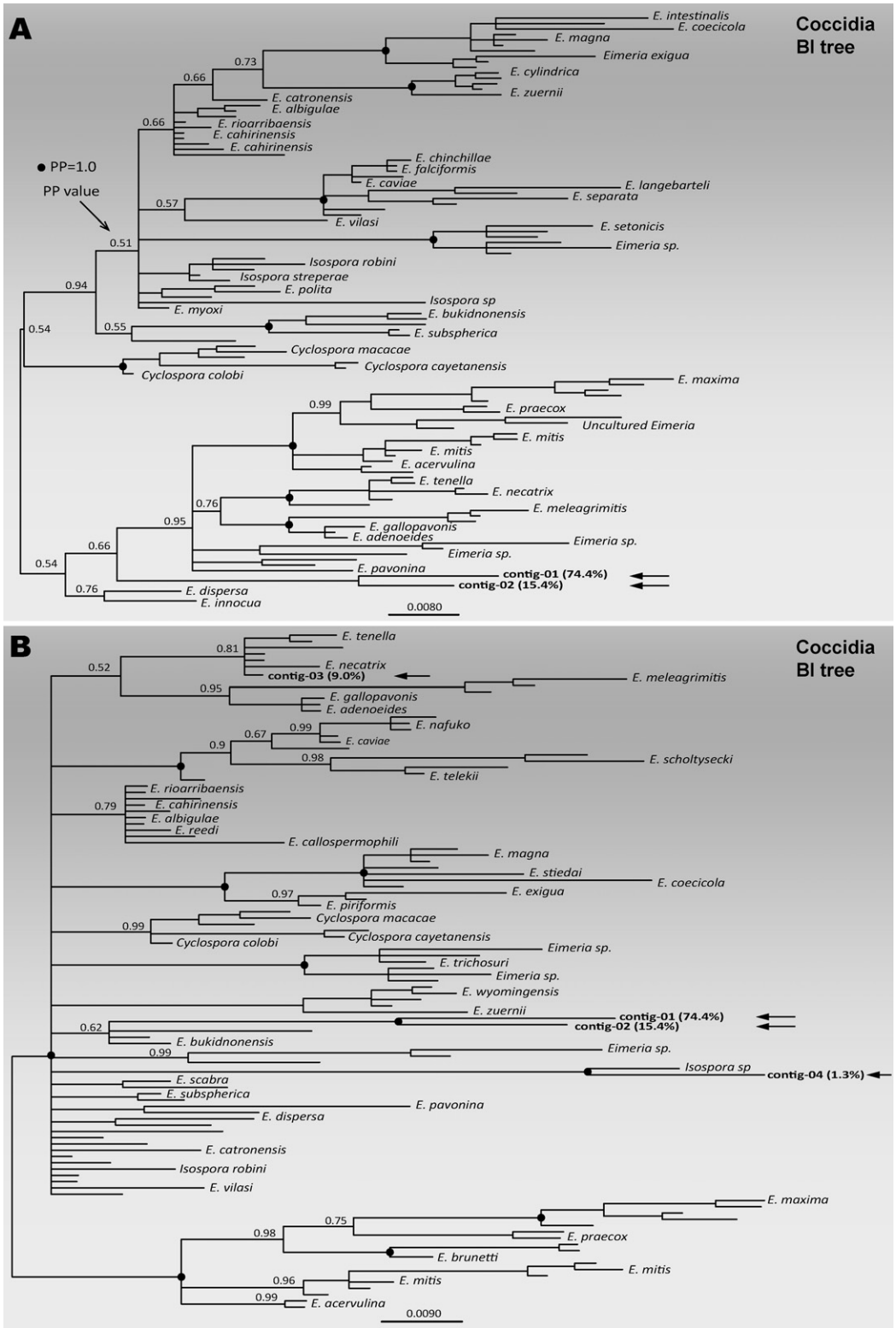

Figure 2 Bayesian inference (BI) trees based on coccidian small subunit ribosomal RNA gene sequences. (**A**) Phylogenetic positions of coccidian contig-01 and contig-02 in the BI tree inferred from long sequences containing 106 taxa and 679 nucleotide positions. (**B**) Phylogenetic positions of all four coccidian contigs in the BI tree inferred from short sequences containing 108 taxa and 267 positions. Posterior probability (PP) values are only shown at the major nodes. Coccidian contigs derived from this study are followed by the percentage of high-quality sequencing reads represented by each contig and indicated by arrows.

mortality in birds (Donovan et al. 2008; Dunn et al. 2013; Howe et al. 2012; Schoener et al. 2014). In United States, *H. lophortyx* was reported to cause infection and death in captive bobwhites in California (Cardona et al. 2002). *Haemoproteus* sp. and *Plasmodium juxtanucleare*-like parasites were also detected in a flock of masked bobwhite quail (*C. virginianus ridgwayi*) maintained in a zoo in Arivaca, Arizona (Pacheco et al. 2011). We examined 400 quail blood smears (100, 100, and 200 smears collected in 2011, 2012, and 2013, respectively). However, we failed to detect blood parasites and observed no apparent hematological abnormality. Among all samples examined, we observed only one red blood cell containing a single *Plasmodium*-like object—its identity could not be firmly established and its possibility as a staining artifact could not be ruled out because of the lack of any additional evidence in the same smear. On the other hand, even if it was a *Plasmodium* meront, intensity of infection was extremely low and probably insignificant to the quail health. Our data agree with an earlier report on the "apparent absence" of hematozoa in north Florida bobwhite populations (Crook et al. 2009).

DISCUSSIONS

Challenges in studying the impact of infectious diseases on the wild quail conservation

The study on the impact of parasitic diseases on wild quail faced a number of technical challenges. The first major one was the availability of limited amounts of fecal samples that could be collected overnight, for which any enrichment protocols such as floatation methods are not practical. Therefore, highly sensitive protocols needed to be developed and optimized. The second was the lack of reference sequences of protozoan parasites in quail, so that PCR primers could only be developed according to the available sequences of related species. The detection also needed to be theoretically capable of detecting any potential species at desired taxonomic levels (e.g. all intestinal coccidia with the exclusion of cryptosporidia, all cryptosporidia and all microsporidia). On the other hand, sensitive molecular detection protocols when developed would allow the detection of multiple pathogens from the same sets of limited amount of fecal samples.

In this study, we developed and optimized nested PCR protocols capable of detecting several groups of protozoan parasites at desired sensitivity and specificity. The PCR detection results were further validated by sequencing. We also modified the standard fecal DNA isolation protocol by adding a freeze/thaw cycle and a homogenization step with mini-glass beads in lysis buffer to release DNA from oocysts and spores. However, despite that the detection protocols were optimized, and nested PCR was known to be much more sensitive than traditional microscopic examination in detecting pathogens, our study might still under-estimate the infection rates. The actual positive rates of all parasite groups could be higher than the reported, because we were only able to collect the fecal pellets once, rather than multiple times over several days from individual wild quail in both RPQRR trap-and-release program and OID operation, in which some birds might be in a period of producing low or no oocysts or spores in a particular day.

The ultimate challenge is the unavailability to access dead birds in the field to directly evaluate the factors that contributed to the death. Many factors may contribute to the population size of wild quail, including birth and survival rates. While production may be relatively consistent in a given population of birds, survival rates are variable and determined by environmental and biological factors (e.g. weather factors, abundance of predators, and/or outbreaks of infectious diseases). However, unlike pen-raised or captive flocks for which sick birds can be isolated for evaluating the cause of the death, sick quail in the field will be taken by predators before or after they die, or simply hidden deep in the bushes, making them unavailable for assessing the factors contributing to the death. In other words, we can only sample healthy birds or survivors to gain clues on the factors contributing to the pending mortality events. On the other hand, epidemiological study would provide certain clues on the impact of infections on the quail populations, because infectious agents would likely also present in the surviving birds.

Impact of protozoan parasites on wild quail

Coccidiosis is the most significant group of apicomplexan parasites in the poultry industry. However, the impact of coccidiosis in wild quail has been poorly understood. Based on the study of other birds including chickens and farm-reared bobwhites, coccidiosis could lead to severe morbidity and mortality when heavily infected. An earlier study showed that *E. lettyae* was highly pathogenic to bobwhites and could produce 25–100% mortality in 5-day old birds and up to 83% mortality in 18-day old birds at doses of 5×10^5 and 1×10^6 of oocysts (Ruff and Wilkins 1987). Together with the fact that the prevalence of coccidia in wild quail was high (i.e. ~ 46% or higher actual prevalence discussed above), we conclude coccidia as one of the potential biotic factors contributing to the health of wild quail in the Rolling Plains ecoregion. The negative impact could be much higher on young chicks whose survival rates are critical to the maintaining and growth of wild quail populations.

Cryptosporidia are the second most significant group of apicomplexan parasites in quail, known to infect both digestive and respiratory tracts, causing conjunctivitis, sinusitis, tracheitis, and enteritis that could be severe or fatal in farm-reared quail and other avian species (Guy et al. 1987; Hoerr et al. 1986; Lindsay et al. 1991; Murakami et al. 2002; van Zeeland et al. 2008). However, the prevalence of cryptosporidia in bobwhite and scaled quail revealed by this study was relatively low (i.e. ~ 12% infection rate, vs. ~ 46% for coccidia), suggesting that cryptosporidiosis might pose a lesser threat on wild quail.

Microsporidian are most closely related to the fungi, but customarily discussed among the protozoa (protists). We

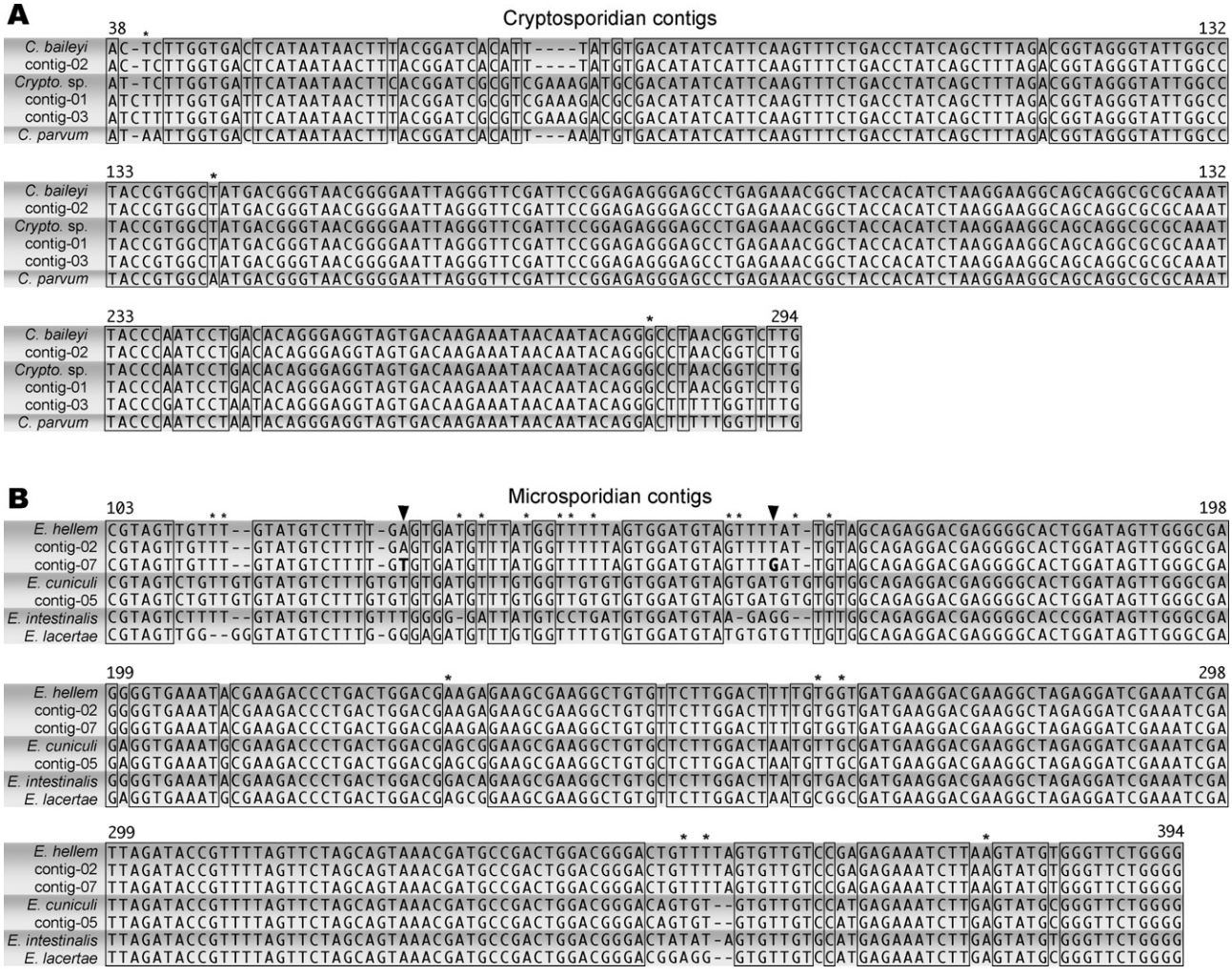

Figure 3 Multiple nucleotide sequence alignments of cryptosporidian and microsporidian contigs with reference sequences. (**A**) Alignment of all three cryptosporidian contigs with those from *Cryptosporidium baileyi* (GenBank: EU717828), an environmental *Cryptosporidium* (*Crypto*) (AF316626) and *Cryptosporidium parvum* (AF040725), in which contig-02 is 100% identical that of *C. baileyi*. Numbers indicate nucleotide positions in EU717828. (**B**) Alignment of selected microsporidian contigs (i.e. contig-02, contig-05, and contig-07) with those from *Encephalitozoon hellem* (KM058744), *Encephalitozoon cuniculi* (DQ453123), *Encephalitozoon intestinalis* (KM058742), and *Encephalitozoon lacertae* (AF067144), in which contig-02 is 100% identical to that of *E. hellem*, contig-07 differs from that of *E. hellem* by two nucleotides as indicated by arrowheads and bold nucleotides, and contig-05 is 100% identical to that of *E. cuniculi*. Numbers indicate nucleotide positions in KM058744. In both alignments, degenerate primer sequences have been removed. In each alignment, nucleotide position conserved in all sequences are boxed, while those conserved among closely related sequences are pointed by asterisks (i.e. conserved between contigs, *C. baileyi* and an environmental avian *Cryptosporidium* sequences, but divergent from the mammalian *C. parvum* in (A) and conserved between contigs, *E. hellem* and *E. cuniculi*, but divergent from *E. lacertae* in (B).

detected high "positive rates" of microsporidia in wild quail (~ 44%), but estimated that only ~ 11% were caused by *E. hellem* and *E. cuniculi* known to truly infect birds. This is the first time that *E. hellem* and *E. cuniculi* are reported in bobwhite and scaled quail. The remaining microsporidia were of insect-origin, but carried by birds. The effects of microsporidiosis in birds could range from no clinical signs as observed in chronically infected budgerigars to outbreak and death in falcons (Muller et al. 2008; Sak et al. 2010). However, the estimated low infection rates by native avian microsporidia suggested that, similar to cryptosporidiosis, microsporidiosis was probably not a significant disease in wild quail under the investigation.

Histomoniasis and hematozoa might cause sickness and death in birds. However, these two groups of parasites were not detected in samples collected in this study, indicating that they were less likely a concern in the wild quail in the Rolling Plains ecoregion.

While coccidiosis could be a significant risk factor, we cannot rule out the significance of cryptosporidiosis and microsporidiosis on the health of wild quail, as we did not

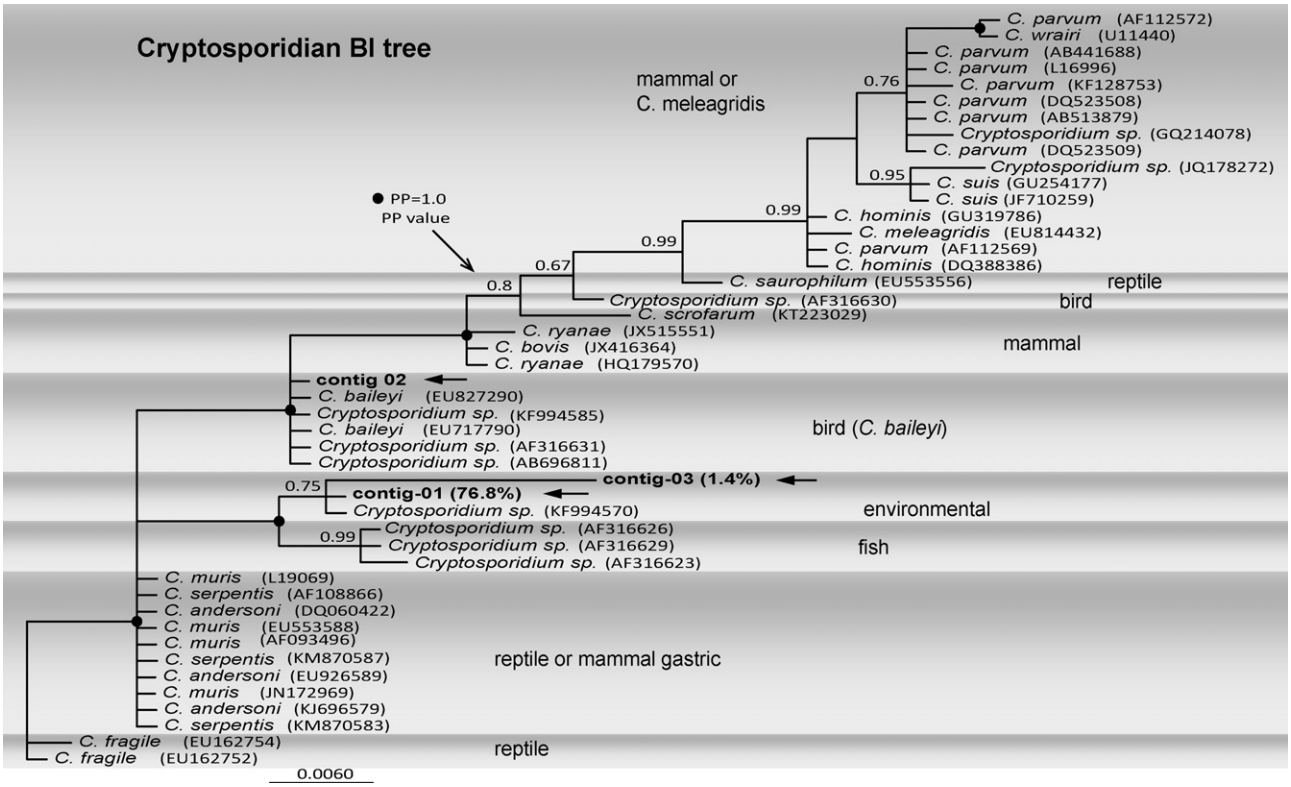

Figure 4 Bayesian inference (BI) trees based on cryptosporidian small subunit ribosomal RNA gene sequences containing 46 taxa and 270 nucleotide positions. Numbers at the nodes indicate posterior probability (PP) values. GenBank accession numbers are shown in parentheses. Cryptosporidian contigs derived from this study are followed by the percentage of high-quality sequencing reads represented by each contig and indicated by arrows.

have the access to the dead birds to determine the true factors contributing to the death. This multiyear survey only represents the first step toward the understanding of impact of protozoan parasites on wild quail. Further investigations are needed to determine: (i) the qualitative and quantitative information on the species compositions of protozoan parasites, particularly the major coccidian species; (ii) the reference sequences for future species-specific survey and monitoring; (iii) the virulence of major protozoan parasites; and (iv) the efficacy of drugs against the field strains found in wild quail.

Implication on public health

Wild animals and humans share many infectious diseases, inducing those caused by parasites. For the parasites under this investigation, coccidia are known to be reasonably host-specific, and cross-infections between avian and mammalian hosts have not been reported. However, some avian cryptosporidian and microsporidian species or genotypes are capable of infecting humans, causing severe to deadly opportunistic infections (OIs) in individuals with compromised or weakened immunity (e.g. AIDS patients, organ transplant recipients, children, and elderly). Among cryptosporidia, *C. meleagridis* is a well-documented OI pathogen, but undetected in wild quail. Another common avian species *C. baileyi* was present, but it is not considered as a significant OI pathogen although there was an earlier report of this species in man. A wide-range of microsporidian species are known to cause human OIs, in which *Enterocytozoon bieneusi* and the *Encephalitozoon* species (e.g. *E. hellem*, *E. incuniculi*, and *Encephalitozoon intestinalis*) are more common OIs, whereas some other species are less usual (e.g. *Anncaliia algerae* and *Trachipleistophora hominis*) (Didier 2005; Didier and Weiss 2006, 2011; Mathis et al. 2005; Nagpal et al. 2013). In this survey, we detected *E. hellem* and *E. incuniculi* (or closely related species), indicating that wild quail are a potential source of microsporidian OIs. Therefore, individuals with compromised or weakened immunity should take the appropriate precautions to avoid becoming infected when participating in the game bird-hunting activities and handling wild birds.

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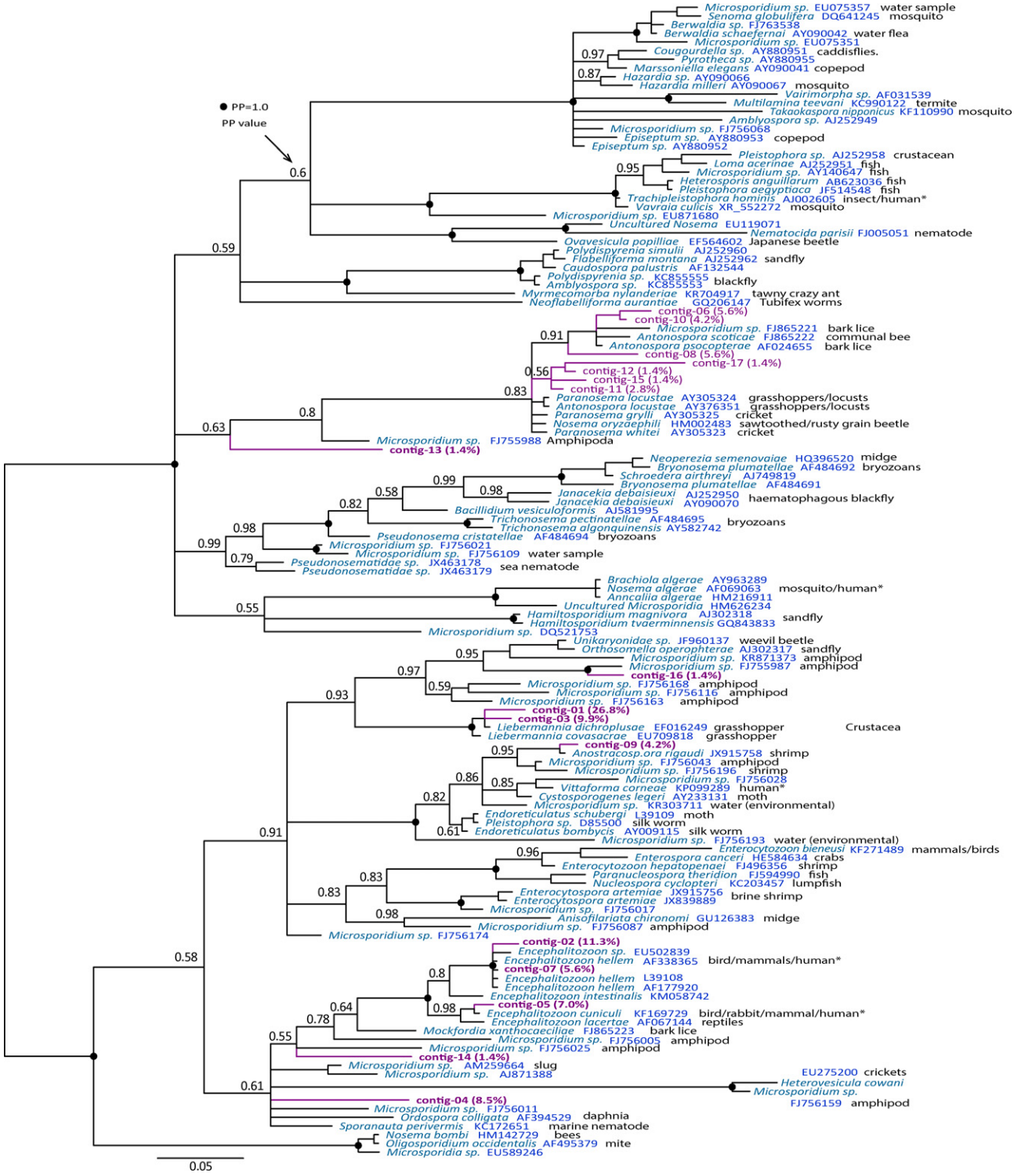

Figure 5 Bayesian inference (BI) trees based on microsporidian small subunit ribosomal RNA gene sequences containing 133 taxa and 234 nucleotide positions. Numbers at the nodes indicate posterior probability (PP) values. GenBank accession numbers are shown in blue fonts. Hosts of selected microsporidian species are shown in black fonts. Microsporidian contigs derived from this study are indicated by maroon fonts followed by the percentage of high-quality sequencing reads represented by each contig.

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