2009

Molecular and morphological description of a hepatozoon species in reptiles and their ticks in the Northern Territory, Australia

Inger-Marie E. Vilcins
Macquarie University

Beata Ujvari
University of Wollongong, beatau@uow.edu.au

Julie M. Old
University of Western Sydney

Elizabeth Deane
Australian National University

Molecular and morphological description of a hepatozoon species in reptiles and their ticks in the Northern Territory, Australia

Abstract

Ticks, representing 3 species of Amblyomma, were collected from the water python (Liasis fuscus) and 3 additional reptile species in the Northern Territory, Australia, and tested for the presence of Hepatozoon sp., the most common blood parasites of snakes. In addition, blood smears were collected from 5 reptiles, including the water python, and examined for the presence of the parasite. Hepatozoon sp. DNA was detected in all tick and reptile species, with 57.7% of tick samples (n = 187) and 35.6% of blood smears (n = 35) showing evidence of infection. Phylogenetic analysis of the 18S rRNA gene demonstrated that half of the sequences obtained from positive tick samples matched closest with a Hepatozoon species previously identified in the water python population. The remaining sequences were found to be more closely related to mammalian and amphibian Hepatozoon species. This study confirms that species of Amblyomma harbor DNA of the same Hepatozoon species detected in the water pythons. The detection of an additional genotype suggests the ticks may be exposed to 2 Hepatozoon species, providing further opportunity to study multiple hostvectorparasite relationships. 2009 American Society of Parasitologists.

Keywords

Molecular, morphological, description, hepatozoon, species, reptiles, their, ticks, Northern, Territory, Australia

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Vilcins, I. E., Ujvari, B., Old, J. M. and Deane, E. (2009). Molecular and morphological description of a hepatozoon species in reptiles and their ticks in the Northern Territory, Australia. Journal of Parasitology, 95 (2), 434-442.

J. Parasitol., 95(2), 2009, pp. 434–442

MOLECULAR AND MORPHOLOGICAL DESCRIPTION OF A *HEPATOZOON* SPECIES IN REPTILES AND THEIR TICKS IN THE NORTHERN TERRITORY, AUSTRALIA

Inger-Marie E. Vilcins, Beata Ujvari*, Julie M. Old†, and Elizabeth Deane‡§
Department of Biological Sciences, Division of Environmental and Life Sciences, E8A, Macquarie University, North Ryde, New South Wales, 2109, Australia. *e-mail: elizabeth.deane@anu.edu.au*

ABSTRACT: Ticks, representing 3 species of *Amblyomma*, were collected from the water python (*Liasis fuscus*) and 3 additional reptile species in the Northern Territory, Australia, and tested for the presence of *Hepatozoon* sp., the most common blood parasites of snakes. In addition, blood smears were collected from 5 reptiles, including the water python, and examined for the presence of the parasite. *Hepatozoon* sp. DNA was detected in all tick and reptile species, with 57.7% of tick samples (n = 187) and 35.6% of blood smears (n = 35) showing evidence of infection. Phylogenetic analysis of the 18S rRNA gene demonstrated that half of the sequences obtained from positive tick samples matched closest with a *Hepatozoon* species previously identified in the water python population. The remaining sequences were found to be more closely related to mammalian and amphibian *Hepatozoon* species. This study confirms that species of *Amblyomma* harbor DNA of the same *Hepatozoon* species detected in the water pythons. The detection of an additional genotype suggests the ticks may be exposed to 2 *Hepatozoon* species, providing further opportunity to study multiple host–vector–parasite relationships.

Species of *Hepatozoon* (Miller, 1908), belonging to the Hepatozoidae (Barta, 1989), are intraerythrocytic and intraleukocytic apicomplexan parasites, and they are the most common intracellular protozoa found in reptiles (Telford, 1984; Wozniak et al., 1996). Members of the genus have a heteroxenous life cycle, with sporogony taking place within an invertebrate vector in which they form large oocysts, each capable of containing hundreds of sporozoite-filled sporocysts (Telford, 1984; Wozniak et al., 1996; Kim et al., 1998). Transmission of *Hepatozoon* spp. occurs via the ingestion of the infected invertebrate, liberating sporozoites and enabling merogenic and gamontogenic development to take place in the hepatocytes, endothelial cells, and visceral organs of a wide range of vertebrate hosts (Telford, 1984; Wozniak et al., 1996; Kim et al., 1998). Secondary transmission, via the predation of infected vertebrate prey, as well as congenital transmission, has also been recorded (Telford, 1984; Lowichik and Yaeger, 1987; Wozniak et al., 1996). Invertebrate vectors recorded for *Hepatozoon* species are broad in nature and include ectoparasites, biting flies, mosquitoes, and leeches (Smith, 1996). Members of the genus have also been described as infecting a range of mammalian, avian, reptilian, and amphibian species (Levine, 1988; Smith, 1996).

Little is known about Australian *Hepatozoon* species, with all blood-borne parasites described in native reptiles being placed within *Haemogregarina* and they are yet to be adequately classified (Mackerras, 1959; O'Donoghue and Adlard, 2000). Only 2 Australian species have been described in any detail, i.e., *H. breinli*, known for its preference for infecting varanid lizards and vectored by a culicine mosquito (O'Donoghue and Adlard, 2000), and *H. peramelis*, prevalent in approximately 25% of its hosts, the bandicoots (*Perameles* and *Isoodon* spp.) (Wicks et al., 2006). However, because many *Hepatozoon* species are virtually indistinguishable morphologically and are capable of infecting several unrelated hosts and vectors, the true number of species in existence is unknown, although it has been estimated at being in excess of 300 (Telford, 1984; Smith, 1996). This estimate is itself questionable given the similar morphologies and frequent practice of naming new species for new geographic locations (Telford, 1984; Smith, 1996). Most recently, molecular techniques, particularly targeting the 18s ribosomal RNA gene, have greatly enhanced the ability to characterize and detect new species, and to identify suspected vectors (Mathew et al., 2000).

The impact of *Hepatozoon* spp. infection in vertebrates varies greatly. In mammalian hosts, severe pathology and death have been recorded, such as with the *H. americanum* infection of canids, where cysts formed in bone marrow and muscle cause significant morbidity and mortality (Baneth et al., 2003; Ewing and Panciera, 2003). In reptiles, pathology associated with *Hepatozoon* spp. infection is increasingly recognized (Wozniak et al., 1996). Although many reptiles are capable of tolerating the presence of infection and suffer little pathogenic affect (Nadler and Miller, 1984; Wozniak et al., 1996), in some species, heavy parasitemia can lead to anemia and blood cell abnormalities, resulting in immunosuppression (Telford, 1984). The first records of *Hepatozoon* spp. infections resulting in clinical disease in reptiles came from laboratory experiments, including studies on captive snakes (Griner, 1983; Wozniak et al., 1996) and in 3 lizard species, infected with *H. mocassini*, normally a parasite of cottonmouth snakes (*Agkistrodon piscivorus*), in which hepatocellular necrosis was seen (Wozniak et al., 1996). In the Northern Territory, several studies on the water python (*Liasis fuscus*) and other reptiles have recorded both a high prevalence of *Hepatozoon* sp. infection (Ujvari et al., 2004), as well as negative impacts associated with long-term parasitemia (Madsen et al., 2005; Ujvari and Madsen, 2005). Snakes with heavy parasitemia were found to have impaired growth, body condition, reproductive output, and juvenile survival, with only those exhibiting low levels of infection reaching old age (Madsen et al., 2005; Ujvari and Madsen, 2005). As is the case with many *Hepatozoon* species, the vectors responsible for the transmission of the species infecting these reptiles remain unknown.

The present study sought to identify a possible ectoparasite vector for *Hepatozoon* species in the water python and other reptiles in the Northern Territory, using molecular polymerase

Received 30 May 2008; revised 2 August 2008; accepted 7 August 2008.
* School of Biological Sciences, Institute of Conservation Biology, University of Wollongong, New South Wales, 2522, Australia.
† School of Natural Sciences, Building K2, Hawkesbury campus, University of Western Sydney, Locked bag 1797, Penrith South DC, 1797, New South Wales, 1797, Australia.
‡ The Chancelry, Australian National University, Canberra, Australian Capital Territory, 0200, Australia.
§ To whom correspondence should be addressed.

TABLE I. Host animals and ticks collected in this study.

Host animal	No. of ticks (%)	Tick species	♀	♂
Yellow-spotted monitor	149 (79.7)	*A. fimbriatum*	137	11
(*Varanus panoptes*)		*A. limbatum*	0	0
n = 33		*A. moreliae*	1	0
		Nymph	0	
Water python	25 (13.4)	*A. fimbriatum*	10	5
(*Liasis fuscus*)		*A. limbatum*	1	5
n = 8		*A. moreliae*	1	1
		Nymph	2	
Frilled-neck lizard	6 (3.2)	*A. fimbriatum*	0	0
(*Chlamydosaurus kingii*)		*A. limbatum*	0	3
n = 6		*A. moreliae*	0	0
		Nymph	3	
Green tree snake	7 (3.7)	*A. fimbriatum*	1	2
(*Dendrelaphis punctulatus*)		*A. limbatum*	1	2
n = 4		*A. moreliae*	0	0
		Nymph	1	
		Total	152	29
Total = 51	187 (100)		187	

chain reaction (PCR) protocols targeting the 18S ribosomal RNA gene.

MATERIALS AND METHODS

Sample site, tick, and blood collection

In October 2005 and January 2006, 252 adult and nymphal ticks, representing 3 *Amblyomma* species, were collected from 4 Squamata taxa at the Djukbinj National Park and Fogg Dam Nature Reserve, near Humpty Doo, Northern Territory, Australia. The ticks were collected randomly during routine reptile trapping and placed immediately into 70% ethanol. Ticks were pooled according to sex and host animal (Table I). The reptile hosts sampled included 33 yellow-spotted monitors (*Varanus panoptes*), 8 water pythons, 6 frilled-neck lizards (*Chlamydosaurus kingii*), and 4 green tree snakes (*Dendrelaphis punctulatus*). Pooling for molecular analysis resulted in 187 samples consisting of 1–5 ticks (Table I).

In addition, at the time of trapping, blood smears were collected for microscopic examination from 45 individual reptiles, representing 5 species, including 4 green tree snakes, 31 yellow-spotted monitors, 4 water pythons, 1 blue-tongued lizard (*Tiliqua scincoides*), and 5 frilled-neck lizards. Blood smears were stained using routine May-Grunwald-Giesma protocols and examined for the presence or absence of intra-erythrocytic parasites and free gametocytes using a compound microscope. Ticks were identified morphologically with the key of Roberts (1970).

DNA extraction

Before DNA extraction, ticks were repeatedly washed with 70% ethanol and allowed to air dry for 10 min on sterile paper. Each tick was finely diced with a new sterile scalpel blade and processed using the DNeasy tissue kit (QIAGEN, Germantown, Maryland) according to the manufacturer's protocol. During the digestion period, which was extended to 24 hr, samples were frequently crushed with separate sterile plastic homogenizers to aid DNA extraction.

PCR and sequencing methods

Initial detection of the presence of *Hepatozoon* species was undertaken using PCR with primers (HepF300, GTT TCT GAC CTA TCA GCT TTC GAC G; HepR900, C AAA TCT AAG AAT TTC ACC TCT GAC) targeting the 18S ribosomal RNA gene described by Ujvari et al. (2004), generating a 600-bp fragment. Selected positives were used to amplify larger portions of the 18S rRNA gene using the following primer sets: 4558F, GCT AAT ACA TGA GCA AAA TCT CAA; 2773R, CGG AAT TAA CCA GAC AAA T (Mathew et al., 2000) and HAM-1F, GCC AGT AGT CAT ATG CTT GTC; HPF-2r, GAC TTC TCC TTC GTC TAA G (Criado-Fornelio et al., 2006). The PCR reaction mix involved the use of 1 μl of extracted tick DNA amplified in a 25-μl reaction mix containing 10 pmol of each primer; 10 mM each of dGTP, dATP, dTTP, and dCTP; 10× buffer A (Promega, Madison, Wisconsin); 25 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Promega); and autoclaved sterile Milli-Q water (Millipore, Billerica, Massachusetts). Amplification was performed in an Eppendorf Mastercycler Personal (Eppendorf, Hamburg, Germany). A negative control consisting of autoclaved Milli-Q water in place of the template was included, and to demonstrate that the primers did not amplify host DNA and thus gave rise to false-positive results, negative controls consisting of known *Hepatozoon* sp.-free tick DNA were run with each set of reactions. (All other reagent concentrations remained the same.) A positive control was included and consisted of *Hepatozoon* sp. DNA collected in the previous study (Ujvari et al., 2004). Resultant PCR products were separated on a 1% agarose gel run in Tris borate-EDTA buffer and stained with ethidium bromide for examination with UV transillumination. PCR products were purified for sequencing using a QIAquik purification kit (QIAGEN). A 12-μl sequencing mix, containing between 30 and 90 nmol of DNA, Milli-Q water, and 3.2 pmol of either forward or reverse primer was prepared for each positive sample selected. The PCR products were purified, and sequenced from both directions on an ABI 3130xl Genetic Analyzer using BigDye Terminator V.3.1 kit (Applied Biosystems, Foster City, California).

Phylogenetic analysis

Consensus sequences were created with manual alignments of the forward and reverse sequences and submitted to GenBank (NCBI, BLAST) to identify similar *Hepatozoon* species and related taxon sequences. Sequences available for members of the genus and related Apicomplexa were selected for phylogenetic comparison. The sequences were aligned using the Clustal algorithm available in the Mega 3.1 program (Kumar et al., 2004). Phylogenetic relationships were determined using distance matrices under the assumption of Juke-Cantor, P-distance, Tamurae-Nei, and Kimura-2. Each matrix was used as a basis for the formation of dendrograms using the neighbor-joining, unweighted pair group method with arithmetic mean, minimum evolution, and maximum parsimony methods. The reliability of each tree branch node was estimated via the performance of 1,000 bootstrap replicates.

RESULTS

Tick infection

In total, 252 adult (n = 246) and nymphal (n = 6) ticks were collected from 4 reptile taxa. The observed ticks belonged to 3 different species of *Amblyomma* and are well known parasites of reptiles, i.e., *Amblyomma fimbriatum* is distributed across Australia, Papua New Guinea, and the Philippines, whereas *Amblyomma limbatum* and *Amblyomma moreliae* are only found in Australia (Roberts, 1970). Pooling of the 252 ticks into same sex/host yielded a total of 187 samples for PCR analysis. From the 33 yellow-spotted monitors sampled, 149 tick samples were collected; 25 samples were obtained from the 8 water pythons; 6 tick samples originated from each of the 6 frilled-neck lizards; and 7 samples were hosted by the 4 green tree snakes (Table I). Of the 187 samples for molecular analysis, 166 were *A. fimbriatum* ticks (88.8% of the entire tick collection), 12 (6.4%) were *A. limbatum* ticks, 3 (1.6%) were *A. moreliae*, and 6 (3.2%) were *Amblyomma* nymphs that were not identified to species level (Table I).

Ticks sampled from the monitor lizards were primarily female *A. fimbriatum* (n = 137, and 11 males), and 1 female *A. moreliae*. All 3 species of *Amblyomma* parasitized the water

TABLE II. *Hepatozoon* detection in tick samples.

Tick species	No. of ticks collected			*Hepatozoon* DNA positive ticks		
A. fimbriatum	166	148♀	18♂	95	93♀	2♂
A. limbatum	12	2♀	10♂	7	1♀	6♂
A. moreliae	3	2♀	1♂	3	2♀	1♂
Nymphs	6			3		
Total	187	152♀	29♂	108	96♀	9♂

pythons, with the majority being *A. fimbriatum* ticks (n = 15), followed by *A. limbatum* (n = 6) and *A. moreliae* (n = 2) ticks. An additional 2 water python tick samples contained *Amblyomma* sp. nymphs. Of the 6 samples collected from frilled-neck lizards, half were nymphs and half were male *A. limbatum*. Green tree snakes generated 7 samples, of which 1 was an *Amblyomma* sp. nymph (14.3%) and the remaining 6 were equally divided between *A. fimbriatum* and *A. limbatum*.

18S ribosomal RNA gene amplification

PCR screening targeting the 18S ribosomal RNA gene detected *Hepatozoon* sp. positivity in 57.7% (n = 108) of tick samples, from all 3 tick species (Table II). The majority of the *Hepatozoon* sp. positive tick samples were composed of *A. fimbriatum* 87.9% (n = 95). Seven (6.5%) of the positive samples originated from the *A. limbatum* ticks, of which 6 were male and 1 was female. All of the *A. moreliae* ticks (n =3) and half of the 6 nymphal tick samples (n = 3) harbored the *Hepatozoon* sp. protozoa (Table II).

In terms of the host origin of positive ticks, 27 (81.8%) of the 33 monitors were infested with 1, or more, samples; 88 (81.5%) of the 108 total positive samples originating from this reptile. Five of the 8 (62.5%) water pythons sampled had 1, or more, positive samples, representing 13 (12%) of the total samples testing positive. Only 3 (2.8%) positive samples originated from frilled-neck lizards; however, this represented 75% of the 4 animals sampled. Of the 6 green tree snakes, 4 (66.7%) had positive tick samples representing 3.7% of the total.

Hepatozoon sp. sequence analysis

Six DNA samples extracted from ticks (1 *A. moreliae* from a water python, 1 *A. fimbriatum* from another water python, and 4 *A. fimbriatum* from monitors) were successfully sequenced using forward and reverse primers. The resultant sequences were aligned and then truncated where necessary to form consensus sequences and used in phylogenetic analysis. The sequences were submitted to NCBI GenBank, with accession numbers as follows: EU430231, *A. fimbriatum* from *V. panoptes* (577 bp); EU430232, *A. fimbriatum* from *V. panoptes* (574 bp); EU430233, *A. moreliae* from *L. fuscus* (523 bp); EU430234, *A. fimbriatum* from *V. panoptes* (924 bp); EU430235, *A. fimbriatum* from *V. panoptes* (570 bp); and EU430236, *A. fimbriatum* from *L. fuscus* (945 bp). BLAST analysis of each sequence varied. Two of the 4 sequences from *A. fimbriatum* ticks collected from a monitor host (EU430231 and EU430232) shared highest sequence identity to *Hepatozoon ayorgbor* (>98%) and *Hepatozoon felis* Spain 1 and 2 (>97%). The remaining 2 (EU430234 and EU430235) were most closely matched to the *Hepatozoon* species previously identified in reptiles (>99%), followed by *H. ayorgbor* (>98%). The *A. fimbriatum* sample collected from the water python host (EU430236) also matched closest with *H. felis* Spain 1 and 2 (>97%) and *H. ayorgbor* (>96%). The *A. moreliae* sample taken from a water python host (EU430233) shared closest sequence identity to a *Hepatozoon* species previously identified in reptiles (>99%), followed by *H. ayorgbor* (>98%). Three of the samples that matched closest to the Northern Territory *Hepatozoon* species detected in reptiles all shared high sequence identity with one another, as did the remaining 3 that matched with *H. ayorgbor* and *H. felis* Spain 1 and 2, indicating that 2 distinct *Hepatozoon* species or genotypes were present within these ticks.

The phylodendrogram topology of the sequences using all distance matrices and parsimony methods showed similar scenario (Figs. 1, 2). Comparisons between published 18S rRNA gene sequences of *Hepatozoon* sp. and related protozoan species confirm that the sequences belonged to *Hepatozoon* (Fig. 1). In the tree generated for the 18S rRNA gene comparing the 6 reptile tick genotypes with other *Hepatozoon* and protozoan species, the 3 genotypes matched most closely to *H. ayorgbor* and *H. felis* Spain 1 and 2, forming a monophyletic lineage in between a monophyletic sister group containing the 2 *H. felis* sequences and another composed of *H. americanum* and the Curupira strain 2 of *H. canis* (Fig. 1). The remaining 3 sequences that shared close sequence identity to the Northern Territory reptile *Hepatozoon* species were present within 2 sister lineages composed entirely of the reptile *Hepatozoon* genotypes (Fig. 1). Two of the sequences originating from *A. moreliae* and *A. fimbriatum* ticks collected from a monitor and water python (EU430233 and EU430234) were grouped in 1 phyletic line and the other, from *A. fimbriatum* collected from a water python (EU430235), was grouped with the remaining sequences available for the Northern Territory reptile *Hepatozoon* species (Fig. 1).

When comparing the *Hepatozoon* sp. 18S rRNA sequences obtained from the reptile ticks with members of the genus only, the topology of the tree was similar, but with some variations (Fig. 2). Once again the first genotype group matching closest to *H. ayorgbor* and the Spain 1 and 2 *H. felis* species formed a distinct monophyletic lineage group with high bootstrap support (Fig. 2). The location of this phyletic line did not vary and was positioned between the same monophyletic groups containing the frog species *H. catesbianae* in one, the *H. americanum* and the related *H. canis* Curupira 2 strain in another (Fig. 2). The genotypes matching closest to the Northern Territory reptile *Hepatozoon* genotypes (EU430233–EU430235) differed in their placement, compared with the previous tree containing both *Hepatozoon* species and other protozoa (Figs. 1, 2). Another genotype (*A. fimbriatum*/*V. panoptes*, EU430234) formed a monophyletic group with *H. ayorgbor* and a species identified in the cotton rat (*Sigmodon hispidus*) (Fig. 2). Another genotype (*A. moreliae*/*L. fuscus*, EU430233) was grouped in a sister lineage to the first described, branching off at the base, with 1 of the Northern Territory reptile *Hepatozoon* sequences and 2 identified in bank voles (*Clethrionomys glareolus*) (Fig. 2). The last of the reptile-related genotypes (*A. fimbriatum*/*V. panoptes*, EU430235) was positioned in a monophyletic group containing only Northern Territory reptile sequences (Fig. 2).

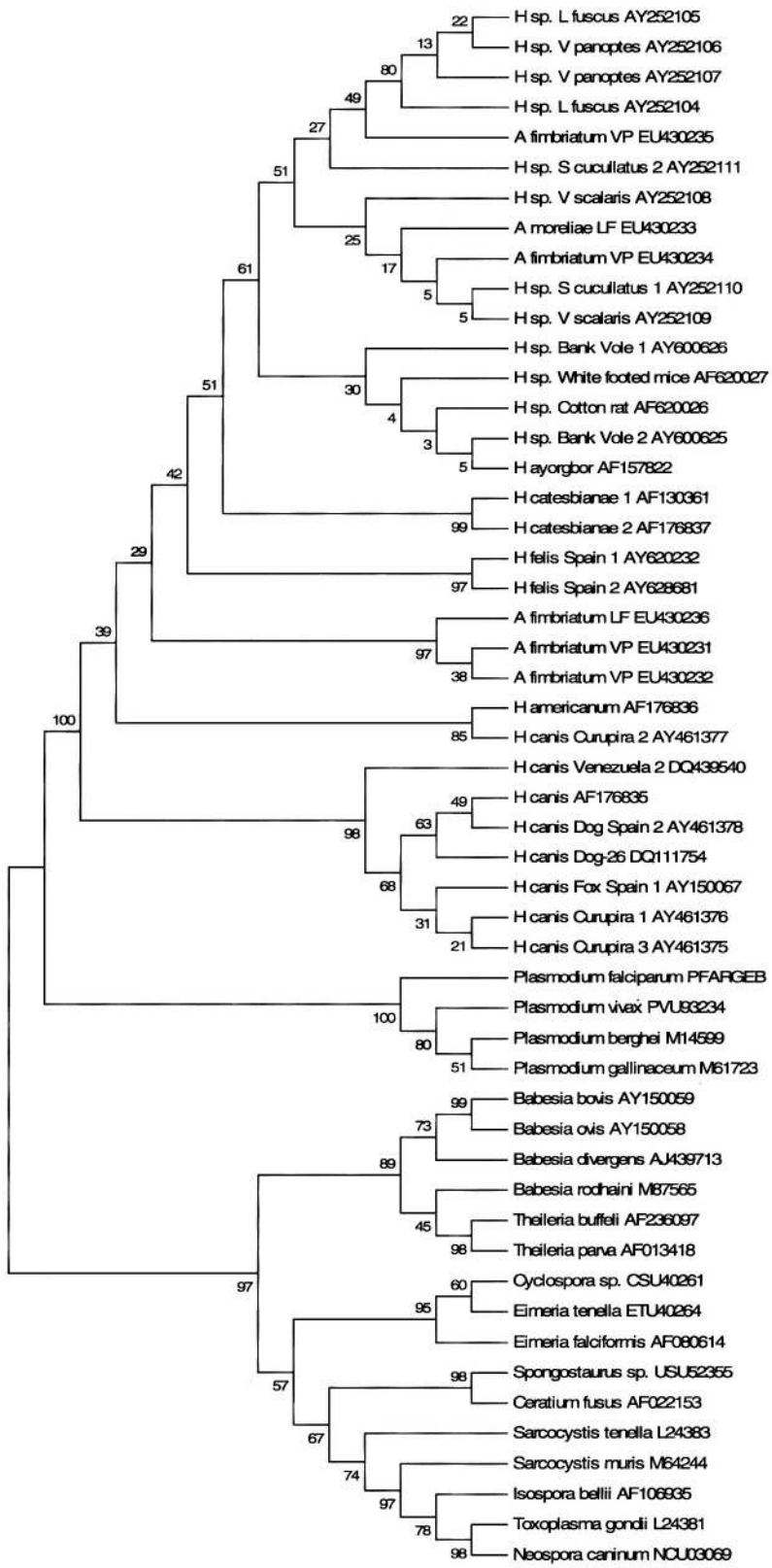


FIGURE 1. Phylogenetic tree showing the classification of the *Hepatozoon* species detected in *Amblyomma* spp. ticks, collected from reptile hosts, among published *Hepatozoon* and protistan species. The closest relatives to the *Hepatozoon* sp. were used to confirm the placement of the species identified in this study within the genus (GenBank accession numbers shown). 18S ribosomal RNA gene sequences were aligned before analysis using neighbor-joining algorithm under the assumption of Kimura-2. All bootstrap values from 1,000 replications are shown on interior branch nodes.

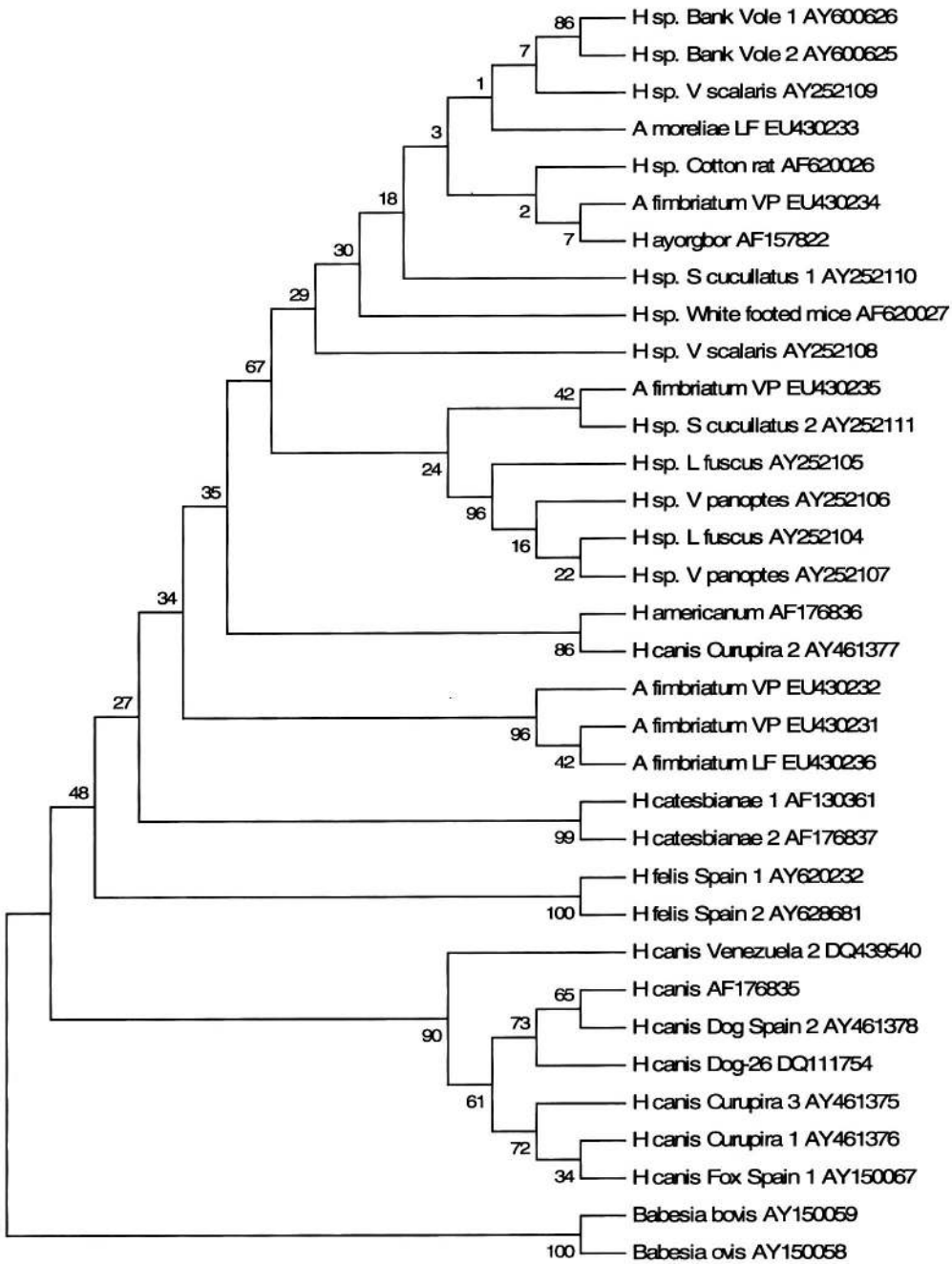
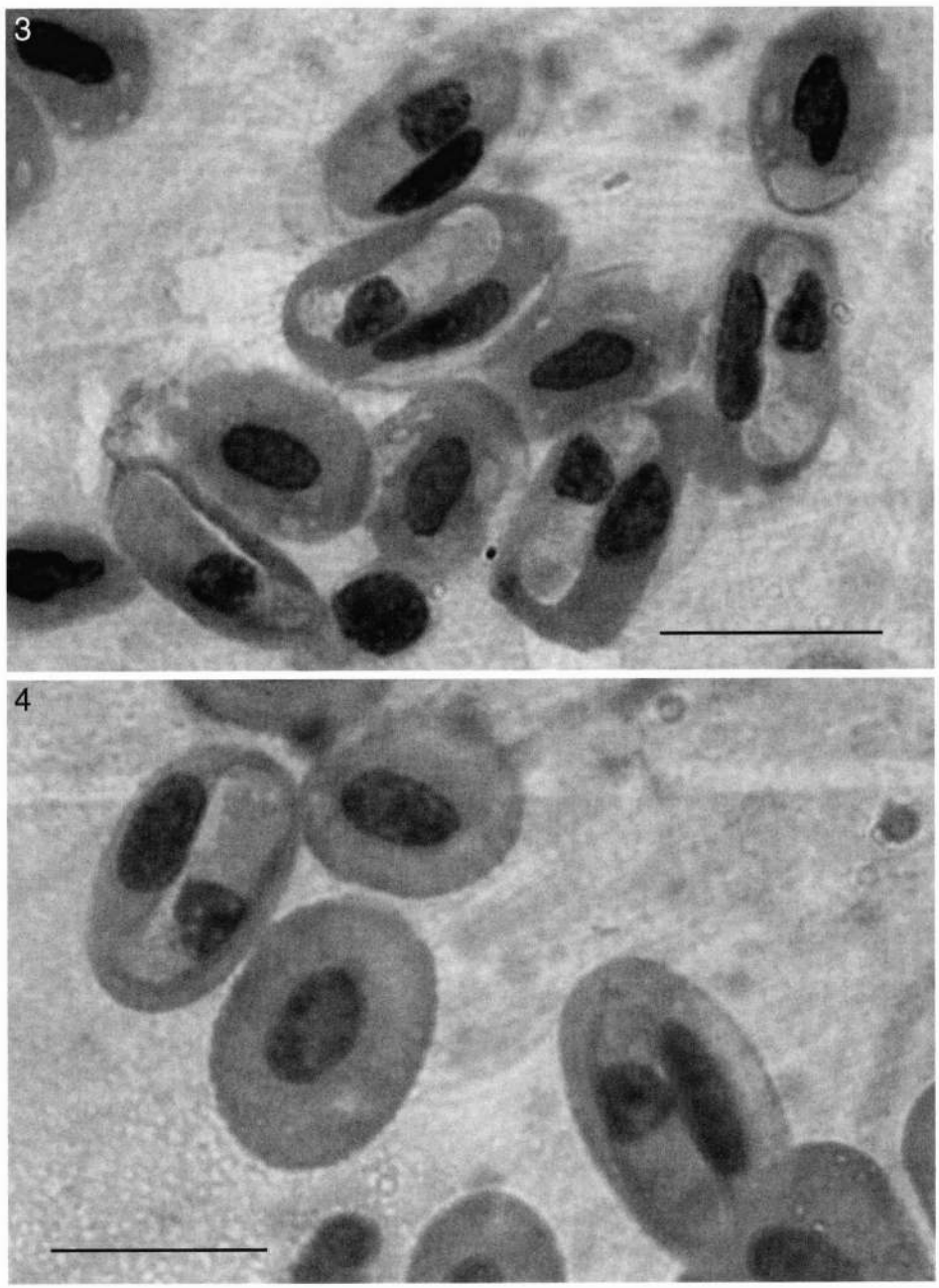


FIGURE 2. Phylogenetic analysis of the *Hepatozoon* species identified within *Amblyomma* spp. ticks collected from reptile hosts, using the neighbor-joining algorithm under the assumption of Kimura-2 are shown for the 18S rRNA gene. The tree compares the *Ixodes tasmani* species with only species of *Hepatozoon*, rooted with *Toxoplasma gondii*. All bootstrap values from 1,000 replications are shown on interior branch nodes.

Morphological analysis

Of the blood smears from 45 different reptile specimens, 16 had intraerythrocytic parasites (35.6%). The majority of the blood smears originated from the yellow-spotted monitors (n = 31), and, of these, 11 (35.5%) had erythrocytic parasites. The parasites were observed occupying the longitudinal length of the infected erythrocytes. In all 5 reptile species, only 1 parasite was visible per infected cell (Fig. 3A, B). In monitors, the erythrocyte morphology was altered and often distended longitudinally by parasite presence. The nuclei of the parasites were circular in shape and took up their entire width (Table III). Four blood smears originated from water pythons, of which 2 contained intraerythrocytic parasites. In the latter cases, the parasites were also observed occupying the longitudinal length of the infected erythrocytes and, in some cases, distorting the cell size (Table III). Three of 4 green tree snake blood smears exhibited parasite infection (Fig. 3A, B). The longitudinal length of infected cells was also affected by the parasites, often pushing the red cell nucleus to one side (Table I; Fig. 3A, B). The



FIGURES 3. (**A, B**) Gametocytes are visible within the erythrocytes of naturally infected green tree snakes (*Dendrelaphis punctulatus*). No multiple infections were observed in any smears. Bar = 10 μm.

infecting parasites were also observed distorting the size of the erythrocytes, but, unlike infections in the other 2 reptiles, the nuclei did not occupy the width of the observed parasites. Gametocytes were at no stage observed emerging, or free, from erythrocytes (exoerythrocytic) in any of the blood smears.

DISCUSSION

The present study reports the presence of *Hepatozoon* sp. DNA in ticks collected from 4 reptile hosts in the Northern Territory, Australia. Sequence analysis demonstrated high levels of identity (>99%) with that previously reported for a *Hepatozoon* species found infecting the same reptile population in the same region (Ujvari et al., 2004). Species of *Hepatozoon* are considered to be the most frequent hemoparasites of reptiles (Smith, 1996), and transmission is believed to occur via either a hematophagous vector or ingestion of an infected host (Smith, 1996). However, host specificity and method of transmission has been greatly debated (Telford et al., 2001) and has been plagued by morphological inconsistencies and absence of genetic information (Mathew et al., 2000; Sloboda et al., 2007). The only confirmed case of tick-associated *Hepatozoon* sp. transmission has been that of *H. kisrae*, which infects *Agema stellio*, and is transmitted by the tick *Hyalomma* cf. *aegyptium* (Paperna et al., 2002). Mosquitoes are considered to be the pri-

TABLE III. Measurements (micrometers) of gametocytes observed in 3 reptile species.

Host	Yellow-spotted monitor (*Varanus panoptes*)	Water python (*Liasis fuscus*)	Green tree snake (*Dendrelaphis punctulatus*)
Infected cell	Erythrocyte	Erythrocyte	Erythrocyte
Gametocyte length	10.0–10.6	9.4–10.6	10.0–11.2
Gametocyte width	2.5–3.7	1.2–1.9	3.2–3.7
Nucleus length	2.8	2.5–2.8	2.7
Nucleus width	2.5	2.5	2.4–2.5

mary vectors for *Hepatozoon* species in ophidian hosts, with low specificity being observed for a range of mosquito genera via experimental transmission (Sloboda et al., 2007).

The detection of *Hepatozoon* sp. DNA in 57.7% of the 3 *Amblyomma* species sampled in this study, coupled with close sequence identity (>99%) with the *Hepatozoon* species previously found infecting the Northern Territory reptiles (Ujvari et al., 2004; Ujvari and Madsen, 2005; Madsen et al., 2005), suggests the source of the reptile infection needs to be clarified to assess whether it originated from the ticks (either through tick feeding or ingestion), or other invertebrates, or via the ingestion of intermediate prey items (such as frogs, lizards, or small mammals). Both females and males of the 3 *Amblyomma* tick species in this study were found to be harboring *Hepatozoon* sp., with 81.8% of monitors (n = 33), 75% of frilled-neck lizards (n = 6), 66.7% of green tree snakes (n = 4), and 62.5% of water pythons (n = 8) hosts sampled infested with 1, or more, positive tick samples. The lowest prevalence of *Hepatozoon* sp. detection in the tick samples were collected from the 8 water pythons, although still high at 62.5%. Establishing cyst formation in the ticks screened in this study and *Hepatozoon* sp. detection in other invertebrates would assist in establishing the source of water python infection. Screening of the dusky rat (*Rattus colletti*), the preferred mammalian prey item composing up to 95% of the water python diet (Shine and Madsen, 1997), would assist in establishing secondary transmission.

Blood smears confirmed parasitic infection in the 4 reptiles from which the ticks were collected for this study, as well as in an additional species, the blue-tongued lizard. Although the observed level of parasitemia (35.6%) was lower than the 66.0% infection of *Hepatozoon hinulinae* observed in the large Australian skink (*Eulamprus quoyii*) in central Queensland, Australia (Salkeld and Schwarzkopf, 2005). Previously, molecular screening by Madsen et al. (2005) and Ujvari et al. (2004) in the same animals sampled in this study confirmed the parasite responsible for observed parasitemia as being a species of *Hepatozoon*.

Although not proven that the *Hepatozoon* sp. observed in the reptiles originated from the ticks, it is of note that the highest detection of *Hepatozoon* sp. in the ticks and parasitemia in the host, was observed in the green tree snakes, with 66.7% (n = 4/6) of the snakes harboring positive ticks and 75% (n = 3/4) of snakes possessing observable intraerythrocytic gametocytes (Fig. 3A, B). In the water python, 62.5% of the snakes had *Hepatozoon* sp. positive ticks and 50% possessed intraerythrocytic parasites. One of 4 blood smears originated from a python from which ticks had also been collected, and in this case *Hepatozoon* sp. was detected in both the ticks and blood.

Genetic information for the 18S rRNA gene of *Hepatozoon* sp. is available for very few species, making phylogenetic comparison difficult (Mathew et al., 2000; Sloboda et al., 2007). Six positive sequences obtained in this study represented 2 distinct genotypes, with 3 sequences in each. The first 3 in group 1, shared greatest sequence similarity to *Hepatozoon* sequences obtained from the same reptile population described by Ujvari et al. (2004) (Figs. 1, 2). The second closest match was to *H. ayorgbor* (>98%), a recently identified parasite, detected in 78.2% (n = 53) of blood smears from *Python regius* from Ghana, Africa (Sloboda et al., 2007). In contrast to *H. ayorgbor*, the Northern Territorian reptile *Hepatozoon* species was characterized by the presence of only 1 gamont in infected erythrocytes, with a much longer gametocyte in all infected cells (Sloboda et al., 2007).

The effect of the *Hepatozoon* species in water pythons, although considered to be a rare phenomenon in snakes (Santos et al., 2005), is similar to that seen in accidental infections in reptile hosts kept in captivity (Wozniak et al., 1996). Because prevalence is so high in the water python population (Ujvari et al., 2004; Ujvari and Madsen, 2005; Madsen et al., 2005), it is unlikely that water pythons are accidental hosts. Rather, the infection is most likely the result of a recent introduction, explaining the observed virulence. Future molecular analysis could resolve the origin of this *Hepatozoon* species and shed light on the level and timeframe of the infection in water pythons. Phylogenetic comparison of the 3 genotypes within group 1 to one another, and with the sequences in GenBank, firmly group them with the previously detected Northern Territory reptile *Hepatozoon* species, followed by *H. ayorgbor* and rodent *Hepatozoon* species (Figs. 1, 2). The grouping of rodent *Hepatozoon* species with these reptile species has a valid explanation, as small mammals, including rodents, are often preferred prey items for large reptile predators. Although sequence identity was very high (99.8% by pairwise comparison), all 3 genotypes in the group showed small sequence polymorphisms compared to one another and the 18S rRNA *Hepatozoon* sp. sequences obtained in the earlier study by Ujvari et al. (2004) from the reptile hosts. Despite the slight polymorphisms observed between the sequences from the hosts' blood and from the vector ticks, they were much more similar to one another compared with the 2 second closest matches, and this may be a reflection of the low host specificity observed for the *Hepatozoon* species.

The other 3 sequences in group 2 showed a much greater level of identity (>99.9 or 100%) to each other and compared with group 1 genotypes, they differed considerably, indicating the presence of 2 *Hepatozoon* sp. in the *A. fimbriatum* ticks collected from the reptiles. These genotypes shared greatest sequence identity with *H. ayorgbor* and *H. felis* Spain 1 and 2, found in domestic cats (Criado-Fornelio et al., 2006). *Hepatozoon felis* has been morphologically identified as infecting a wide range of domestic and wild felids worldwide, but it is rarely associated with severe pathology (Perez et al., 2004). However, very little is known of *H. felis* hosts, its life cycle, or which vectors and/or intermediate hosts may be involved in its transmission (Perez et al., 2004). The phylogenetic trees generated, comparing group 2 with species of *Hepatozoon* and oth-

er protozoans (Fig. 1), as well as within the genus itself (Fig. 2), partly agrees with the BLAST analysis. In both cases, the trees grouped the second genotype with the 2 *H. felis* strains with high bootstrap support (Figs. 1, 2), as well as with *H. americanum* and *H. canis* Curupira 2, a pair of well studied pathogens of canids, both capable of causing fatal disease (Shaw et al., 2001; Ewing et al., 2002). These analyses support the suggestion that the members of group 2 are a different species than those capable of infecting the reptile hosts. Furthermore, it is possible that these parasites infect prey species of the larger reptiles, such as small mammals (rodents or marsupials) and amphibians. This is further supported by the absence of these genotypes in the previous study, which directly screened the reptile blood (Ujvari et al., 2004). The lack of overall genetic material available for most of the *Hepatozoon* species makes it difficult to undertake a comprehensive comparison and characterization of the 2 genotypes identified in this study; additional genes need to be targeted and sequenced to allow the relationships between species within the genus to be clarified.

The origin of the reptile infection identified in the present study is an interesting question. As identified here, *Hepatozoon* sp. was detected in all 3 ticks species, including 50% of nymph samples tested. The high prevalence of infection in the reptiles in the Northern Territory observed here and in previous research (Ujvari et al., 2004) provides an opportunity to investigate the host suitability of these tick species. The infection of so many different reptiles with the same *Hepatozoon* species strengthens the hypothesis that some members of the genus are capable of exhibiting low host specificity, inhibited only by host geographic distribution and access, rather than host genetic relation (Sloboda et al., 2007). The findings of the present study highlight the opportunities that exist in the Northern Territory environment to research the interaction of 3 *Amblyomma* tick species with a range of *Hepatozoon* sp.-infected reptilian hosts. These interactions also allow for the study of differing host responses to *Hepatozoon* sp. infection, including those associated with pathology (Ujvari et al., 2004; Madsen et al., 2005) and whether the ticks involved are capable of developing an infection and playing a role in the parasite's life cycle.

LITERATURE CITED

BANETH, G., J. S. MATHEW, V. SHAKAP, D. K. MACINTIRE, J. R. BARTA, AND S. A. EWING. 2003. Canine hepatozoonosis: Two disease syndromes caused by separate Hepatozoon spp. Trends in Parasitology **19:** 27–31.

BARTA, J. R. 1989. Phylogenetic analysis of the class Sporozoea (Phylum Apicomplexa Levine, 1970): Evidence for the independent evolution of heteroxenous life cycles. Journal of Parasitology **75:** 195–206.

CRIADO-FORNELLO, A., J. L. RUAS, N. CASADO, N. A. R. FARIAS, M. P. SOARES, G. MULLER, J. G. W. BRUM, M. E. A. BERNE, A. BULING-SARANA, AND J. C. BARBA-CARRETERO. 2006. New molecular data on mammalian *Hepatozoon* species (Apicomplexa: Adeleorina) from Brazil and Spain. Journal of Parasitology **92:** 93–99.

EWING, S. A., J. S. MATHEW, AND R. J. PANCIERA. 2002. Transmission of *Hepatozoon americanum* (Apicomplexa: Adeleorina) by ixodids (Acari: Ixodidae). Journal of Medical Entomology **39:** 631–634.

———, AND R. J. PANCIERA. 2003. American canine hepatozoonosis. Clinical Microbiology Reviews **16:** 688–697.

GRINER, L. A. 1983. Pathology of zoo animals. Zoological Society of San Diego, San Diego, California, 608 p.

KIM, B. J., T. G. SMITH, AND S. S. DESSER. 1998. The life history and host specificity of *Hepatozoon clamatae* (Apicomplexa: Adeleorina) and ITS-1 nucleotide sequence variation of *Hepatozoon* species of frogs and mosquitoes from Ontario. Journal of Parasitology **84:** 789–797.

KUMAR, S., K. TAMURA, AND M. NEI. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics **5:** 150–163.

LEVINE, N. D. 1988. The protozoan phylum Apicomplexa. CRC Press, Boca Raton, Florida, 224 p.

LOWICHIK, A., AND R. G. YAEGER. 1987. Ecological aspects of snake haemogregarine infections from two habitats in southeastern Louisiana. Journal of Parasitology **73:** 1109–1115.

MACKERRAS, M. J. 1959. The haematozoa of Australian reptiles. Australian Journal of Zoology **9:** 61–122.

MADSEN, M., B. UJAVARI, AND M. OLSSON. 2005. Old pythons stay fit; effects of haematozoan infections on life history traits of a tropical predator. Oecologia **142:** 407–412.

MATHEW, J. S., R. A. VAN DEN BUSSCHE, S. A. EWING, J. R. MALAYER, B. R. LATHA, AND R. J. PANCIERA. 2000. Phylogenetic relationships of *Hepatozoon* (Apicomplexa: Adeleorina) based on molecular, morphologic and life-cycle characters. Journal of Parasitology **86:** 366–372.

MILLER, W. W. 1908. *Hepatozoon perniciosum* (n. g., n. sp.), a haemogregarine pathogenic for white rats; with a brief description of the sexual cycle in the intermediate host, a mite (*Laelaps echidninus* Berlese). Zeitschrift fur Parasitenkunde **62:** 165–178.

NADLER, S. A., AND J. H. MILLER. 1984. A redescription of *Hepatozoon mocassini* (Leveran 1902) from *Agkistrodon piscivorus leucostoma* Troust, 1836. Journal of Protozoology **31:** 321–324.

O'DONOGHUE, P. J., AND R. D. ADLARD. 2000. Catalogue of protozoan parasites recorded in Australia. Memoirs of the Queensland Museum **45:** 1–163.

PAPERNA, I., T. KREMER-MECABELL, AND S. FINKELMAN. 2002. *Hepatozoon kisrae* n. sp. infecting the lizard *Agama stellia* is transmitted by the tick *Hyalomma* cf. *aegyptium*. Parasite **9:** 17–27.

PEREZ, R. R., A. S. RUBINI, AND L. H. O'DWYER. 2004. The first report of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) in domestic cats from Sao Paulo state, Brazil. Parasitology Research **94:** 83–85.

ROBERTS, F. H. S. 1970. Australian ticks. Commonwealth Scientific and Industrial Research Organization, Melbourne, Victoria, Australia, 267 p.

SALKELD, D. J., AND L. SCHWARZKOPF. 2005. Epizootiology of blood parasites in an Australian lizard: A mark-recapture study of a natural population. International Journal for Parasitology **35:** 11–18.

SANTOS, M. M. V., L. H. O'DWYER, AND J. DE SILVA. 2005. Seasonal variation of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) parasitemia from *Boa constrictor amaralii* (Serpentes, Boidae) and *Hydrodynastes gigas* (Serpentes, Colubridiae). Parasitology Research **97:** 94–97.

SHAW, S. E., M. J. DAY, R. J. BIRTLES, AND E. B. BREITSCHWERDT. 2001. Tick-borne infectious diseases of dogs. Trends in Parasitology **17:** 74–80.

SHINE, R., AND T. MADSEN. 1997. Prey abundance and predator reproduction: Rats and pythons on a tropical Australian floodplain. Ecology **78:** 1078–1086.

SLOBODA, M., M. KAMLER, J. BULANTOVA, J. VOTYPKA, AND D. MODRY. 2007. A new species of *Hepatozoon* (Apicomplexa: Adeleorina) from *Python regius* (Serpentes: Pythonidae) and its experimental transmission by a mosquito vector. Journal of Parasitology **93:** 1189–1198.

SMITH, T. G. 1996. The genus *Hepatozoon* (Apicomplexa: Adeleina). Journal of Parasitology **82:** 565–585.

TELFORD, S. R. 1984. Haemoparasites of reptiles. *In* Diseases of amphibians and reptiles, G. L. Hoff, F. L. Frye, and E. R. Jacobson (eds.). Plenum Press, New York, New York, p. 385–517.

TELFORD, S. R., JR., E. J. WOZNIAK, AND J. F. BUTLER. 2001. Haemogregarine specificity in two communities of Florida snakes with descriptions of six new species of *Hepatozoon* (Apicomplexa: Hepatozoidae) and a possible species of *Haemogregarina* (Apicomplexa: Haemogregarinidae). Journal of Parasitology **87:** 890–905.

UJVARI, B., T. MADSEN, AND M. OLSSON. 2004. High prevalence of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) infection in water py-

thons (*Liasis fuscus*) from tropical Australia. Journal of Parasitology **90:** 670–672.

———, AND ———. 2005. Age, parasites, and condition affect humoral immune response in tropical pythons. Behavioural Ecology **17:** 20–24.

WICKS, R. M., P. B. S. SPENCER, P. MOOLHUJZEN, AND P. CLARK. 2006. Morphological and molecular characteristics of a species of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) from the blood of *Isoodon obesulus* (Marsupialia: Peramelidae) in Western Australia. Systematic Parasitology **65:** 19–25.

WOZNIAK, E. J., K. R. KAZACOS, S. R. TELFORD, AND G. L. MCLAUGHLIN. 1996. Characterization of the clinical and anatomical pathological changes associated with *Hepatozoon mocassini* infections in unnatural reptilian hosts. International Journal for Parasitology **26:** 141–146.