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PHYLOGENETIC ANALYSIS OF *HEPATOZOON* SPECIES (APICOMPLEXA: ADELEORINA) INFECTING FROGS OF NOVA SCOTIA, CANADA, DETERMINED BY ITS-1 SEQUENCES

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ABSTRACT: Species of *Hepatozoon* are apicomplexan parasites infecting tetrapod vertebrates and hematophagous arthropods. Two species, *Hepatozoon catesbianae* and *Hepatozoon clamatae*, have been described inhabiting the erythrocytes of bullfrogs and green frogs. A number of characteristics typically used to distinguish between members of this genus are shared between these 2 species, prompting speculation as to whether or not these organisms are in fact distinct species. To test the species distinction, bullfrogs and green frogs were captured at various sites across Nova Scotia, blood samples were collected, and DNA was extracted from samples containing parasites. The internal transcribed spacer 1 (ITS-1) from geographically diverse samples of both species was amplified by PCR, sequenced, and analyzed. ITS-1 sequences from the 2 species revealed single-nucleotide polymorphisms at 6 sites. Phylogenetic analysis of these molecular data and cytopathological features place isolates of each species in separate monophyletic groups. Comparison of the ITS-1 sequences between isolates from Nova Scotia and Ontario revealed that ITS-1 sequences of *H. catesbianae* from a previous study were mischaracterized as being those of *H. clamatae*. Phylogenetic data based on molecular variation and cytopathological features from this study provide the strongest evidence to date supporting the distinction between these 2 species.

Species of *Hepatozoon* are apicomplexan parasites that infect the viscera and blood of a wide range of vertebrates including amphibians, mammals, reptiles, and birds, which serve as intermediate hosts, and a variety of hematophagous arthropods including mosquitoes, ticks, mites, and flies, which serve both as vectors and as definitive hosts of the parasite (Smith, 1996). *Hepatozoon* species are haemogregarines, an informal grouping of 6 genera of adeleorin Apicomplexa that infect the erythrocytes of their vertebrate hosts and share many morphological and life cycle traits. Morphologically, the intraerythrocytic gamonts of one *Hepatozoon* species often bear a strong resemblance to those of other species of the genus, as well as those of species of other haemogregarine species. However, these parasites exhibit complex life cycles in 2 or more hosts. Thus, life cycle characteristics, such as the location of sporogony within the definitive host and features of the cystic stages, are critical in determining the systematics of these parasites (Siddall, 1995; Smith, 1996).

Stebbins described *Haemogregarina catesbianae* (1903) and *Karyolysus clamata* (1905) in the blood of bullfrogs, *Rana catesbeiana*, and green frogs, *Rana clamitans*, respectively, in New York. Upon experimental determination of the life cycle of *Haemogregarina catesbianae* in ranid frogs and in the mosquito, *Culex territans*, the species was redescribed as *Hepatozoon catesbianae* based on shared life cycle characteristics with fully described *Hepatozoon* species (Desser et al., 1995). Smith (1996), in his revision of *Hepatozoon*, reclassified *Haemogregarina clamatae* (Lehmann, 1960), a redescription of *Karyolysus clamata*, as *Hepatozoon clamatae*. Determination of the life cycle of *H. clamatae* (Kim et al., 1998) supported this reclassification, revealing this species to exhibit a life history characteristic of *Hepatozoon*.

The study by Kim et al. (1998) revealed a number of similarities between *H. clamatae* and *H. catesbianae*. Both species infect the same hosts, have the same host ranges, and exhibit highly similar life cycles. Traits normally used to distinguish among *Hepatozoon* species were found to be indistinguishable between these 2 species. Analysis of the internal transcribed spacer 1 (ITS-1) sequence from isolates of both species in Ontario did not reveal any shared nucleotide polymorphisms that could serve to distinguish between the 2 (Kim et al., 1998).

Kim et al. (1998) maintained the distinction between these species on the basis that gamonts of *H. clamatae* fragment the nuclei of their host erythrocytes, a character not seen in erythrocytes infected with *H. catesbianae* (Kim et al., 1998). Other traits used to support this distinction include the inability of *H. catesbianae* to infect leopard frogs (*Rana pipiens*) and that *H. clamatae* exhibited a higher parasitemia in green frogs than in bullfrogs, whereas the opposite was seen for *H. catesbianae* (Kim et al., 1998).

However, a number of factors raise doubts about the species distinction, most notably the reliability of using cytopathological features alone to differentiate among the species. ITS-1, a noncoding spacer region located between the 18S and 5.8S rRNA genes, has been used previously for comparative studies among apicomplexans including species of *Eimeria* (Lew et al., 2003; Su et al., 2003), *Toxoplasma*, *Neospora* (Homan et al., 1997), and *Hepatozoon* (Smith et al., 1999). Because ITS-1 sequences have been successfully used in the past to distinguish between closely related species, the absence of synapomorphies in the ITS-1 sequences between *H. catesbianae* and *H. clamatae* in the study by Kim et al. (1998) undermines the current species distinction.

In the present study, we evaluate the geographic distribution and prevalence of these 2 *Hepatozoon* species infecting frogs in Nova Scotia. In addition, the species distinction between these 2 parasites is reevaluated using the ITS-1 sequences of Nova Scotian isolates of both species to test the hypothesized species distinction maintained by Kim et al. (1998). Prevalence of the parasites among their 2 primary vertebrate hosts, green frogs and bullfrogs, was also analyzed for comparison with the host affinity observations made by Kim et al. (1998).

MATERIALS AND METHODS

Six species of ranid frogs (green frog, *R. clamitans*; bullfrog, *R. catesbeiana*; leopard frog, *R. pipiens*; wood frog, *Rana sylvatica*; pickerel frog, *Rana palustris*; mink frog, *Rana septentrionalis*) were captured from various aquatic habitats across Nova Scotia between June and August 2005 (Fig. 1; Table I). Approximately 20–40 μl of blood was collected on site from frogs by quick insertion and withdrawal of a 27-

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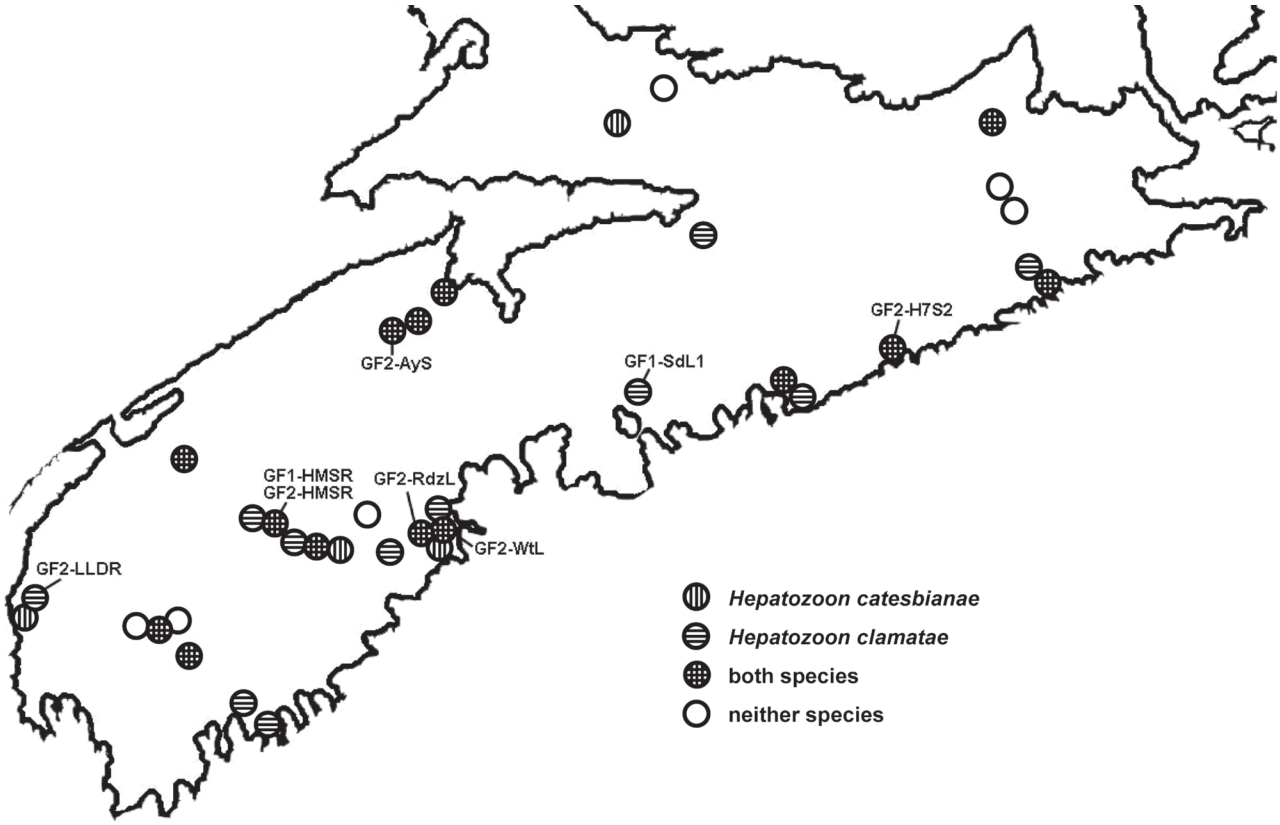

FIGURE 1. Map of the prevalence of *Hepatozoon* species infecting frogs in Nova Scotia. Circles with horizontal hatching indicate the presence of *Hepatozoon clamatae*, circles with vertical hatching indicate the presence of *Hepatozoon catesbianae*, and circles that are hatched both vertically and horizontally are indicative of the presence of both species (in the same region or even the same frog). White circles represent sites where *Hepatozoon* infections were not found in any frogs sampled. Locations of the 3 *H. catesbianae* isolates (GF2-WtL, GF2-AyS, GF2-H7S2) and five *H. clamatae* isolates (GF1-HMSR, GF2-HMSR, GF2-LLDR, GF2-RdzL, GF1-SdL1) used for ITS-1 sequencing analysis are indicated on the map.

gauge needle through the skin into the maxillary vein between the upper jaw line and the anterior side of the tympanum. The blood that welled up was drawn into 2 heparinized hematocrit tubes. The contents of 1 hematocrit tube containing 10–20 μl of blood were transferred into a vial. Sample vials were kept in an insulated container at 0 C until they were transferred to a −20 C freezer within 12 hr. The contents of the second hematocrit tube, containing 10–20 μl of blood, were transferred onto a glass microscope slide and smeared to form a thin blood film. Blood smears were stained using Diff-Quik® (Dade Behring, Düdingen, Switzerland). Frogs were treated with Bactine® antiseptic at the puncture site and released. All frogs were handled and sampled according to guidelines approved by the Animal Care Committee of Acadia University.

Blood smears from all samples were examined using bright-field microscopy and analyzed for intraerythrocytic gamonts of *Hepatozoon* species (Fig. 2). Distinction between the presence of gamonts of *H. catesbianae* and *H. clamatae* was made on the basis of nuclear disruption of host erythrocytes as described by Kim et al. (1998).

Infection patterns among and within host species were compared using contingency table analysis in SAS (SAS Institute, Inc., 2000). A chi-square test was used for analysis except for analyses where expected values for any cells of the table were less than 5, in which case a Fisher exact test was used.

DNA from 8 geographically diverse samples of green frog blood positive for *Hepatozoon* spp. were isolated using a Qiagen DNeasy® DNA extraction kit (Mississauga, Ontario, Canada). Three samples of *H. catesbianae* (GF2-AyS, GF2-WtL, and GF2-H7S2) and 5 samples of *H. clamatae* (GF1-HMSR, GF2-HMSR, GF1-SdL1, GF2-RdzL, and

TABLE I. Prevalence of infections of *Hepatozoon* species in sampled frogs.

Host species	*Hepatozoon catesbianae*	*Hepatozoon clamatae*	Mixed infection	Not infected	Total
Green frog (*Rana clamitans*)	9	41	11	8	69
Bullfrog (*Rana catesbeiana*)	3	1	0	13	17
Leopard frog (*Rana pipiens*)	0	0	0	1	1
Mink frog (*Rana septentrionalis*)	0	0	0	1	1
Wood frog (*Rana sylvatica*)	0	0	0	1	1
Pickerel frog (*Rana palustris*)	0	0	0	2	2
Total	12	42	11	26	91

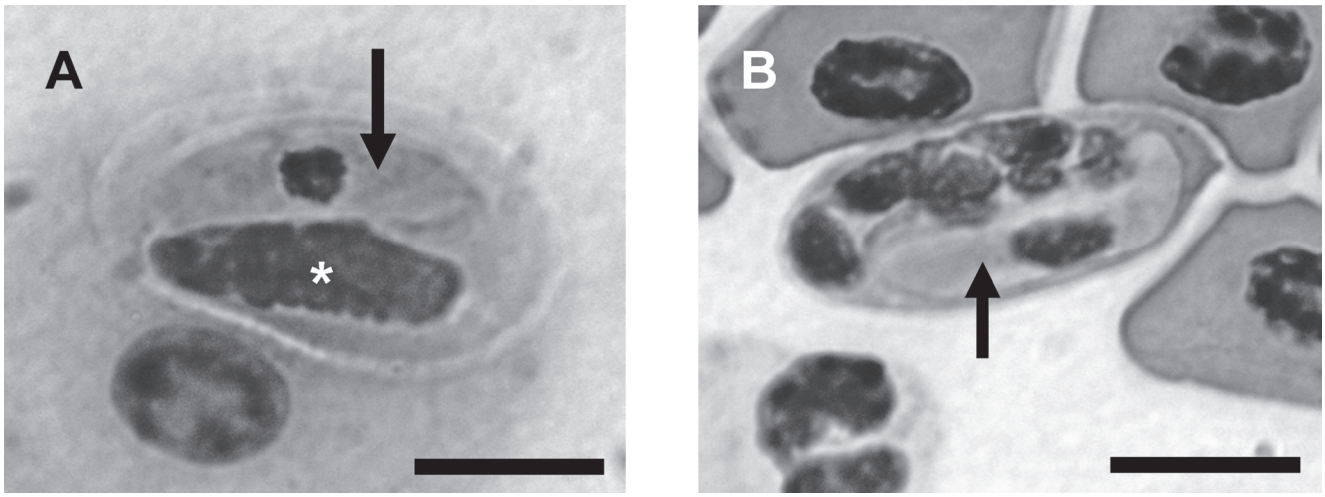

FIGURE 2. Gamonts of *Hepatozoon* species inhabiting erythrocytes of the green frog, *Rana clamitans*. (**A**) Gamont of *Hepatozoon catesbianae* (arrow) from the GF2-WtL isolate, used for sequencing analysis, inside a green-frog erythrocyte. The host nucleus is displaced, but remains intact (asterisk). (**B**) Gamont of *Hepatozoon clamatae* (arrow) from the GF2-RdzL isolate, used for sequencing analysis, inside a green-frog erythrocyte. Note the dark-staining fragments of the host nucleus surrounding the gamont. Scale bar = 10 μm.

GF2-LLDR) were selected for molecular analysis. All samples were pure infections of 1 species of parasite except for GF2-AyS (~70% *H. catesbianae* and ~30% *H. clamatae*) and GF2-H7S2 (~63% *H. catesbianae* and ~37% *H. clamatae*).

Specific primers were synthesized by Operon Biotechnologies, Inc. (Huntsville, Alabama) and designed from known *Hepatozoon* spp. ITS-1 sequences (Smith et al., 1999). The forward primer, herpfor (5′-GCGGAAGGATCATTCACATT-3′), was located in the 18S rRNA sequence upstream from ITS-1 and the reverse primer, herprev (5′-TC CTTCATCGATGCACAAAC-3′) was located in the 5.8S rRNA sequence downstream from ITS-1. PCR reactions were done in 25-μl volumes and comprised 1 unit of *Taq* DNA polymerase, 1.25 μl of 10 mM herpfor primer, 1.25 μl of 10 mM herprev primer, 2.0 μl of 0.2 mM dNTP solution, 1.5 μl of 50 mM $MgCl_2$, 2.5 μl of 10× PCR buffer solution, 13.3 μl of ddH_20, and 3.0 μl of template DNA. Solutions were heated to 94 C for 3 min, then subjected to 44 amplification cycles (94 C for 1 min, 56 C for 1 min, 72 C for 2 min) followed by a final extension at 72 C for 10 min.

PCR products were electrophoresed in a 0.7% agarose gel and bands putatively containing ITS-1 (~200 base pairs) were cut from the gel and extracted using a Qiagen MinElute® gel extraction kit. Extracted PCR products were sequenced at the University of Maine Sequencing Center.

Sequences were edited using Sequence Navigator, version 1.0 (Applied Biosystems, Foster City, California) and aligned by eye using SeAl, version 2.0a11 (Rambaut, 1996). The ITS-1 sequences from Nova Scotia isolates were aligned with those of 4 *H. clamatae* isolates (SM4, SM7, S34, G20) and 2 *H. catesbianae* isolates (GF8, B3E) from Ontario (Kim et al., 1998) for comparison along with those of 2 isolates of *Hepatozoon sipedon* from Ontario (Smith et al., 1999) for use as outgroups (Fig. 3). The cytopathological character of nuclear disruption of host erythrocytes was also included in the data matrix. Parsimony trees were then generated using PAUP, version 4.0 (Swofford, 2001).

RESULTS

Prevalence of *H. catesbianae* and *H. clamatae*

Gamonts of both *H. catesbianae* and *H. clamatae* were found in blood films from sampled frogs (Fig. 2; Table I). Gamonts of *H. catesbianae* were found infecting erythrocytes in 3 of 17 (17.6%) bullfrogs and in 20 of 69 (29.0%) green frogs. Gamonts of *H. clamatae* were found infecting erythrocytes in 1 of 17 (5.9%) bullfrogs and in 52 of 69 (75.4%) green frogs. Of the 69 green frogs, 11 were found to be infected by gamonts of both species. Mixed infections were not observed in any bullfrogs. Infections with either species of *Hepatozoon* were more common among green frogs (88.4%) than among bullfrogs (23.5%). Gamonts of *Hepatozoon* species were not found in the blood of any of the mink, wood, leopard, or pickerel frogs sampled. Infections of *H. clamatae* were much more common among green frogs than were infections of *H. catesbianae* ($\chi^2 = 6.3$, $P = 0.01$). Infected bullfrogs were not numerous enough for statistical analysis, but the observation that of the 4 infected frogs, 3 were infected with *H. catesbianae* and 1 with *H. clamatae* suggests that an *H. catesbianae* infection bias could be verified by more sampling. Analysis of the pattern of *Hepatozoon* spp. infections among all infected frogs found the bias to be statistically significant as well (Fisher exact test, $P = 0.04$).

Geographically, both species of *Hepatozoon* were found sympatrically across Nova Scotia (Fig. 1). Both species of *Hepatozoon* were found in the same geographical areas, in the same bodies of water, and, in some cases, in the same frog (Table I).

Comparison of ITS-1 sequences among *Hepatozoon* isolates of Nova Scotia

Aligned sequences were 230 base pairs in length following insertions to account for the longer ITS-1 sequences of the outgroups used for phylogenetic analysis (Fig. 3). Comparisons among the 8 sequences showed 6 nucleotide polymorphisms that provided useful characters for determining relationships among *Hepatozoon* species in infected frogs (Fig. 3). These 6 nucleotide polymorphisms, accounting for a 4.7% difference in the ITS-1 sequence, were shared among isolates of the same species.

Comparison of ITS-1 sequences among isolates of *Hepatozoon* species

Analysis using PAUP version 4.0 generated 2 most-parsimonious trees. A strict consensus of the 2 trees revealed 2 sister clades. One clade comprises all Nova Scotia *H. clamatae* and

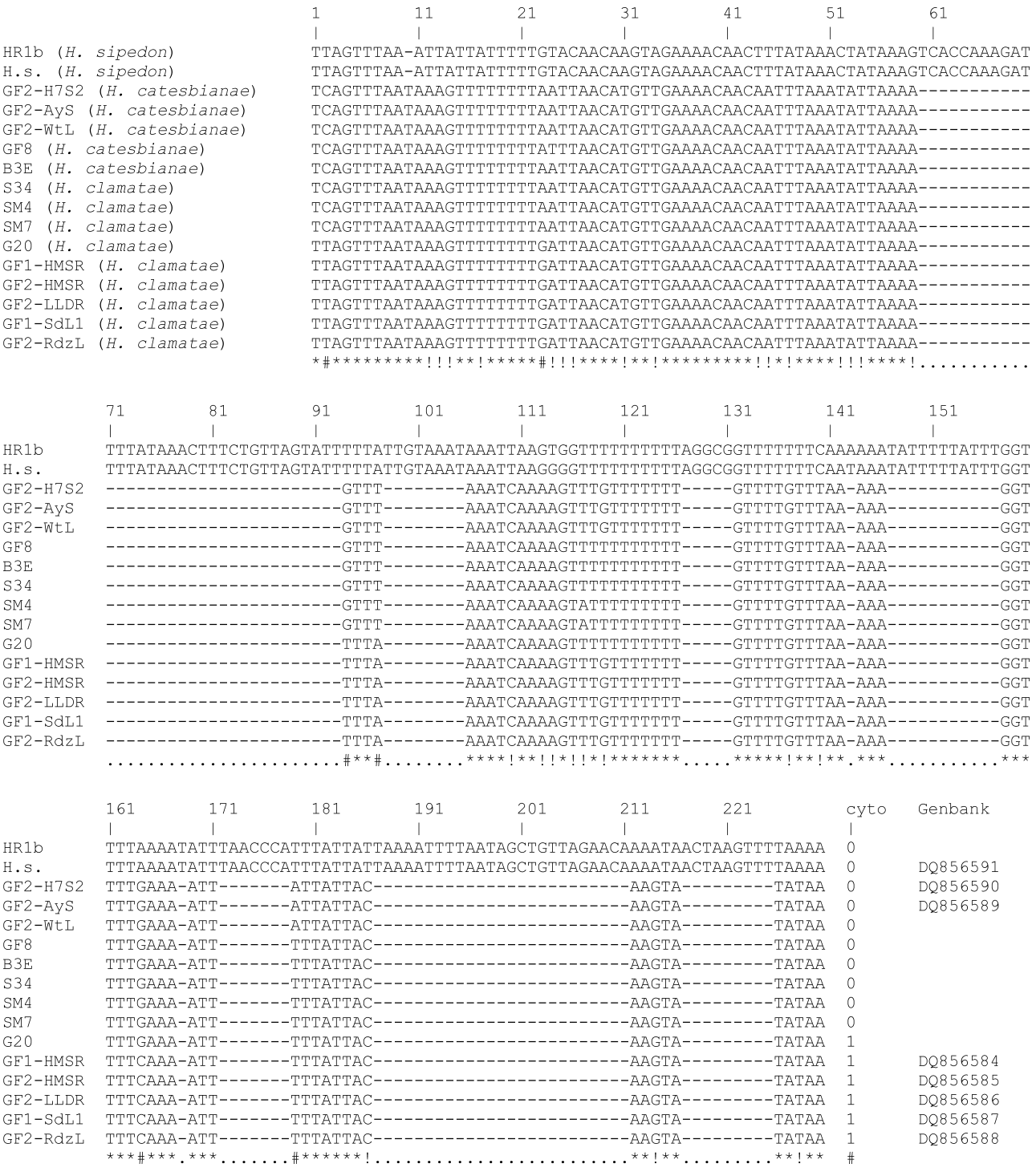

FIGURE 3. Alignments of ITS-1 sequences of *Hepatozoon* species. The ITS-1 sequences of the out-group species, *Hepatozoon sipedon*, are considerably longer than those of *Hepatozoon catesbianae* and *Hepatozoon clamatae*, and therefore gaps are present. The 6 variable sites, located at sites 2, 23, 94, 97, 164, and 179, that were found to be informative for distinguishing between *H. catesbianae* and *H. clamatae* are denoted at the base of the alignments by '#', whereas other polymorphisms are denoted by '!'. Sites that are invariant among all sequences are indicated by '*', and indels between snake isolates and frog isolates are denoted by '•'. The morphological character of nuclear disruption of host erythrocytes, indicated as 'cyto' in the figure, is also included, as it is used in the phylogenetic analysis (Fig. 4). In this case, '0' represents a lack of cytopathology, whereas '1' indicates that cytopathology is present. GenBank accession numbers for novel ITS-1 sequences of Nova Scotia isolates are listed.

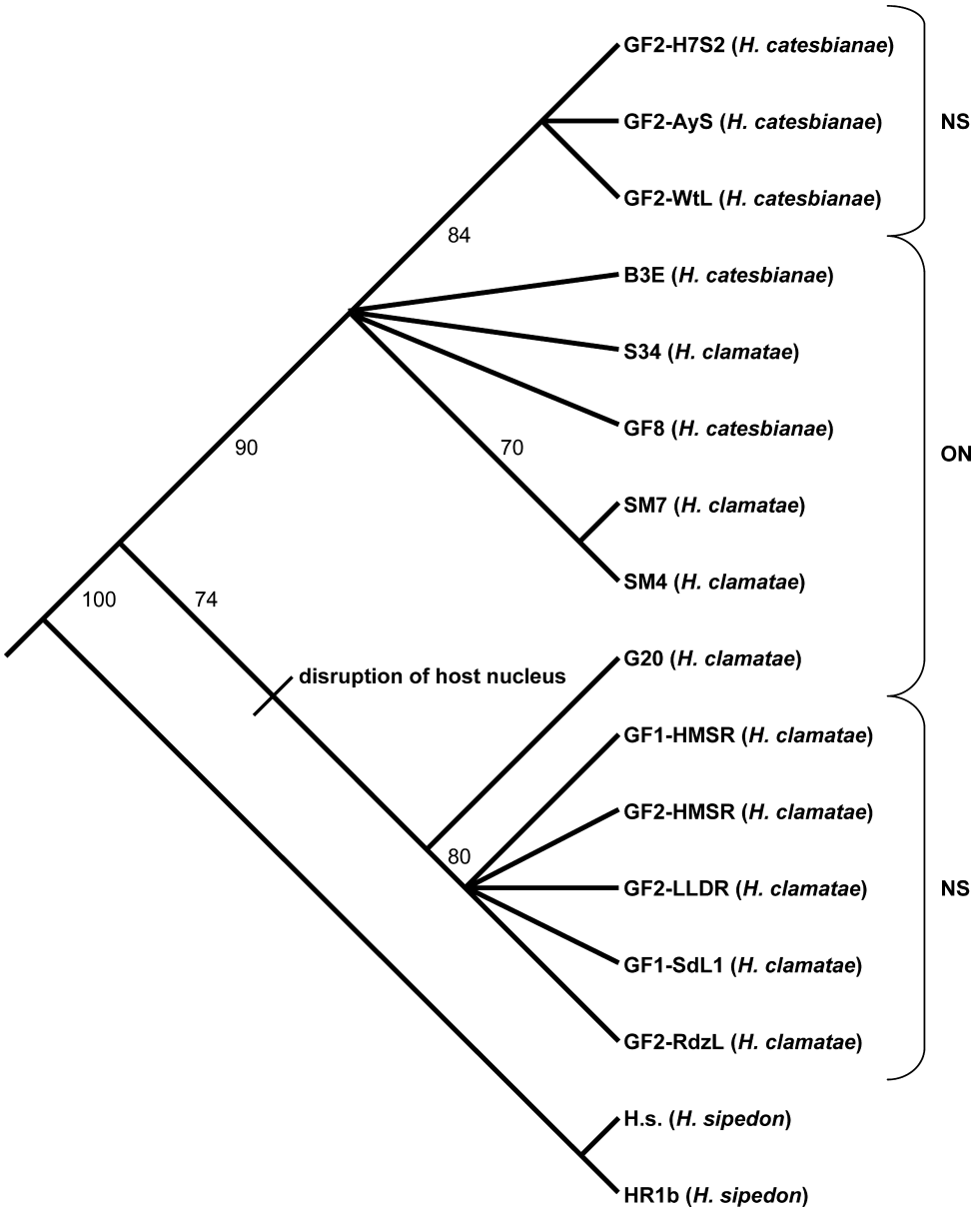

FIGURE 4. Phylogenetic relationships of isolates of *Hepatozoon* species from Nova Scotia and Ontario. This tree represents the consensus of the 2 most parsimonious trees generated using comparison of ITS-1 sequences and cytopathological features. The out-group species is *Hepatozoon sipedon*, a closely related species infecting mosquitoes, frogs, and snakes. Brackets indicate the province from which the isolate was taken. Ontario isolates were sampled by Kim et al. (1998). Bootstrap values, shown next to corresponding clades, were generated from the following options in PAUP version 4.0 (Swofford, 2001): search—full heuristic, branch swap—on all multiple starting trees, addition sequence—simple, and character resampling—all.

1 of the Ontario *H. clamatae* isolates. The second clade comprises the Nova Scotia *H. catesbianae* isolates and the remainder of the Ontario *Hepatozoon* spp. isolates (Fig. 4). Within the *H. clamatae* clade, the Nova Scotia isolates form a monophyletic group separate from the Ontario isolate. Within the second clade, the Nova Scotia *H. catesbianae* isolates form a monophyletic group apart from the other isolates, which include both *H. catesbianae* and *H. clamatae* isolates from Ontario.

DISCUSSION

The ranges of *H. catesbianae* and *H. clamatae* were extended from Ontario and New York to include all of mainland Nova Scotia (Fig. 1). Both of these species were found sympatrically across the province. High prevalences were found, with 58.2% of all frogs sampled found to be infected with *H. clamatae* and 25.3% with *H. catesbianae*. Furthermore, 12.1% of all frogs sampled contained mixed infections of both parasites. Data from ITS-1 sequences of *Hepatozoon* isolates provided evidence that supports the elevation of these 2 parasites to distinct species.

The presence of *Hepatozoon* species in 71.4% of all frogs sampled indicates that these 2 species are parasites of significance among mosquitoes, green frogs, and bullfrogs of Nova Scotia. Although the single leopard frog and mink frog sampled

were not found to be infected by either *Hepatozoon* species, previous observations of *H. clamatae* gamonts inhabiting leopard frogs (Kim et al., 1998) and *Hepatozoon* species infecting mink frogs (Barta and Desser, 1984) in Ontario suggest that species of this genus are possibly present in these frogs in Nova Scotia.

Patterns of *Hepatozoon* spp. infections among bullfrogs and green frogs in Nova Scotia are consistent with the host affinity observations made by Kim et al. (1998), who noted that *H. clamatae* had a greater affinity for green frogs than for bullfrogs, with the reverse being observed for *H. catesbianae*. Although not enough infected bullfrogs were sampled to allow for statistical analysis, the proportion of *H. catesbianae* infections among the infected bullfrogs that were sampled suggests that a bias for *H. catesbianae* infections may be present among bullfrogs.

Comparison of ITS-1 sequences among the Nova Scotia isolates provides the strongest evidence to date that *H. catesbianae* and *H. clamatae* are 2 distinct species. Among these sequences, 6 variable sites were found at which all *H. catesbianae* sequences shared the same nucleotides, accounting for a 4.7% difference from the *H. clamatae* sequence and uniting them as a monophyletic group (Fig. 4). The isolates of *H. clamatae* were united as a monophyletic group by the trait of nuclear disruption of host erythrocytes, a trait not present in the out-group (Smith, 1996).

Different phylogenetic relationships were initially found when ITS-1 sequences of Nova Scotia were compared to Ontario isolates using phylogenetic analysis. The consensus of the 2 most-parsimonious trees (Fig. 4) at first appears to produce a paraphyletic group containing all of the *H. catesbianae* isolates from both regions along with 3 of the *H. clamatae* isolates from Ontario. However, closer inspection of the aligned sequences (Fig. 3) shows that 5 of the Ontario isolates (GF8, B3E, S34, SM4, SM7), 3 of which are described as isolates *H. clamatae* (SM4, SM7, S34), share the same nucleotide as the Nova Scotia *H. catesbianae* isolates at 5 of the variable sites described above (positions 2, 23, 94, 97, and 164). This accounts for the grouping of the 3 Ontario *H. clamatae* isolates (SM4, SM7, S34) in the same clade as the Ontario and Nova Scotia *H. catesbianae* isolates. The sixth Ontario isolate (G20) shares the same nucleotide at 5 of these sites as the *H. clamatae* isolates from Nova Scotia (positions 2, 23, 94, 97, and 179). Our evidence suggests that 3 of the ITS-1 sequences ascribed to *H. clamatae* by Kim et al. (1998) (SM4, SM7, S34) are in fact ITS-1 sequences of *H. catesbianae*. The sequence from the G20 isolate in the study by Kim et al. (1998) was, therefore, the only ITS-1 sequence from *H. clamatae* used for comparison in that study. Because only 1 *H. clamatae* ITS-1 sequence was unknowingly being compared to that of 5 *H. catesbianae* sequences, the variation at the sites that we have found to be informative appeared to Kim et al. (1998) to be autapomorphies. This explains why Kim et al. (1998) were unable to find useful characters in the ITS-1 sequences to distinguish between the 2 species. If we reassign these 3 sequences to *H. catesbianae*, the tree is then shown to contain a monophyletic group for each species, with the *H. clamatae* clade additionally supported by the character of nuclear disruption of host erythrocytes. The isolates SM4 and SM7, now indicated as *H. catesbianae*, are united as a group apart from the other Ontario *H. catesbianae* isolates within their clade on the basis of a single nucleotide polymorphism. Both of these isolates were collected from the same body of water, and may have inherited a single mutation that arose recently in that specific location.

A likely scenario to explain the mischaracterization by Kim et al. (1998) is that the samples that were identified as *H. clamatae* infections and subsequently used for molecular analysis also contained gamonts of *H. catesbianae*. If the primers used for PCR amplification by Kim et al. (1998) had a greater affinity for the primer binding site (or sites) in *H. catesbianae* sequences than in *H. clamatae* sequences, then *H. catesbianae* ITS-1 would be preferentially amplified. The result would be the sequencing and erroneous characterization of *H. catesbianae* ITS-1 as being *H. clamatae* ITS-1. This is a plausible scenario, as primer-binding affinity has been shown experimentally to result in a bias of PCR product from heterogeneous solutions of template DNA (Polz and Cavanaugh, 1998). This hypothesis is supported by the sequencing results from 2 Nova Scotia *H. catesbianae* isolates (GF2-AyS and GF2-H7S2). These sequences contained significant numbers of *H. clamatae* gamonts, accounting for ~30% and ~37% of the gamonts in GF2-AyS and GF2-H7S2, respectively. Despite the high numbers of *H. clamatae* in each of these samples, sequencing produced ITS-1 sequences that were unambiguously those of *H. catesbianae* as seen in the resulting electropherograms.

The present study also shows that cytopathology is in fact a useful character for distinguishing between gamonts of *H. catesbianae* and *H. clamatae*. Grouping of the isolates using the trait of nuclear disruption of host erythrocytes produces the same 2 groups as does analysis of the molecular characters. This indicates that nuclear disruption is a likely a trait caused by the parasites themselves rather than a phenotypic trait of the hosts, which had been suspected, and is applicable for phylogenetic analysis.

The evidence presented also shows that ITS-1 sequences are useful for providing characters to distinguish among closely related and morphologically similar *Hepatozoon* spp. This is not the first case where rRNA gene sequences have shown organisms that are highly similar in life history and morphological terms to be distinct groups of organisms (Barta, 2001). Use of 18S rRNA sequences to infer phylogenetic relationships among the Apicomplexa revealed that species of *Isospora*, grouped together based on morphological and life history similarities, contained 2 groups of species that in fact belong to different families (Barta, 2001). Analyses of rRNA sequences, including ITS-1, are, therefore, important for determining relationships of highly similar parasites.

Internal transcribed spacers of rRNA genes are present in multiple copies in many apicomplexans and may in fact be evolving independently (Barta, 1997; Rooney, 2004). Thus, there is the possibility that ITS-1 sequences of different isolates of *Hepatozoon* spp. in this study are not homologous, but instead represent 2 orthologous genes descended from a common ancestor. However, the strong correlation of the cytopathological features with the different ITS-1 sequences observed in isolates of *H. catesbianae* and *H. clamatae* does not support the latter scenario. Nonetheless, sequencing and analyzing an additional gene such as single-copy nuclear gene or a mitochondrial gene would strengthen this analysis and future studies on haemogregarine systematics.

Further work needs to be done to elucidate the relationships between *Hepatozoon* species, especially those of anurans. Of the 42 currently described species of *Hepatozoon* infecting anurans, *H. catesbianae* and *H. clamatae* are the only 2 species for which life cycles have been fully described (Desser et al., 1995; Kim et al., 1998). Life cycle studies are highly informative for inferring relationships among *Hepatozoon* species as well as haemogregarines in general, but can fail to distinguish between species so similar in morphology and life history as *H. catesbianae* and *H. clamatae*. The current study shows that ITS-1 sequences provide informative characters for supporting morphological and life history traits in establishing phylogenetic relationships among haemogregarines.

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