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ORIGINAL PAPER

Cryptosporidium galli and novel *Cryptosporidium* avian genotype VI in North American red-winged blackbirds (*Agelaius phoeniceus*)

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Abstract Proventriculus and intestinal samples from 70 North American red-winged blackbirds (*Agelaius phoeniceus*; order Passeriformes) were examined for the presence of *Cryptosporidium* by PCR amplification and sequence analysis of the 18S ribosomal RNA (18S rRNA), actin, and 70-kDa heat shock protein (HSP70) genes. Twelve birds (17.1 %) were positive for the *Cryptosporidium* 18S rRNA gene: six birds were positive at the proventriculus site only and six birds were positive at the proventriculus and intestinal sites. Sequence analysis of the 18S rRNA, actin and HSP70 genes showed the presence of the gastric species *Cryptosporidium galli* in a single proventriculus sample and a closely related genotype, which we have named *Cryptosporidium* avian genotype VI, in all other positive samples. These findings contribute to our understanding of *Cryptosporidium* diversification in passerines, the largest avian order.

Keywords *Cryptosporidium* · Red-winged blackbird · Passerines · *Cryptosporidium galli* · Avian genotype VI · Proventriculus · Intestine

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Introduction

The apicomplexan parasite *Cryptosporidium* infects the gastrointestinal epithelium of all major vertebrate groups (Kváč et al. 2014) and causes the disease cryptosporidiosis, which can be chronic and life threatening (Checkley et al. 2015). Approximately 30 species and 70 genotypes of *Cryptosporidium* form two major clades in nucleotide sequence phylogenies: a smaller, basal clade characterized by specificity for the gastric epithelium and a larger clade comprising members that infect the intestinal epithelium (Xiao et al. 2004).

Cryptosporidium has been identified in 17 of the 26 avian orders, including the largest order, Passeriformes, which contains almost 60 % of the extant avian diversity in 5700 species (Kváč et al. 2014). Passerines are host to three avian-adapted *Cryptosporidium* species, *Cryptosporidium galli*, *Cryptosporidium meleagridis*, and *Cryptosporidium baileyi* and three avian-adapted *Cryptosporidium* genotypes, avian genotypes I, III, and IV (Current et al. 1986; Gomes et al. 2012; Nakamura et al. 2014; Nakamura et al. 2009; Ng et al. 2006; Qi et al. 2011; Ryan 2010; Ryan et al. 2003b; Sevá Ada et al. 2011; Slavin 1955).

Most studies on *Cryptosporidium* in passerines have focused on captive birds, and comparatively little is known about *Cryptosporidium* infecting free-living, wild birds. There has been just one report to date of *Cryptosporidium* in free-living North American passerines: an uncharacterized *Cryptosporidium* species was identified in fledgling cliff swallows (*Petrochelidon pyrrhonota*) with clinical signs of conjunctivitis, rhinitis, and sinusitis (Ley et al. 2012).

Red-winged blackbirds (*Agelaius pheonicius*) are members of the New World passerine family Icteridae, which has 95 species in 23 genera, including oropendolas (*Psarocolius* spp.), caciques (*Cacicus* spp.), orioles (*Icterus*

Table 1 Samples positive for *Cryptosporidium* DNA by PCR analysis of the 18S rRNA, actin, and HSP70 genes

Animal number	Sex	Sample number	Genes			Species/genotype
			18S rRNA	Actin	HSP70	
1	Female	PV-3486	+[a]	–	+	Avian genotype VI
		IN-3487	–[b]	–	–	
2	Female	PV-3492	+	+	+	Avian genotype VI
		IN-3493	–			
3	Male	PV-3543	+	–	–	Avian genotype VI
		IN-3544	–	–	–	
4	Female	PV-3545	+	–	–	Avian genotype VI
		IN-3546	+	–	–	Avian genotype VI
5	Male	PV-3551	+	–	–	Avian genotype VI
		IN-3552	–			
6	Male	PV-3553	+	–	+	Avian genotype VI
		IN-3554	+	–	+	Avian genotype VI
7	Female	PV-3575	+	–	–	Avian genotype VI
		IN-3576	–	–	–	
8	Male	PV-3605	+	+	–	*C. galli*
		IN-3606	–	–	–	
9	Male	PV-3607	+	–	+	Avian genotype VI
		IN-3608	+	–	+	Avian genotype VI
10	Male	PV-3635	+	–	+	Avian genotype VI
		IN-3636	+	–	+	Avian genotype VI
11	Female	PV-18212	+	+	+	Avian genotype VI
		IN-18213	+	–	–	Avian genotype VI
12	Male	PV-18220	+	+	+	Avian genotype VI
		IN-18221	+	+	+	Avian genotype VI

PV proventiculus, *IN* intestine

[a] Positive by PCR analysis

[b] Negative by PCR analysis

spp.), meadowlarks (*Sturnella* spp.), grackles (*Quiscalus* spp., *Hypopyrrhus* spp., *Lampropsar* spp., and *Macroagelius* spp.), and cowbirds (*Molothrus* spp.) (Lowther 1975). They are abundant in North America with a range that extends as far north as Alaska and as far south as Cuba (Yasukawa and Searcy 1995). Higher latitude populations migrate to lower latitudes during winter, where they nest in marshes, wetlands, and hayfields (Ball et al. 1988). To date, the only Icteridae family members identified as hosts of *Cryptosporidium* have been chopi blackbirds (*Gnorimopsar chopi*) from Brazil, which hosted *C. galli* (Nakamura et al. 2014), and a red rumped cacique (*Cacicus haemorrhous*) and crested oropendola (*Psarocolius decumanus*) from the Czech Republic, which hosted *C. baileyi* (Ryan et al. 2003a).

In the present study, *Cryptosporidium* DNA from proventriculus and intestinal contents of red-winged blackbirds caught in the USA was characterized by sequence analysis of the 18S ribosomal RNA (18S rRNA), actin, and heat shock protein 70 (HSP70) genes. These analyses show the presence of *C. galli* and the closely related, novel *Cryptosporidium* avian genotype VI in North American red-winged blackbirds.

Materials and methods

Sample collection and DNA isolation

Seventy red-winged blackbirds, comprising 41 after hatch year males, 26 after hatch year females, and three juveniles of undetermined sex, were captured using live capture (e.g., mist nets, walk-in traps) or lethal methods from areas of Kansas, North Dakota, and Minnesota, USA. Live captured birds were immediately euthanized by over-anesthetizing with halothane. Bird carcasses were dissected and a sample was taken from the proventriculus and intestinal contents. DNA

was isolated from 200 mg of each sample by alkaline digestion and phenol-chloroform extraction and purified using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA) as described previously (Feltus et al. 2006). The capture, handling, and euthanizing of birds in this study were conducted in accordance with Institutional Animal Care and Use Committee of North Dakota State University Protocol #A13006.

PCR amplification

Nested PCR protocols were used to amplify fragments of the 18S rRNA, actin, and HSP70 genes. A fragment of the *Cryptosporidium* 18S rRNA gene was amplified as described by Xiao et al. (2001), with the exception that 0.5× PCR buffer was used (Promega, Madison, WI). A fragment of the actin gene was amplified as previously described by Sulaiman et al. (2002). The protocol to amplify a fragment of the HSP70 gene was developed as part of this study. Nested PCR primers were designed with specificity for HSP70 sequences that are conserved in *C. galli* [accession no. AY168849], *Cryptosporidium* sp. CzechB1 Eurasian woodcock [accession no. AY273773], *Cryptosporidium muris* [accession no. AF221542], *Cryptosporidium andersoni* [accession no. AY954894], *Cryptosporidium serpentis* [accession no. AF221541], and *Cryptosporidium scrofarum* [accession no. JX424842]. In the primary reaction, a fragment of ~750 bp was amplified using 0.1 μM each of the primers HSPAvAF1

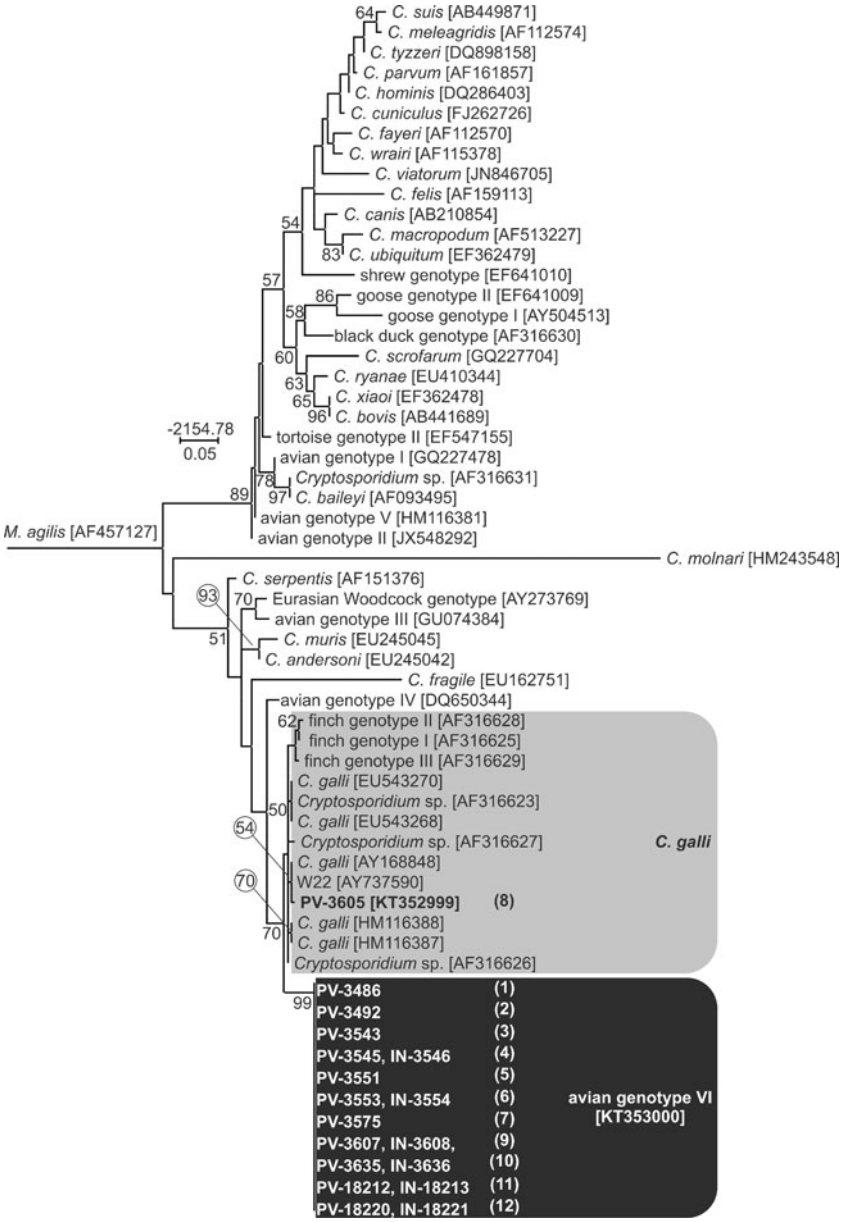

Fig. 1 Maximum likelihood tree of 18S rRNA gene sequences. The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model (Tavaré 1986). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed some sites to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree was rooted with 18S rRNA from *Monocystis agilis* [accession no. AF457127]. The prefix *PV* indicates a sequence obtained from a proventriculus sample. The prefix *IN* indicates a sequence obtained from an intestinal sample. The animal number from Table 1 is presented in parenthesis after the sample number(s)

(5′-GCT CGT GGT CCT AAA GAT AA) and HSPAvAR1 (5′-ACG GGT TGA ACC ACC TAC TAA T), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5U Taq DNA polymerase, 1× PCR buffer, and 0.5–2 μL template DNA in a 100-μL reaction. A secondary fragment of ~515 bp was amplified using 0.1 μM each of the primers HSPAvAF2 (5′-ACA GTT CCT GCC TAT TTC) and HSPAvAR2 (5′-GCT AAT GTA CCA CGG AAA TAA TC), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5U Taq DNA polymerase, 1× PCR buffer, and 2 μL of primary PCR product in a 100-μL secondary reaction. The primary PCR conditions were 35 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 1 min. The first cycle was preceded by an initial denaturation at 94 °C for 5 min, and the last cycle was followed by a final extension at 72 °C for 10 min. The secondary reaction used the same conditions as the primary, with the exception that the annealing temperature was 50 °C.

DNA from *Cryptosporidium hominis* was used as a positive control for the 18S rRNA and actin PCR reactions. Water was included instead of DNA template as a negative control in all reactions. Secondary PCR products were separated on an agarose gel and visualized under UV illumination using ethidium bromide staining.

Sequencing and phylogenetic analysis

PCR products were purified (Wizard SV, Promega, Madison, WI) and sequenced in both directions with secondary primers using a BigDye Terminator v3.1 cycle sequencing kit in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA). Sequences were assembled using SeqMan (DNAStar, Madison, WI) and aligned using the MAFFT version 7 online server with automatic selection of alignment strategy (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013). Alignments were manually edited and phylogenetic analyses were performed using MEGA 6.0 (Tamura et al. 2013). The evolutionary history of aligned sequences

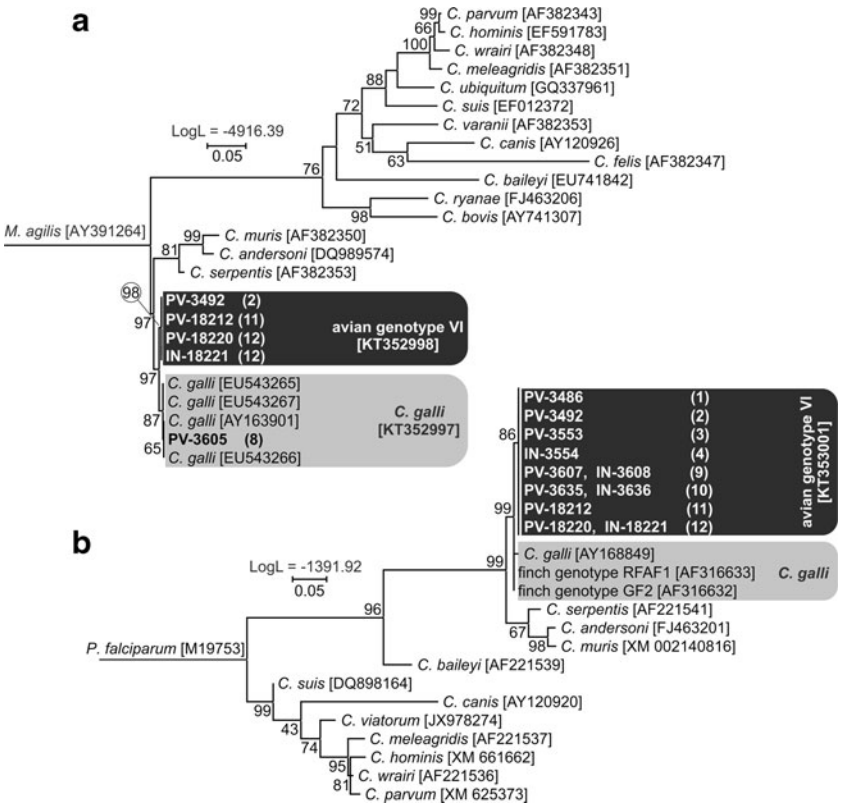

Fig. 2 Maximum likelihood tree of actin (**a**) and HSP70 (**b**) gene sequences. The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model (Tavaré 1986) for the actin tree or the Tamura 3-parameter model (Tamura 1992) for the HSP70 tree. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites in actin sequences. The rate variation model allowed some sites in actin and HSP70 sequences to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The actin tree was rooted with an actin sequence from *Monocystis agilis* [accession no. AY391264]. The HSP70 tree was rooted with a HSP70 sequence from *Plasmodium falciparum* [accession no. M19753]. The prefix *PV* indicates a sequence obtained from a proventriculus sample. The prefix *IN* indicates a sequence obtained from an intestinal sample. The animal number from Table 1 is presented in parenthesis after the sample number(s)

was inferred using the maximum likelihood (ML) method (Saitou and Nei 1987), with the substitution model that best fit the alignment selected using the Bayesian information criterion. The general time reversible model (Tavaré 1986) with a gamma rate distribution and invariant sites was selected for 18S rRNA and actin alignments. The Tamura 3-parameter model (Tamura 1992) with invariant sites was selected for the HSP70 alignment.

Sequences from this study have been deposited in GenBank under the accession numbers KT352997–KT353001.

Results

Prevalence of *Cryptosporidium* in red-winged blackbirds

Twelve out of 70 birds (17.1 %), seven males and five females, were positive for the *Cryptosporidium* 18S rRNA gene (Table 1). Six birds were positive only at the proventriculus site and six birds were positive at both the proventriculus and intestinal sites.

Phylogenetic analysis of *Cryptosporidium* isolates from red-winged blackbirds

A ML tree constructed from 18S rRNA gene sequences in this study and representative sequences in GenBank showed the presence of two closely related genotypes in the gastric *Cryptosporidium* clade (Fig. 1). PV-3605 clustered with *C. galli*, sharing 99.9 % sequence identity with a *C. galli* isolate from storm water in New York, USA [accession no. AY737590]. Sequences from the remaining 17 samples shared 100 % identity with each other, 98.5 % identity with PV-3605, and between 97.7 and 98.3 % identity with *C. galli* sequences published in GenBank. We have named this novel genotype *Cryptosporidium* avian genotype VI.

A fragment of the actin gene was amplified and sequenced from five of the 18 samples that were positive for the 18S rRNA gene (Fig. 2a). PV-3605 shared 100 % sequence identity with *C. galli* from a western capercaillie [accession no. AY163901], chestnut-bellied seed finch [accession no. EU543267], Atlantic canary [accession no. EU543266], and cockatiel [accession no. EU543265]. Actin sequences from *Cryptosporidium* avian genotype VI (PV-3492, PV-18212, PV-18220, and IN-18221) shared 100 % identity with each other and 99.3 % identity with PV-3605 and published actin sequences from *C. galli*.

A fragment of the HSP70 gene was amplified and sequenced from 11 of the 18 samples that were positive for the 18S rRNA gene (Fig. 2b). A HSP70 sequence could not be obtained from the *C. galli* isolate PV-3605. Sequences of HSP70 from *Cryptosporidium* avian genotype VI shared 100 % identity with each other and 98.6 % identity with a published *C. galli* HSP70 sequence [accession no. AY168849].

Discussion

We report on the genotyping of *Cryptosporidium* isolates from red-winged blackbirds in the USA. Data show that free-living North American red-winged blackbirds host the gastric species *C. galli*, and a closely related genotype within the gastric clade, which we have named *Cryptosporidium* avian genotype VI.

Consistent with their phylogenetic positions, *C. galli* and avian genotype VI were found in the proventriculus of all positive red-winged blackbirds. The finding that half of the positive birds also had a positive intestinal sample is not surprising, as oocysts of gastric species pass through the intestine and are shed in the feces. Although we did not perform histopathology to confirm the gastric location, previous work has shown that *C. galli* exclusively infects the proventricular epithelium (Blagburn et al. 1990; Morgan et al. 2001), similar to the Eurasian woodcock genotype (Ryan et al. 2003a) and avian genotype III (Makino et al. 2010). Each of these species/genotypes has been shown to cause clinical disease and mortality in birds (Blagburn et al. 1990; Makino et al. 2010; Morgan et al. 2001; Ryan et al. 2003a). Further studies are required to determine if *Cryptosporidium* avian genotype VI is pathogenic for red-winged blackbirds.

The 18S rRNA gene in *C. galli* exhibits significant sequence heterogeneity (Morgan et al. 2001). In one study, three different 18S rRNA sequences from a single bird diverged by 0.6 ± 0.3 % (Morgan et al. 2001). These sequences were initially named finch genotypes I, II, and III, but were subsequently classified as heterogeneous sequences of *C. galli* (Ryan et al. 2003b). Among the possible explanations for intraspecific 18S rRNA sequence heterogeneity, the occurrence of paralogous gene copies (Le Blancq et al. 1997; Stenger et al. 2015) is most problematic for the inference of evolutionary relationships because paralogs are not related by descent (Koonin 2005). The mean evolutionary divergence of 18S rRNA sequences from avian genotype VI and *C. galli* (2.0 ± 0.5 %) was greater than that of heterogeneous sequences from *C. galli* (0.7 ± 0.2 %; calculated using Mega 6.0 from sequences reported in Fig. 1), supporting our decision to categorize avian genotype VI separately from *C. galli*. The finding that of actin and HSP70 sequences also diverged further supported this decision.

The present study has contributed to the understanding of *Cryptosporidium* diversity in passerine hosts. Further sampling of passerines in their natural habitat will help to determine how factors such as host speciation and geographic

isolation have influenced *Cryptosporidium* diversification in birds.

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