



Multilocus sequencing of *Hepatozoon* cf. *griseisciuri* infections in Ontario eastern gray squirrels (*Sciurus carolinensis*) uncovers two genotypically distinct sympatric parasite species

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Abstract

Intra-leukocytic gamonts consistent with the description of *Hepatozoon griseisciuri* Clark, 1958 are reported for the first time in Canadian eastern gray squirrels (*Sciurus carolinensis* Gmelin, 1788). Polymerase chain reaction (PCR) amplification and direct Sanger sequencing identified a pair of distinct genotypes at both a nuclear and mitochondrial locus; two 18S ribosomal RNA gene sequences (rDNA; genotype A and genotype B: 1816 base pairs (bp); 98.8% pairwise identity) and 2 distinct complete mitochondrial genome sequences (genotype A: 6311 bp; genotype B: 6114 bp; 89.1% pairwise identity) were obtained from 3 *H. griseisciuri*-infected squirrels sampled in Guelph, Ontario. The genetic content of both circular-mapping mitochondrial genomes was conventional for apicomplexan protists; each encoded for 3 protein-coding genes (cytochrome *c* oxidase subunit I (COI); cytochrome *c* oxidase subunit III (COIII); and cytochrome B (CytB)), 14 fragmented large subunit rDNA, 10 fragmented small subunit rDNA, and 8 unassigned rDNA. These genotypes, based on sequences obtained from a pair of loci from two parasite genomes, confirm the presence of at least two *Hepatozoon* species infecting Ontario eastern gray squirrels, one of which is likely to be conspecific with *H. griseisciuri*.

Keywords *Hepatozoon griseisciuri* · Squirrel · Apicomplexa · Adeleorina · Mitochondrion · Ribosomal RNA

Introduction

The genus *Hepatozoon* (Adeleorina) was erected by Miller (1908) to support the description of *Hepatozoon perniciosum* Miller, 1908 (later synonymized as *Hepatozoon muris* (Balfour, 1906) Wenyon, 1926), an apicomplexan parasite of the rodent mite *Laelaps* (subgenus *Echinolaelaps*) *echidnina* Berlese, 1887 that utilized the leukocytes of laboratory rat intermediate hosts. The genus *Hepatozoon* later expanded to include species infecting a vast range of invertebrate definitive hosts (e.g., various dipteran insects, ticks, mites, fleas, lice) and vertebrate intermediate hosts (e.g., mammals,

amphibians, reptiles, birds) (Smith 1996; Barta 2000). Given the breadth of hosts associated with *Hepatozoon* spp., the monophyly of the genus has been put into question (Smith and Desser 1997; Karadjian et al. 2015).

Hepatozoon species have been reported from a number of different sciurid hosts (see Clark 1958) but *Hepatozoon griseisciuri* Clark, 1958 is currently the only *Hepatozoon* species described in the eastern gray squirrel, *Sciurus carolinensis* Gmelin, 1788. First observed in the leukocytes of the eastern gray squirrel, Hermann and Price (1955) identified the gamonts as *Hepatozoon sciuri* based on Coles' (1914) description of a morphologically similar parasite from a Eurasian red squirrel (*Sciurus vulgaris*) in the UK. Based on the description of additional lifecycle stages, Clark (1958) named *H. griseisciuri* and determined the definitive host of *H. griseisciuri* to be a mite commonly identified at that time as *Euhaemogamasus ambulans* (Thorell, 1872); Redington (1970) later concluded in a comprehensive taxonomic revision that the correct identification of this mite definitive host was *Haemogamasus reidi* Ewing, 1925. Clark (1958) concluded that *H. griseisciuri* could “be distinguished from all other adequately known species of *Hepatozoon* by the fact that the

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sporocysts regularly contain only 4 large sporozoites.” In addition, Clark (1958) found that sporogonic development was possible in laboratory-reared spiny rat mites, *L. echidnina*, that are not typically found on eastern gray squirrels. *Hepatozoon griseisciuri* has a two-host lifecycle starting in the squirrel as merogonic replication in the tissues (e.g., lung, spleen, liver, bone marrow) followed by gamonts that mature in leukocytes (Clark 1958; Hendricks 1975; Davidson and Calpin 1976; Watkins and Nowell 1991). The remainder of the lifecycle occurs in blood-feeding *H. reidi* that ingests gamonts that then undergo syzygy, gamete maturation, syngamy, and sporogony in this definitive mite host. Large, polysporocystic oocysts typical of the genus *Hepatozoon* mature in the hemocoel of the mite definitive host and are presumably ingested by the squirrel host to initiate infection (Clark 1958; Redington and Jachowski 1971, 1972).

Infections with *H. griseisciuri* in eastern gray squirrels have been reported throughout the geographical range of the host, including the southeastern United States (Herman and Price 1955; Clark 1958; Parker 1968; Davidson and Calpin 1976), Rhode Island (Weidanz and Hyland 1958), and Wisconsin (Dorney and Todd 1959). The parasite has also been reported in the UK where the eastern gray squirrel has been introduced (Britt and Molyneux 1979; Watkins and Nowell 1991). It is unclear if these authors have all reported on the same *Hepatozoon* species. Perhaps several *Hepatozoon* species can utilize the eastern gray squirrel as an intermediate host. Classically, full characterization of a *Hepatozoon* sp. required detailed observations on parasite development in its definitive host to differentiate it from similar species. More recently, molecular data have been used to explore the diversity and relationships among *Hepatozoon* spp. and these data have revealed the existence of putative sibling species (e.g. Baneth et al. 2000). Currently, the molecular target from the adeleorinid coccidia with the most numerous publically available sequences is the nuclear 18S ribosomal RNA (rRNA) gene (rDNA). Extrachromosomal genome sequences from the mitochondrion and the apicoplast have become available recently from some members of the Adeleorina (Léveillé et al. 2014, 2019a, 2019b). There are currently no publicly available DNA sequences for *H. griseisciuri*.

We report the presence of intra-leukocytic gamonts consistent with the description of *H. griseisciuri* in Canadian eastern gray squirrels for the first time and describe the amplification and sequencing of 2 unique complete 18S rDNA sequences and 2 unique complete mitochondrial genome sequences from 3 infected eastern gray squirrels. These are the first DNA sequences reported for this parasite and allude to the presence of a pair of genetically distinct *Hepatozoon* species infecting Ontario eastern gray squirrels.

Materials and methods

Case material

Three adult road-killed eastern gray squirrels (*S. carolinensis*) were collected in Guelph, ON, Canada, necropsied and found to be infected with *Hepatozoon* cf. *griseisciuri*. Squirrel 1 was collected in June 2017; heart tissue containing coagulated blood was retained for DNA extraction. Squirrel 2 was collected in September 2017 and blood, liver, and spleen tissues were retained and frozen for DNA extraction. Squirrel 3 was collected in March 2018 and fresh samples of blood, liver, heart, kidney, spleen, and lung tissues were retained for histological observation and DNA extraction.

Histological slide preparation

During post-mortem examination, free-flowing blood was collected by syringe from the hearts of squirrel 2 and squirrel 3, and was used to make thin blood smears. Slides were air-dried, fixed, and stained with eosin and methylene blue (Shandon Kwik-Diff Stain, Thermo Fisher Scientific, Waltham MA, USA). Micrographs of gamont stages of *Hepatozoon* cf. *griseisciuri* were taken with a $\times 100$ objective lens using bright-field illumination with an Olympus Provis AX70 microscope (Olympus, Tokyo, Japan) fitted with an Infinity 3-1C microscopy camera (Lumenera Corp., Ottawa, Canada).

Fresh liver, heart, kidney, spleen, and lung tissue collected from squirrel 3 were processed routinely after fixation in 10% buffered formalin for a 24–48 h period and were paraffin embedded. Briefly, 5- μ m sections were deparaffinized with xylene and rehydrated by a series of graded baths in alcohol and phosphate-buffered saline buffer. All sections were stained with hematoxylin and eosin by the Animal Health Laboratory at the University of Guelph (Guelph ON, Canada).

DNA isolation

DNA was extracted from frozen blood-rich tissues (squirrel 1, heart; squirrel 2, lung; squirrel 3, liver) using a DNAzol reagent according to the manufacturer’s protocol (Thermo Fisher Scientific, Waltham MA, USA). After isolation, DNA was quantified spectrophotometrically using a Nanodrop 2000 instrument (Thermo Fisher Scientific, Waltham MA, USA).

Primer design

All existing and newly generated polymerase chain reaction (PCR) and sequencing primers (see Tables 1, 2, 3, and 4) were developed using the methods described in Léveillé et al. (2019b).

Table 1 PCR amplification primers used to amplify the nuclear 18S ribosomal RNA sequence and the complete mitochondrial genome sequence of *Hepatozoon cf. griseisciuri* genotype A

Amplicon	Size (bp)	Primer names	Primer Sequences	Anneal Temperature ^a
Mitochondrial amplicon A1	2765	Api_LSUF_F ^b Api_LSUG_R ^b	5'-GTWCGCCGGGATAACAGGT-3' 5'-AGATAGGGAACAACTGYCTCAA-3'	62 °C
Mitochondrial amplicon A2	3589	Api_LSUG_F ^b Api_LSUF_R ^b	5'-TTGAGRCAGTTTGTTCCTATCT-3' 5'-ACCTGTTATCCCCGGCGWA-3'	62 °C
Mitochondrial amplicon A3	761	Adel_RNA13_R Hep_SSUB_F	5'-GCTWACTTCCCGGTCAAAC-3' 5'-AGCCTGGTGGTAGTGTCAT-3'	61 °C
Mitochondrial amplicon A4	1504	Hep_CytB_391F BarHep_COI_274R	5'-CCWTGGGGTCAGATGAGTT-3' 5'-CCACTTCRGACGAGCCACA-3'	62 °C
Nuclear 18S rDNA amplicon A1	1375	Medlin_A (F) ^c Adel_18S_1522R ^b	5'-AACCTGGTTGATCCTGCCAGT-3' 5'-AYCCTATTTAGCAGGTTAAGGT-3'	60 °C
Nuclear 18S rDNA amplicon A2	794	Adel_18S_1000F ^b Medlin_B (R) ^c	5'-AGATACCGTCGTAGTCTTAACT-3' 5'-GATCCTTCTGCAGGTTACCTAC-3'	61 °C

^a Estimated for high-fidelity PCR using Thermo Fisher Tm calculator for Platinum SuperFi DNA polymerase (www.thermofisher.com)

^b Léveillé et al. (2019b)

^c Universal forward and reverse primers from Medlin et al. (1988)

PCR amplification and amplicon isolation

High-fidelity PCR protocols and PCR amplicon isolation were carried out as described in Léveillé et al. (2019b). Complete nuclear 18S rDNA sequences were amplified from squirrel 1 (genotype A) and squirrel 3 (genotype B) samples as two overlapping amplicons generated using whole-gene universal primers Medlin_A and Medlin_B (forward and reverse primers, respectively, from Medlin et al., 1988) paired with Adeleorina-specific internal primers Adel_18S_1522R and Adel_18S_1000F respectively (Tables 1 and 2). A partial 18S rDNA sequence was amplified from squirrel 2 using the Medlin_A and Adel_18S_1522R primer pair.

The mitochondrial genome sequence of *Hepatozoon cf. griseisciuri* genotype A was generated from squirrel 1 from 4 overlapping amplicons. The first 2 amplicons were generated by a trial and error method that involved using all 4

combinations of forward and reverse primers targeting the highly conserved regions LSUF and LSUG (primers: Api_LSUF_F; Api_LSUF_R; Api_LSUG_F; Api_LSUG_R; primers cited in Table 1). Successful amplifications resulted in mitochondrial amplicon A1 generated from Api_LSUF_F and Api_LSUG_R and mitochondrial amplicon A2 generated from Api_LSUG_F with Api_LSUF_R (Table 1). After sequencing mitochondrial amplicon A1 and mitochondrial amplicon A2, primers Adel_RNA13_R and Hep_SSUB_F were selected to generate mitochondrial amplicon A3 that would span the LSUF region (Table 1). Primers Hep_CytB_391F and BarHep_COI_274R were selected to generate mitochondrial amplicon A4 to span the LSUG region (Table 1).

Initial attempts to amplify and sequence *Hepatozoon cf. griseisciuri* mitochondrial genome sequences from squirrel 2 yielded mixed sequences containing genotype A as well as a

Table 2 PCR amplification primers used to amplify the nuclear 18S ribosomal RNA sequence and the complete mitochondrial genome sequence of *Hepatozoon cf. griseisciuri* genotype B

Amplicon	Size (bp)	Primer names	Primer sequences	Anneal temperature ^a
Mitochondrial amplicon B1	5960	HepSq_B_RNA23t_F Api_LSUG_R ^b	5'-CTAGGCCAATAACCCAAATCTCTG-3' 5'-AGATAGGGAACAACTGYCTCAA-3'	62 °C
Mitochondrial amplicon B2	1282	Hep_CytB_391F HepSq_B_COIR	5'-CCWTGGGGTCAGATGAGTT-3' 5'-AGCCATGATTCCAAATAGCAAC-3'	64 °C
Nuclear 18S rDNA amplicon B1	1375	Medlin_A (F) ^c Adel_18S_1522R ^b	5'-AACCTGGTTGATCCTGCCAGT-3' 5'-AYCCTATTTAGCAGGTTAAGGT-3'	60 °C
Nuclear 18S rDNA amplicon B2	794	Adel_18S_1000F ^b Medlin_B (R) ^c	5'-AGATACCGTCGTAGTCTTAACT-3' 5'-GATCCTTCTGCAGGTTACCTAC-3'	61 °C

^a Estimated for high-fidelity PCR using Thermo Fisher Tm calculator for Platinum SuperFi DNA polymerase (www.thermofisher.com)

^b Léveillé et al. (2019b)

^c Universal forward and reverse primers from Medlin et al. (1988)

Table 3 Sequencing primers used for Sanger sequencing of the nuclear 18S ribosomal RNA sequence and the complete mitochondrial genome sequence of *Hepatozoon cf. griseisciuri* genotype A

Amplicon	Primer name	Primer sequence	Position from start
Mitochondrial amplicon A1	Api_LSUG_R ^a	5'-AGATAGGGAACAACTGYCTCAA-3'	6156 → 6178
	H_COI_613R	5'-ACCATGAATTAAGGTCATAATACCCA-3'	571 → 545
	H_COI_613F	5'-TGGGTATTATGACCTTAAATTCATGGT-3'	545 → 571
	BHep_COIII_238F	5'-ATTCTGGGGAGYVTTTCAGCT-3'	1961 → 1940
	Api_LSUF_F ^a	5'-GTWCGCCGGGATAACAGGT-3'	2609 → 2590
Mitochondrial amplicon A2	Api_LSUF_R ^a	5'-ACCTGTTATCCCCGGCGWA-3'	2590 → 2608
	Haem_RNA14F ^a	5'-GAAACCTCCTGAKCGACTC-3'	2855 → 2874
	Api_LSUE_F ^a	5'-GGTAAGACCCTGAGCACCT-3'	3632 → 3614
	Api_LSUE_R ^a	5'-AGGTGCTCAGGGTCTTACCG-3'	3614 → 3633
	Hep_CytB_416R	5'-GCCCAGAAACTCATCTGA-3'	5122 → 5105
	Hep_COB_1101R ^b	5'-ACTGAGGTAATTGGCTACC-3'	5724 → 5706
	Api_LSUG_F ^a	5'-TTGAGRCAGTTTGTCCCTATCT-3'	6178 → 6156
Mitochondrial amplicon A3	Hep_CytB_391F	5'-CCWTGGGGTCAGATGAGTT-3'	5097 → 5115
	BHep_COI_274R	5'-CCACTTCRGACGAGCCACA-3'	289 → 271
	Adel_RNA13_R	5'-GCTWACTTCCCGGTCAAAC-3'	2233 → 2251
Mitochondrial amplicon A4	Adel_RNA13_R	5'-GCTWACTTCCCGGTCAAAC-3'	2233 → 2251
Nuclear 18S rDNA amplicon A1	Medlin_A (F) ^c	5'-AACCTGGTTGATCCTGCCAGT-3'	1 → 21
	18S_Adel_461F	5'-AGCTTTCGAYGGTADRGA-3'	321 → 339
	18S_696R ^d	5'-TACGAGCYTTYTAAGTCAAC-3'	641 → 621
	Adel_18S_1522R ^a	5'-AYCCTATTTAGCAGGTTAAGGT-3'	1375 → 1354
	Adel_18S_1000F ^a	5'-AGATACCGTCGTAGTCTTAACT-3'	1023 → 1044
Nuclear 18S rDNA amplicon A2	Medlin_B (R) ^c	5'-GATCCTTCTGCAGGTTACCTAC-3'	1816 → 1794

^a Léveillé et al. (2019b)^b Léveillé et al. (2014)^c Universal forward and reverse primers from Medlin et al. (1988)^d Ogedengbe (2015)**Table 4** Sequencing primers used for Sanger sequencing of the nuclear 18S ribosomal RNA sequence and the complete mitochondrial genome sequence of *Hepatozoon cf. griseisciuri* genotype B

Amplicon	Primer name	Primer sequence	Position from start
Mitochondrial amplicon B1	Api_LSUG_R ^a	5'-AGATAGGGAACAACTGYCTCAA-3'	5958 → 5980
	Hep_2112_F	5'-TTCGATGGAAAACCAGAACTAC-3'	150 → 171
	Api_Uni_COI_F	5'-TGGGCHCAYCAYATGATGAC-3'	871 → 890
	Api_LSUF_F ^a	5'-GTWCGCCGGGATAACAGGT-3'	2652 → 2633
	Api_LSUF_R ^a	5'-ACCTGTTATCCCCGGCGWA-3'	2633 → 2651
	Hep_SSUB_F	5'-AGCCTGGTGGTAGTGTCCAT-3'	3008 → 2989
	Adel_RNA15_F	5'-TWTGWAACACACTTCCCTTCT-3'	3016 → 3036
	Api_LSUE_R ^a	5'-AGGTGCTCAGGGTCTTACCG-3'	3598 → 3617
	Api_RNA17_R ^a	5'-ACCAGCCTGGGATCRWAA-3'	4038 → 4055
	Hep_COB_1F ^b	5'-TGGTTTATGACCGTCAGGCT-3'	4471 → 4490
	HepSq_B_RNA23t_F	5'-CTAGGCCAATAACCCAAATCTCTG-3'	5803 → 5780
Mitochondrial amplicon B2	Hep_CytB_391F	5'-CCWTGGGGTCAGATGAGTT-3'	4926 → 4944
	HepSq_B_COI_R	5'-AGCCATGATTCCAAATAGCAAC-3'	93 → 72
Nuclear 18S rDNA amplicon B1	Medlin_A (F) ^c	5'-AACCTGGTTGATCCTGCCAGT-3'	1 → 21
	18S_696R ^d	5'-TACGAGCYTTYTAAGTCAAC-3'	641 → 621
	Adel_18S_1522R ^a	5'-AYCCTATTTAGCAGGTTAAGGT-3'	1375 → 1354
Nuclear 18S rDNA amplicon B2	Adel_18S_1000F ^a	5'-AGATACCGTCGTAGTCTTAACT-3'	1023 → 1044
	Medlin_B (R) ^c	5'-GATCCTTCTGCAGGTTACCTAC-3'	1816 → 1794

^a Léveillé et al. (2019b)^b Léveillé et al. (2014)^c Universal forward and reverse primers from Medlin et al. (1988)^d Ogedengbe (2015)

unique sequence, genotype B (results not shown). The new primer HepSq_B_RNA23t_F was designed to target genotype B specifically; this new primer was paired with Api_LSUG_R to generate mitochondrial amplicon B1 covering the majority of the mitochondrial genome sequence (Table 2). After sequencing mitochondrial amplicon B1, primer HepSq_B_COIR was designed to target genotype B specifically and paired with Hep_CytB_391F to generate mitochondrial amplicon B2 that overlapped both ends of mitochondrial amplicon B1 and completed the genotype B mitochondrial genome sequence (Table 2).

The mitochondrial genotype of squirrel 3 was identified by targeting a region spanning several fragmented rDNA that exhibited considerable variability between the *H. griseisciuri* genotype A and *H. griseisciuri* genotype B mitochondrial genomes (genotype A, 793 bp; genotype B, 747 bp). This region was targeted with universal primers Api_LSUE_F and Haem_RNA14_F (primers cited in Table 3). No additional sequencing of the mitochondrial genome from this isolate was done.

PCR amplicon sequencing and analysis

The purified PCR amplicons generated were then sequenced in both directions with the forward and reverse amplification primers supplemented with internal sequencing primers as needed (Tables 3 and 4), with exception of the partial 18S rDNA amplicon generated from squirrel 2. The latter amplicon was sequenced unidirectionally with the Medlin_A (F) primer (primer cited in Table 1). All Sanger sequencing was accomplished using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems Inc., Foster City CA, USA) by the Molecular Biology Unit of the Laboratory Services Division, University of Guelph (Guelph ON, Canada). Chromatograms received from sequencing reactions were imported and assembled into contigs using the De Novo Sequence Assembler within Geneious (version 6.1 or later, www.geneious.com, Kearsse et al. 2012). All assembled amplicon contigs overlapped one another by at least 250 bp and overlapping regions showed 100% pairwise sequence identity in all cases.

Complete 18S rDNA sequences from genotype A and genotype B were searched against the public sequence databases using the BLAST algorithm (Altschul et al. 1990) to identify similar 18S rDNA sequences.

Annotation and analysis of protein-coding regions

Open reading frames (ORF) in the assembled mitochondrial genome were identified using an ORF search utility within Geneious using transl_table 4 (i.e., mold protozoan mitochondrial translation table). Translated ORFs (amino acid residue (AA) sequences) were searched against the public sequence

databases using the DELTA-BLAST algorithm (Altschul et al. 1990; Boratyn et al. 2012) to identify each ORF in relation to similar protein-coding sequences (CDS).

Annotation of mitochondrial ribosomal DNA sequences

The fragmented mitochondrial rDNA of *H. griseisciuri* genotype A and genotype B were annotated based on similarity to the fragmented rDNA previously annotated in *Plasmodium falciparum* (M76611; Feagin et al. 2012), *Hepatozoon catesbiana* (KF894962; Léveillé et al. 2014), *Klossiella equi* (MH203050; Léveillé et al. 2019b), and *Eimeria tenella* (HQ702484) mitochondrial genomes as described by Léveillé et al. (2014, 2019a, 2019b). Any rDNA that aligned with the *H. griseisciuri* mitochondrial sequence with a pairwise sequence identity below 65% were excluded from further analysis. Putative fragmented rDNA ribosomal annotations (Table 4) follow the nomenclature of Feagin et al. (2012) in their annotation of the mitochondrial genome of *P. falciparum*.

Pairwise comparison of *Hepatozoon cf. griseisciuri* genotype A and genotype B

Pairwise comparisons of 18S rDNA, whole mitochondrial genome sequences and orthologous CDS were analyzed in Geneious using the “Multiple Align” tool (cost matrix: 70%, gap open penalty: 12, gap extension penalty: 3). Pairwise comparisons of translated AA sequences from orthologous CDS were analyzed in Geneious using the “Multiple Align” tool (cost matrix: Blosum62, gap open penalty: 12, gap extension penalty: 3).

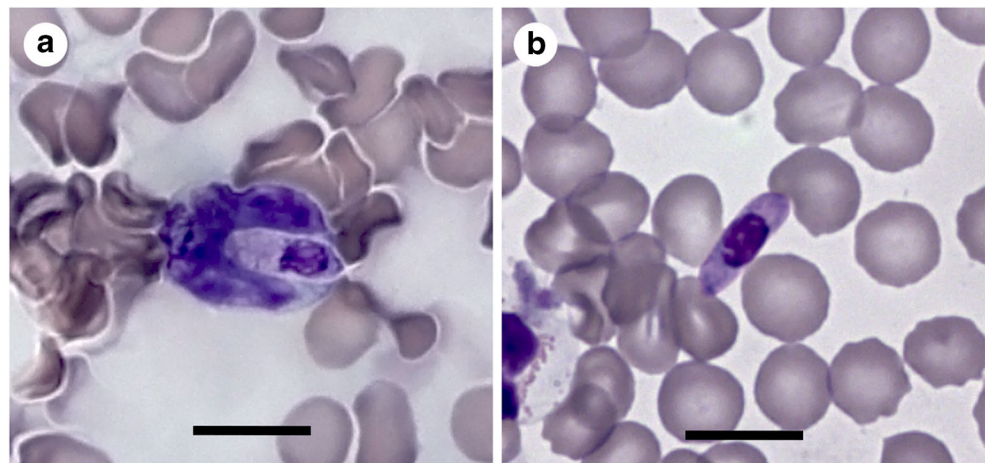
Results

Microscopic observations

Blood smears were not available for Squirrel 1 and therefore microscopic observations of gamonts were not possible. Stained thin blood smears collected from Squirrel 2 and Squirrel 3 contained infrequent *Hepatozoon cf. griseisciuri* gamonts found within blood cells that most resembled monocytes (Fig. 1). Gamonts observed from Squirrel 2 ($n = 8$) measured $10.4\text{--}11.4$ (10.9) $\mu\text{m} \times 3.5\text{--}5.0$ (4.1) μm with gamont nuclei measuring $4.2\text{--}6.9$ (5.4) $\mu\text{m} \times 2.7\text{--}4.1$ (3.1) μm . Gamonts observed from Squirrel 3 ($n = 9$) measured $9.3\text{--}11.3$ (10.6) $\mu\text{m} \times 3.2\text{--}5.0$ (3.9) μm with gamont nuclei measuring $3.9\text{--}7.0$ (5.0) $\mu\text{m} \times 2.5\text{--}3.6$ (3.1) μm .

There were no parasite stages observed from the histological sections of liver, heart, kidney, spleen, and lung tissues recovered from Squirrel 3.

Fig. 1 Micrographs of Giemsa's stained gamonts of *Hepatozoon* cf. *griseisciuri* in a thin blood film from eastern gray squirrel, *Sciurus carolinensis*, located within a monocyte (a) and free (b) in the blood. Scale bar = 10 μ m



Complete nuclear 18S ribosomal DNA sequences

The complete nuclear 18S rDNA sequence of *H. griseisciuri* genotype A amplified from squirrel 1 had a unit length of 1816 bp (MK452252). The complete nuclear 18S rDNA genotype B amplified from squirrel 3 also had a unit length of 1816 bp (MK452253). A pairwise comparison of the 2 sequences had a 98.8% pairwise identity and 22 single-nucleotide differences (SND). Both sequences generated similar BLAST search results with 98–99% pairwise identity (92–98% coverage) to several unnamed *Hepatozoon* sp. 18S rDNA sequences (AB181504, AY600626, FJ719818, FJ719816, JX644997, EF222259) collected from various rodent hosts and 99% pairwise identity (97% coverage) to a *Hepatozoon ayorgbor* 18S rDNA sequence (EF157822) collected from a ball python (*Python regius*).

The partial 18S rDNA amplicon generated from squirrel 2 (829 bp; sequence not published) had 19 ambiguous base calls (caused by multiple base signals contributing to the chromatogram at these sites). The 19 ambiguous base calls documented in the squirrel 2 partial 18S rDNA sequence corresponded to the SNDs observed between genotype A and genotype B 18S rDNA sequences. This confirmed that squirrel 2 was infected with both genotype A and genotype B of *H. griseisciuri* with no other genotypes detected.

Complete mitochondrial genome sequences

The complete mitochondrial genome of *H. griseisciuri* genotype A amplified from squirrel 1 had a unit length of 6311 bp with an overall G+C content of 39.2% (MK452388). The circular-mapping mitochondrial genome organization (Fig. 2) consisted of three protein-coding genes cytochrome *c* oxidase subunit I (COI), cytochrome *c* oxidase subunit III (COIII), and cytochrome *b* (CytB) (Table 5). The G+C content of these protein-coding genes was 37.6% for COI, 36.4% for COIII, and 37.0% for CytB. The complete mitochondrial genome of *H. griseisciuri* genotype B selectively amplified from squirrel 2 had a unit

length of 6114 bp with an overall G+C content of 38.6% (MK452389). The mitochondrial genome organization was nearly identical to that of genotype A and also contained three protein-coding genes (COI, COIII, and CytB); the G+C content of these protein-coding genes was 37.1% for COI, 34.4% for COIII, and 36.6% for CytB. As is typical of apicomplexan mitochondrial genomes, many putative fragmented rDNA were identified in both genome sequences: 14 fragmented large subunit (LSU) rDNA; 10 fragmented small subunit (SSU) rDNA; and 8 unassigned fragmented rDNA (Table 6). Each putative fragmented rDNA had a pairwise identity greater than 65% to a corresponding fragmented rDNA functionally annotated previously in the mitochondrial genome of *P. falciparum* (M76611; see Feagin et al. 2012). As observed in the mitochondrial genome of *K. equi* (Léveillé et al. 2019b), the sequence for LSUA was fragmented in 2 pieces with the start and the end of the gene occurring at different locations. The sequence identified as RNA16 overlapped with the 3'-end of the COI CDS in both genomes. Fragmented rDNA encoding RNA8, RNA11, and RNA12 in the *P. falciparum* mitochondrial genome were not identified in either of the mitochondrial genotypes from *Hepatozoon* cf. *griseisciuri* (Table 6).

The small (747 bp) mitochondrial genome target amplified from squirrel 3 using primers that target both genotypes had 100% pairwise identity to the genotype B sequence selectively amplified from squirrel 2. This sequence contained no ambiguous base calls (caused by competing chromatogram readings) indicating that the squirrel 3 sample contained genotype B exclusively.

Although genotype A and genotype B mitochondrial genomes had the same CDS and rDNA elements, they differed slightly in their organization. RNA2 mapped near the start of CytB in genotype A but was located upstream of COIII and reading in the opposite direction in genotype B (Fig. 2, Table 6). There was an 8-bp insert (TAAGTAAG; location: 1418–1425 bp) near the end of the genotype B COI CDS that resulted in a frameshift mutation and caused the COI CDS to be

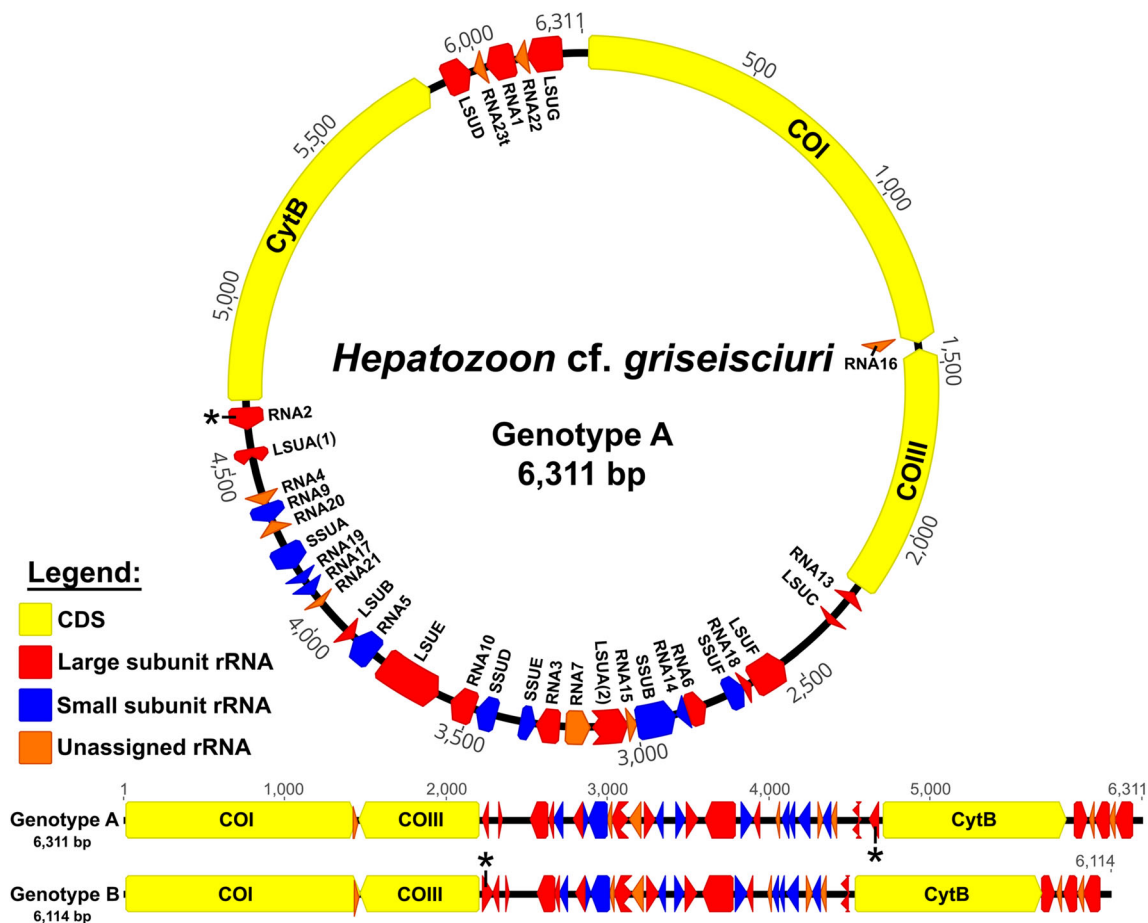


Fig. 2 Annotated complete mitochondrial genome sequences of *Hepatozoon cf. griseisciuri* genotype A (MK452388), illustrated in both circular and linear forms, and genotype B (MK452389), illustrated in linear form only. Three protein-coding regions (CDS; cytochrome *c* oxidase subunit I (COI), cytochrome *c* oxidase subunit III (COIII), and cytochrome *b* (CytB)) were encoded by the genotype A and genotype B mitochondrial genomes. Numerous putative fragmented ribosomal

RNA (rRNA) genes (rDNA) were identified in both genomes; fragmented rDNA nomenclature follows Feagin et al. (2012). The arrangements of CDS and fragmented rDNA were identical in the genotype A and genotype B mitochondrial genomes with the exception of RNA2 that was found in different locations on each (*). Initial genome images were produced with Geneious (www.geneious.com; Kearse et al. 2012)

longer than the genotype A COI CDS (Table 5). The genotype B COI CDS had a 27-bp overlap with the end of the COIII CDS.

There was also a 26-bp insert near the end of the genotype A CytB CDS that terminated the coding region earlier than the

Table 5 Features associated with the protein-coding regions of *Hepatozoon cf. griseisciuri* genotype A and genotype B mitochondrial genome sequences

Protein-coding region	Sequence size (bp)	Sequence position (bp)	Sequence pairwise identity	Translation start codon	Translation stop codon	Translation pairwise identity
Cytochrome <i>c</i> oxidase subunit I (COI)						
Genotype A	1422	16 → 1437	92.0%	TTA	TAG	98.3%
Genotype B	1473	16 → 1488		TTA	TAA	
Cytochrome <i>c</i> oxidase subunit III (COIII)						
Genotype A	741	2199 → 1459	89.9%	TTA	TAA	98.4%
Genotype B	741	2202 → 1462		TTA	TAA	
Cytochrome <i>b</i> (CytB)						
Genotype A	1134	4707 → 5840	90.5%	TTA	TAG	95.8%
Genotype B	1158	4536 → 5693		TTA	TAG	

Table 6 Annotation of fragmented ribosomal RNA (rRNA) genes (rDNA) encoded in the mitochondrial genomes of *Hepatozoon cf. griseisciuri* genotype A and genotype B based on pairwise comparisons with conserved annotated genes from *Plasmodium falciparum* (M76611, see Feagin et al. 2012), *Hepatozoon catesbiana* (KF894962), *Klossiella equi* (MH203050), and *Eimeria tenella* (HQ702484)

Fragmented rDNA ^a	rRNA assignment ^b	Position in genotype A (bp)	Position in genotype B (bp)	Sequence length (bp)
SSUA	SSU	4252 → 4181	4184 → 4113	72 (partial)
SSUB	SSU	2998 → 2885	3013 → 2900	114
SSUD	SSU	3422 → 3482	3414 → 3474	61
SSUE	SSU	3344 → 3303	3358 → 3317	42
SSUF	SSU	2719 → 2665	2753 → 2699	55
LSUA	LSU	4559 → 4519	4492 → 4452	41
		3130 → 3027	3145 → 3042	104
LSUB	LSU	3941 → 3908	3898 → 3865	34
LSUC	LSU	2324 → 2345	2367 → 2388	22
LSUD	LSU	5893 → 5972	5693 → 5772	80
LSUE	LSU	3793 → 3604	3777 → 3588	190
LSUF	LSU	2630 → 2522	2673 → 2565	109
LSUG	LSU	6252 → 6147	6054 → 5949	106
RNA1	LSU	6108 → 6025	5910 → 5827	84
RNA2	LSU	4681 → 4620	2227 → 2288	62
RNA3	LSU	3230 → 3296	3246 → 3311	67(A)/66(B)
RNA4	–	4421 → 4384	4354 → 4317	38 (partial)
RNA5	SSU	3828 → 3902	3789 → 3863	75
RNA6	LSU	2843 → 2788	2858 → 2803	56
RNA7	–	3208 → 3138	3220 → 3149	71(A)/72(B)
RNA8	SSU	Not found	Not found	–
RNA9	SSU	4386 → 4335	4319 → 4268	52
RNA10	LSU	3489 → 3560	3481 → 3552	72
RNA11	LSU	Not found	Not found	–
RNA12	SSU	Not found	Not found	–
RNA13	LSU	2257 → 2228	2322 → 2293	30
RNA14	SSU	2848 → 2879	2863 → 2894	32
RNA15	–	3025 → 2999	3040 → 3014	27
RNA16	–	1420 → 1448	1428 → 1456	29
RNA17	SSU	4120 → 4083	4057 → 4020	38
RNA18	LSU	2664 → 2640	2698 → 2674	25
RNA19	SSU	4156 → 4127	4094 → 4065	30
RNA20	–	4304 → 4338	4236 → 4271	35(A)/36(B)
RNA21	–	4064 → 4043	4015 → 3994	22
RNA22	–	6148 → 6113	5950 → 5915	36
RNA23t	–	6014 → 5984	5816 → 5786	31

^a Nomenclature follows Feagin et al. (2012)

^b Based on assignment by Feagin et al. (2012)

genotype B CytB CDS (Table 5). Pairwise alignment of genotype A and genotype B complete mitochondrial genome sequences had a pairwise identity of 89.1%. Pairwise identity values of protein CDS and translation alignments are shown in Table 5.

When searched against public databases using the DELTA-BLAST algorithm (Altschul et al. 1990; Boratyn et al. 2012), the translation predictions of mitochondrial protein-coding sequences from *H. griseisciuri* genotype A and genotype B were found to be similar to the few currently published adeleorinid and various hemosporinid mitochondrial genome sequences (see Table 7).

Discussion

We report the first molecular data (complete nuclear 18S rDNA and the complete mitochondrial genome) from

Hepatozoon spp. that is consistent with the only *Hepatozoon* species described to infect the eastern gray squirrel, *Hepatozoon griseisciuri* Clark, 1958. The pairwise differences observed between 18S rDNA and mitochondrial genome sequences of genotype A and genotype B indicate that these genotypes likely represent two different *Hepatozoon* spp. infecting eastern gray squirrels in Ontario. The 0.08 divergence at the COI locus versus the 0.012 divergence between 18S rDNA sequences observed in the present study supports the use of the former genetic locus for species-level analyses, particularly given the proclivity of 18S rDNA in the nuclear genomes of apicomplexan parasites to have multiple paralogous copies (see Léveillé et al. 2019a). The superiority of COI sequences over nuclear 18S rDNA for species delimitation has been demonstrated previously for eimeriid coccidia (Ogedengbe et al. 2011); drawing the same conclusion for adeleorinid coccidia will require additional sequence data from the mitochondrial genomes of these parasites. Pending

Table 7 Translation predictions of mitochondrial protein-coding sequences from *Hepatozoon* cf. *griseisciuri* genotype A and genotype B were searched against public databases using the DELTA-BLAST algorithm (Altschul et al. 1990; Boratyn et al. 2012)

Translated protein-coding sequences	DELTA-BLAST search results		
Cytochrome <i>c</i> oxidase subunit I (COI)	<i>H. catesbiana</i> e (AIG55088)	<i>K. equi</i> (AZL31646)	Various hemosporinid species
Genotype A	96.4%	77.1%	66–67%
Genotype B	95.5%	76.8%	
Cytochrome <i>c</i> oxidase subunit III (COIII)	<i>H. catesbiana</i> e (AIG55090)	<i>K. equi</i> (AZL31645)	Various hemosporinid species
Genotype A	90.7%	52.9%	45–49%
Genotype B	90.2%	53.3%	
Cytochrome <i>b</i> (CytB)	<i>H. catesbiana</i> e (AIG55089)	<i>K. equi</i> (AZL31647)	Various hemosporinid species
Genotype A	95.9%	66.4%	58–61%
Genotype B	95.6%	65.8%	

further investigations on the biology and lifecycles of these parasites, we have taken the position that the two genotypes described herein should both retain the name of *Hepatozoon* cf. *griseisciuri*. Only after their biologies can be linked unequivocally with a specific genotype can one, both, or neither genotype be associated unequivocally with *H. griseisciuri*. The presence of both *H. griseisciuri* genotypes infecting squirrel 2 reveal that these related species are capable of infecting the same intermediate host concurrently.

The morphometrics of gamonts within monocytes from squirrel 2 (genotype A and genotype B) and squirrel 3 (genotype B) were not significantly different from one another (data not shown) and both corresponded to the dimensions ($9.9\text{--}11.8 \times 3.3\text{--}4 \mu\text{m}$; nuclei, $4\text{--}5.2 \times 3.3 \mu\text{m}$ ($n = 5$)) provided by Clark (1958) in his description of *H. griseisciuri*. No merogonic stages were observed histologically in the tissues collected from squirrel 3.

Searching the 18S rDNA sequences of *H. griseisciuri* genotype A and genotype B against public databases using the BLAST algorithm (Altschul et al. 1990) confirmed that there are several unnamed *Hepatozoon* spp. sampled from various rodent intermediate hosts worldwide with high pairwise sequence identities (98–99%). Surprisingly, the 18S rDNA sequences from *H. griseisciuri* also had high pairwise sequence identity (99%) to an 18S rDNA sequence from *H. ayorgbor* infecting a ball python. *Hepatozoon ayorgbor* was demonstrated experimentally to have a 3-host lifecycle involving a mosquito definitive host, a rodent first intermediate host, and a serpent second intermediate host (Sloboda et al. 2007, 2008).

The complete mitochondrial genomes of the *H. griseisciuri* (genotype A and genotype B) infecting the eastern gray squirrel were structurally similar to the majority of described mitochondrial genomes from the Apicomplexa: circular mapping; 3 CDS (COI, COIII, CytB); and multiple fragmented small and large subunit rDNAs (e.g., Hikosaka et al. 2011a, 2013; Feagin et al. 2012; Léveillé et al. 2014, 2019b). As with many apicomplexan mitochondrial genomes, the physical form of these circular-mapping genomes of these *H. griseisciuri* genotypes could be either linear concatemers of several to many

genome copies or single physically circular genomes (see Hikosaka et al. 2013).

Beyond basic gene content comprising each of the mitochondrial genomes, the organization of those genes in the *H. griseisciuri* (genotype A and genotype B) mitochondrial genomes was unique among the Apicomplexa, including mitochondrial genomes from other adeleorinid coccidia (Léveillé et al. 2014, 2019a, b) and, surprisingly, the two genotypes sequenced in the present study had minor differences in organization. This is in contrast to the usual conservation of mitochondrial genome organization (i.e., the order and orientation of these genome components) observed for members within each of several major apicomplexan groups (e.g., the hemosporinids (see Hikosaka et al. 2011b) or eimeriids) but not between major groups. Among eimeriid coccidia, the mitochondrial genome organization and content is virtually identical among 5 different genera (i.e., the genera *Eimeria*, *Isospora*, *Caryospora*, *Cyclospora*, and *Lankesterella* (see Ogedengbe 2015)); the degree of reorganization observed between the two *Hepatozoon* genotypes in the present paper exceeded the variation reported among these eimeriid parasites. Among other sequenced apicomplexan mitochondrial genomes, only the piroplasms (*Babesia* spp. and *Theileria* spp.) seem to display a diversity of mitochondrial genome structures within single genera (Hikosaka et al. 2010).

Despite the observed organizational diversity, the mitochondrial genomes of *Hepatozoon* cf. *griseisciuri* did share similarities with those of *H. catesbiana*e and *K. equi*. The mitochondrial genome of genotype A had a 76-bp repeat region from 21-bp upstream to 55 bp into the CDS of both COIII and CytB, and the genotype B mitochondrial genome had an 85 bp from 30-bp upstream to 55 bp into the CDS of both COIII and CytB. Similar repeated regions were observed at the starts of the COIII and CytB CDS of *H. catesbiana*e (70 bp; see Léveillé et al. 2014) and *K. equi* (107 bp; see Léveillé et al. 2019b). In all cases, these repeated stretches did not seem to alter the predicted transmembrane folding structures of the resulting translated AA sequences (not shown). There was also a common cluster of fragmented

rDNA that were conserved among the genomes. A cluster of fragmented rDNA (LSUD-RNA23t-RNA1-RNA22-LSUG) was conserved in *H. griseisciuri*, *H. catesbiana*, and *K. equi* mitochondrial genomes. Further, the mitochondrial genome of *H. catesbiana* shared several long stretches of fragmented rDNA (LSUC-LSUF-RNA18-SSUF; RNA6-RNA14-SSUB-RNA15-LSUA(2)-RNA7; RNA3-SSUD-RNA10-LSUE; RNA20-RNA9-RNA4-LSUA(1)-CytB) with the two *H. griseisciuri* genotypes. Some of these elements (SSUF, LSU, LSUC, RNA14, SSUB) clustered closely together in the mitochondrial genome of *K. equi*, but were not in the same arrangement. This suggests that fewer rearrangement events have occurred within the mitochondrial genomes of *H. griseisciuri* and *H. catesbiana* compared with the mitochondrial genome of *K. equi*. These similarities and the higher pairwise identities of the nuclear 18S rDNA and mitochondrial CDS sequences of *H. catesbiana* and *H. griseisciuri* compared with *K. equi* reinforces that these *Hepatozoon* spp. are more closely related to one another than to *K. equi*.

The mitochondrial genomes of *Hepatozoon* spp. sequenced to date display staggering diversity (see Chapter 8 of Léveillé 2019). This is reinforced by the apparent absence of a complete mitochondrial genome sequence in *Hepatozoon canis* (see Léveillé et al. 2019a). While we have only begun to scratch the surface of this diversity, observations to date suggest that taxa currently in the genus *Hepatozoon* are likely distributed into multiple genera that have yet to be defined. The likely necessity to split the genus *Hepatozoon* has been recognized previously (see Smith and Desser 1997). Karadjian et al. (2015) started the required taxonomic revisions based on a combined analysis of the biology of multiple hemogregarines (sensu lato) and a large-scale phylogenetic analysis of adeleorinid 18S rDNA sequences. As a result, Karadjian et al. (2015) described a new genus, *Bartazoon*, to hold all members of the “hemogregarines type 4” clade in their phylogenetic tree. Our data suggest that their proposed taxonomic solution does not sufficiently address the breadth of diversity observed among these heteroxenous adeleorinid parasites; the number of genera may need to be increased to accommodate this diversity.

Our preliminary analysis of the new 18S rDNA sequences obtained herein suggests that *H. griseisciuri* (genotype A and genotype B) would also cluster within the “hemogregarines type 4” clade of Karadjian et al. (2015) (data not shown, see Figure 8.2 from Léveillé 2019). The biology of *H. griseisciuri* involves a rodent intermediate host and mite definitive host with transmission presumably via ingestion of mites containing sporulated oocysts (Clark 1958; Redington and Jachowski 1971). Although *Hepatozoon* species have widely varying lifecycles in a range of biologically diverse definitive and intermediate hosts (Smith 1996; Karadjian et al. 2015), the biology of *H. griseisciuri* (see Clark 1958) is most similar to the *Hepatozoon* species used to define the genus *Hepatozoon*

by Miller (1908), with which it shares both geography (eastern North America) and a definitive acarine host, at least experimentally (Clark 1958). If the adeleorinid nuclear and mitochondrial genotypes explored in the present paper are truly congeneric with the type species of the genus *Hepatozoon* as described by Miller (1908); then, the assignment of all species that clustered in “hemogregarines type 4” to the genus *Bartazoon* by Karadjian et al. (2015) becomes problematic. This was supported by the conclusions of Maia et al. (2016) that reinforced the need for a systematic revision of the Adeleorina but warned that it would be preferable to wait to name distinct lineages formally until additional molecular data became available, particularly sequences from the currently accepted type species, *H. muris*.

The amplification and sequencing of partial 18S rDNA and complete mitochondrial genome sequences from *Hepatozoon* cf. *griseisciuri* uncovered two, genotypically distinct *Hepatozoon* species found infecting eastern gray squirrels both singly and concurrently; this unexpected genetic diversity foreshadows the diversity among adeleorinid blood parasites that remains to be uncovered. This reinforces that the taxonomic revision of this group continues to be complicated by the comparable lack of molecular data linked to well-defined biological characteristics (Smith 1996). The majority of adeleorinid species have only ever been observed in one (of perhaps several) intermediate host and, frequently, solely from the easily observable stage in the lifecycle, the intracellular gamont. Moreover, the definitive hosts, within which the majority of the biologically distinctive lifecycle stages (gametogonic and sporogonic) are found, can be challenging to collect and are labor intensive to study in the laboratory. As sequences from multiple genetic loci from adeleorinid parasites become increasingly available, these molecular markers can be applied to link morphological and biological observations among the various hosts and tissues infected during these complex heteroxenous lifecycles. Only then will we be able to begin to understand the biology and evolutionary relationships among these diverse adeleorinid taxa.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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