

Molecular and morphological characterization of a haemogregarine in the alligator snapping turtle, *Macrochelys temminckii* (Testudines: Chelydridae)

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Abstract A severely underweight alligator snapping turtle *Macrochelys temminckii* Troost in Harlan, 1835, was found near Tyler, Texas, and taken to the Caldwell Zoo. Blood films were submitted to Texas A&M University, College Station, Texas, for morphological and molecular identification of haemogregarine-like inclusions in the red blood cells. Intraerythrocytic *Haemogregarina* sp. forms were found on microscopic examination at a parasitemia of <1 %. The morphology and morphometric data for the forms indicate similarity to *Haemogregarina macrochelysi* n. sp. Telford et al., 2009, previously reported in alligator snapping turtles in Florida and Georgia, but two characteristic stage forms were not shared between *H. macrochelysi* n. sp. and the parasite found in this report. The haemogregarine 18S ribosomal RNA gene (1555-bp fragment) was amplified and cloned, and five clones sequenced. The sequences were deposited in the NCBI GenBank database. All five showed ~96 % identity to *Haemogregarina balli* Paterson and Desser, 1976, *Hepatozoon* sp., and *Hemolivia stellata* Petit et al., 1990. A 774-bp segment shared 98–99 % identity with the corresponding *Haemogregarina* sp. rDNA sequence (KR006985) from Caspian turtles (*Mauremys caspica* McDowell, 1964) in Iran. A neighbor-joining phylogenetic tree generated from aligned sequences from the clones, 26 hematozoa, *Adelina dimidiata* Schneider, 1875, and *Cryptosporidium serpentis* Levine, 1980, revealed the cloned sequences clustered on their own branch within the *Haemogregarina* spp. clade. No genetic data are available for *H. macrochelysi* n. sp. at this time, so

it remains unclear if this parasite in a Texas alligator snapping turtle is conspecific with *H. macrochelysi* n. sp.

Keywords Haemogregarine · Alligator snapping turtle · *Macrochelys temminckii* · 18S ribosomal RNA gene · Hematozoa

Introduction

The alligator snapping turtle, *Macrochelys temminckii* Troost in Harlan, 1835 (Testudines: Chelydridae), is characterized by sharp claws, spiked carapacial ridges, and huge head with strongly hooked beak. It is distributed in North America across the southeastern USA and is endemic to river systems that drain into the Gulf of Mexico from the Suwannee River in Georgia and Florida, and from the Brazos River in Texas (Powell et al. 2016). It is the largest species of freshwater turtle in North America, weighing up to 135 kg. It is highly aquatic although oviposition occurs on land. The alligator snapping turtle is non-migratory and a top-trophic-level predator (Boundy and Kennedy 2006; Ernst and Lovich 2009; Ewert 1976).

Haemogregarines are widely distributed blood parasites among aquatic turtles and have been reported in North America, Europe, Asia, and Australia (Telford 2009). Leeches are thought to be the invertebrate hosts and the transmission vectors for aquatic vertebrate hosts, while ticks are the transmission vectors for parasites of terrestrial reptiles (Telford 2009; Cook et al. 2009; Merino et al. 2009; Siddall and Desser 1991). In the USA, *Haemogregarina* spp. have been investigated in freshwater turtle populations in Louisiana, Georgia, Tennessee, Kentucky, and Texas (Acholonu 1974; Davis and Sterrett 2011; Edney 1949; Strohle and Christensen 1984; Wang and Hopkins 1965)

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and particularly in the alligator snapping turtle *M. temminckii* in Arkansas, Georgia, and Florida (McAllister et al. 1995; Telford et al. 2009).

Haemogregarines are intraerythrocytic apicomplexans that belong to the suborder Adeleorina of the family Haemogregarinidae. *Haemogregarina* species have indirect life cycles. They infect a wide variety of vertebrate hosts such as mammals, birds, fishes, snakes, crocodylians, lizards, and turtles in which they undergo asexual cycles of merogony, gametogony, syngamy, and sporogony (Barta et al. 2012; Siddall and Desser 1992). The various types of meronts and merozoites that arise may either initiate further rounds of merogonic replication or differentiate into gamonts (Barta et al. 2012; Smith 1996). The invertebrate transmission vectors in which the sexual stages take place include ticks, mites, other arthropods, and leeches (Telford Jr 2009). Although the complete life cycles for *Haemogregarina balli* in Nearctic snapping turtles and *Haemogregarina stepanowi* Danilewsky, 1885, in the European pond turtle have been described, data regarding the complete life cycles for many of these organisms are lacking (Siddall and Desser 1991, 1992; Telford 2009). However, intraerythrocytic stages for a number of *Haemogregarina* spp. have been described in various hosts from different parts of the world (Barta et al. 2012; Davis and Sterrett 2011).

Nearly 300 haemogregarine species are named in previous studies based on morphological and/or biological features, such as the size and the shape of the stages in the host erythrocytes (Levine 1982; Perkins and Keller 2001). However, morphological similarities and the lack of detected life cycle stages, especially the identification of the vector and morphology of the parasite in the vector, have prevented an accurate identification of the different infective forms (Levine 1982). Numerous *Haemogregarina* spp. have been reported in turtles and tortoises globally, but descriptions of chelonian hematozoa in the USA are limited (Acholonu 1974; McAllister et al. 1995; McAllister 2015; Strohlein and Christensen 1984; Telford et al. 2009; Wang and Hopkins 1965) with some including photomicrographs and selected measurements.

New molecular tools combined with morphological approaches provide acceptable differential diagnoses and taxonomy of *Haemogregarina* spp. (Lv et al. 2015). To date, the 18S ribosomal RNA (rRNA) gene has been utilized in characterization, identification, and prevalence as well as in taxonomic relationship studies among apicomplexan parasites in reptiles, including freshwater turtles (Dvořáková et al. 2015; Kopečná et al. 2006; Kvičerová et al. 2014; Maia et al. 2014; Rakhshandehroo et al. 2016; Telford et al. 2009).

This study entails the first known *Haemogregarina* sp. identified in the alligator snapping turtle (*M. temminckii*) in Texas and includes both morphological and molecular descriptions of this parasite. Moreover, this report provides the first DNA sequence data for a *Haemogregarina* sp. in the USA.

Materials and methods

An alligator snapping turtle was found in early January 2015 laying by the side of a road near Tyler, Texas, and was taken to the Caldwell Zoo in Tyler. The turtle was drastically underweight. No leeches were present although examination of a stained blood film revealed the presence of haemogregarine-like inclusions in the red blood cells. Unfixed blood films were sent to the Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas, for parasite identification.

A blood film was methanol fixed and Giemsa stained (AccuStain, Sigma) for microscopic examination by light microscopy at $\times 1000$ magnification under immersion oil. The parasitemia was estimated based on examination of 1000 erythrocytes. Scanning and morphometric data for the blood stage parasites were obtained, and images were captured using an Olympus IX70 microscope (Olympus America, Inc. Center Valley, PA) equipped with Spot Software 5.2 Macro-Photography© 2016 SPOT Imaging (Diagnostic Instruments, Inc. Avon, MA). The length and width for multiple examples of each developmental stage observed were measured in micrometers and the mean and standard deviation determined. The range in length and width for each stage was recorded. The observed morphology of the blood stage parasites in this study was compared with published morphological data on turtle haemogregarines (McAllister 2015; Telford 2009; Telford et al. 2009).

DNA isolation and amplification

The blood film from one slide was scraped into 100 μ l of Dulbecco's phosphate-buffered saline (PBS) using a sterile scalpel blade. DNA was extracted from the blood following the manufacturer's protocol (FlexiGene DNA Extraction kit, Qiagen, Redwood City, CA, USA). The extracted DNA concentration was determined (NanoDropND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA) and adjusted to approximately 100 ng/ μ l.

Forward primer HemoFN (5'-CCGTGGTAATTCTA GAGCTAATACATGAGC-3') and reverse primer HemoRN (5'-GATAAGGTTTACGAACTTTCTATAT TTA-3') were designed from an alignment of haemogregarine 18S rRNA gene sequences in the GenBank database to amplify a gene fragment of ~1550 bp. The amplification reaction was performed according to the manufacturer's instructions (Phusion® High-Fidelity DNA polymerase, FINNZYMES, New England Biolabs, Inc., Ipswich, MA, USA) in a final volume of 25 µl containing ~100 ng of DNA. A negative control reaction (no DNA) was included. The amplification profile was initial denaturation at 98 °C for 2 min followed by 35 cycles of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 2 min with a final extension at 72 °C for 7 min followed by hold at 4 °C (Labnet MultiGene Thermal Cycler, Woodbridge, NJ, USA). All amplicons were electrophoresed through a 1 % agarose gel alongside a 200-bp marker (BioDL200 BioFlux, Bulldog Bio, Portsmouth, NH, USA), stained with ethidium bromide, and visualized under ultraviolet transillumination.

Cloning and phylogenetic analyses

Appropriately sized amplicons of ~1550 bp were directly ligated within 24 h of PCR into the pJET1.2/blunt plasmid vector (CloneJET PCR Cloning Kit, Fermentas, Inc., Burlington, Ontario, Canada) and chemically competent *Escherichia coli* cells (TOP 10 One Shot® INVαF; Invitrogen, Grand Island, NY, USA) transformed following manufacturers' instructions. Twenty transformed colonies were randomly chosen for colony PCR to verify the insert, and five verified colonies were expanded in overnight cultures. Plasmid DNA was extracted from the cultures (EZ-10 Spin Column Plasmid DNA Minipreps Kit, Bio Basic Inc., Amherst, NY, USA), quantified as above and adjusted to a concentration of approximately 150 ng/µl for automated bi-directional sequencing (Eton Bioscience Inc, San Diego, CA, USA) using pJET1.2 sequencing primers (CloneJET PCR Cloning Kit).

The resulting sequences were analyzed using Sequencher Version 5.1 and manually trimmed to remove ambiguous or unreadable data. Chromatogram-based contiguous sequences were generated for each of the five clones, and similarities to 18S rRNA gene sequences in the GenBank database were determined using the NCBI Basic Local Alignment Tool (BLAST) (Altschul et al. 1990). The obtained sequences were deposited in the NCBI GenBank database.

The sequences were aligned using Muscle (<http://www.ebi.ac.uk/Tools/msa/muscle/>) with additional sequences of *Haemogregarina* species and those of closely related genera, and *Cryptosporidium serpentis* as the outgroup, selected from the NCBI GenBank database (Table 1). The sequences in the Muscle alignment were trimmed to equivalent lengths BioEdit (Hall 1999), and the final alignment was used to construct a maximum likelihood tree in DIVEIN (<https://indra.mullins.microbiol.washington.edu/DIVEIN/diver.html>) under the default settings. The resulting tree was viewed and appropriately labelled using Mega 6.0 (Tamura et al. 2013).

Results

Host

The alligator snapping turtle was not treated. It is currently in good health (Fig. 1) and remains in residence at the Caldwell Zoo, Tyler, Texas.

Morphology

Intraerythrocytic *Haemogregarina* sp. forms with morphology typical of different blood stages were found on microscopic examination of a Giemsa-stained blood film (Fig. 2). The parasitemia was <1 %. No extracellular organisms were identified, nor were organisms found within white blood cells.

Among the intraerythrocytic stages observed were trophozoites, premeronts, meronts, and gamonts (Figs. 2 and 3). Trophozoites were the smallest forms and occurred individually in the erythrocyte with length × width dimensions of $8.75 \pm 0.5 \times 3.75 \pm 1.4$ µm (range, 8–9 × 3–5; $n = 4$) (Fig. 2a–c). Trophozoites possessed a prominent eccentric nucleus and vacuolated cytoplasm. Premeronts were the most common form seen in the blood film (Fig. 2d–f). The parasites were elongated with a central or slightly eccentric nucleus located toward the side of the parasite. Premeronts measured $13.42 \pm 0.80 \times 5.95 \pm 0.41$ µm (range, 12–15 × 5–7; $n = 25$). Meronts were elongated and slightly curved, measuring $11.1 \pm 1.7 \times 4.9 \pm 0.7$ µm (range, 10–14 × 4–6, $n = 10$), and contained a single nucleus (Fig. 3a–b) or multiple irregular nuclei (Fig. 3c–f). The immature gamont possessed a nucleus located toward or at one end (Fig. 3g–i) and were 11.6 ± 0.8 µm × 5.1 ± 0.4 µm (range 11–13 × 5–6, $n = 6$). Gamonts were slender and recurved with twin folded approximately equal limbs. The gamont total length was $33 \pm 7.02 \times 3.3 \pm 1.4$ µm (range, 26–40 × 3–4, $n = 4$) with LW 133.75

Table 1 List of taxa used to generate phylogenetic tree

Taxon	GenBank accession no	Reference
<i>Adelina dimidiata</i>	DQ096835	Kopečná et al. 2006
<i>Cryptosporidium serpentis</i>	AF093499	Xiao et al. 1999
<i>Dactylosoma ranarum</i>	HQ224957	Barta et al. 2012
<i>Dactylosoma ranarum</i>	HQ224958	Barta et al. 2012
<i>Haemogregarina balli</i>	HQ224959	Barta et al. 2012
<i>Haemogregarina pellegrini</i>	KM887508	Dvořáková et al. 2015
<i>Haemogregarina pellegrini</i>	KM887509	Dvořáková et al. 2015
<i>Haemogregarina sacaliae</i>	KM887507	Dvořáková et al. 2015
<i>Haemogregarina stepanowi</i>	KF257929	Dvořáková et al. 2014
<i>Haemogregarina stepanowi</i>	KT749877	Dvořáková et al. 2014
<i>Haemogregarina stepanowi</i> (Syria)	KF257927	Dvořáková et al. 2014
<i>Haemogregarina</i> sp.	KF257923	Dvořáková et al. 2014
<i>Haemogregarina</i> sp. (Gabon)	KF257924	Dvořáková et al. 2014
<i>Haemogregarina</i> sp.	KF257925	Dvořáková et al. 2014
<i>Haemogregarina</i> sp. clone 2	KX507246	Current study
<i>Haemogregarina</i> sp. clone 4	KX507247	Current study
<i>Haemogregarina</i> sp. clone 6	KX507248	Current study
<i>Haemogregarina</i> sp. clone 7	KX507249	Current study
<i>Haemogregarina</i> sp. clone 8	KX507250	Current study
<i>Hemolivia mariae</i>	KF992711	KvičEROVÁ et al. 2014
<i>Hemolivia mariae</i>	KF992712	KvičEROVÁ et al. 2014
<i>Hemolivia mauritanica</i>	KF992705	KvičEROVÁ et al. 2014
<i>Hemolivia mauritanica</i>	KF992706	KvičEROVÁ et al. 2014
<i>Hemolivia mauritanica</i>	KF992710	KvičEROVÁ et al. 2014
<i>Hemolivia stellata</i>	KP881349	Barta et al. 2012
<i>Hemolivia</i> sp.	KF992714	KvičEROVÁ et al. 2014
<i>Hepatozoon ayorgbor</i>	EF157822	Sloboda et al. 2007
<i>Hepatozoon</i> cf. <i>clamatae</i>	HQ224963	Barta et al. 2012
<i>Hepatozoon domerguei</i>	KM234648	Barta et al. 2012
<i>Hepatozoon felis</i>	AY620232	Criado-Fornelio et al. 2006
<i>Hepatozoon</i> sp.	HQ224960	Barta et al. 2012
<i>Hepatozoon</i> sp.	FJ719813	Merino et al. 2009

GenBank accession numbers and relevant references are given

$\pm 39.4 \mu\text{m}^2$ (78–160) and L/W 9.6 ± 0.9 (8.6–10.8). The length of the gamont folded limbs ranged from 14 to 15 μm with a width across both limbs of 6–8 μm (Fig. 3j, k). The nucleus was located at one end of the structure in the bend of the limbs. Encapsulated gamonts were rare and appeared as non-staining bodies (Fig. 3l).

A voucher Giemsa-stained specimen slide has been deposited in the Systematics Research Collections, University of Nebraska State Museum, Lincoln, NE, under Accession Number P-2016-057.

Molecular characterization

Genomic DNA from the alligator snapping turtle blood film was obtained with a concentration of 696 ng/ μl . The primary PCR successfully amplified the 18S rRNA gene region with the designed primers, yielding amplicons of the expected size of ~1550 bp. Twenty transformed colonies were verified to contain the proper insert by PCR and five (clones 2, 4, 6, 7, and 8) were selected for sequence analysis. The cloned sequences were 1555 bp

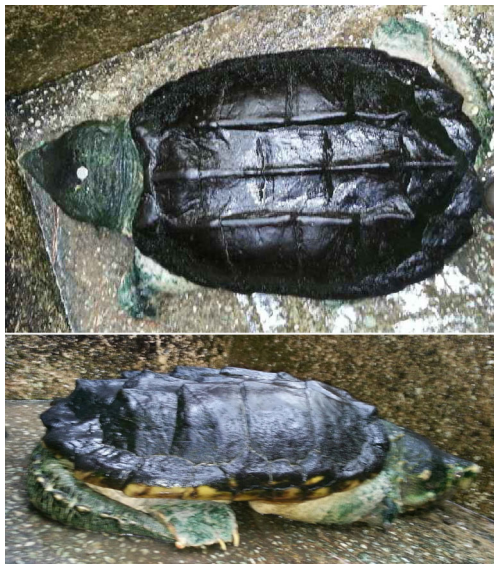


Fig. 1 Dorsal (*top panel*) and lateral (*bottom panel*) views of the alligator snapping turtle *Macrochelys temminckii* infected with *Haemogregarina* sp. A quarter placed on the turtle's head demonstrates his size (*top panel*)

in length and varied in sequence from one another, ranging from 99.3 to 99.9 % identity (Table 2). Clone 6 was the most variable, differing from the other clones in 8 to 10 nucleotide positions (99.3–99.5 % identity).

The 18S rDNA 1555-bp insert sequence of the five clones all showed ~96 % identity to *H. balli* Paterson and Desser, 1976, *Hepatozoon* sp., and *Hemolivia stellata* Petit et al., 1990 (GenBank accession nos. HQ224959, FJ719813, and KP881349, respectively) by BLAST analysis. Higher identity was found between *Haemogregarina* sp. (KR006985) from Iran (99 % identity with clones 2, 6, and 8; 98 % identity with clones 4 and 7) by BLAST analysis of a 774-bp

segment corresponding to that deposited for KR006985. The obtained sequences were deposited in the NCBI Genbank database under accession numbers KX507246, KX507247, KX507248, KX507249, and KX507250 for *Haemogregarina* sp. clones 2, 4, 6, 7, and 8, respectively.

The five cloned sequences were aligned with 26 18S rRNA gene sequences from closely related taxa, and from *Adelina dimidiata* and *C. serpentis* (Table 1). The final aligned length was 1382 bp after trimming sequences as needed to uniform corresponding fragments. The maximum likelihood phylogenetic tree generated from the final alignment shows three well-supported clades of the adeleorinid parasites infecting a variety of hosts (amphibians, reptiles, and mammals) (Fig. 4). The first clade contains two isolates of *Dactylosoma ranarum* (Kruse, 1890), the second contains *Haemogregarina* spp., and the third clade includes both *Hemolivia* spp. and *Hepatozoon* spp. but branches into two separate clusters, one holding the seven *Hemolivia* spp. isolates and one holding the five *Hepatozoon* spp. isolates. The tree topology shows the *Haemogregarina* sp. cloned sequences from this study within the *Haemogregarina* spp. clade, but in a separate branch clustered together, distinct from the previously reported sequences (Fig. 4).

Discussion

The alligator snapping turtle in this report was found in the winter in an emaciated state. The finding of intraerythrocytic parasites caused speculation regarding the possible impact of the infection on the turtle's health. However, generally, infections by haemogregarine blood parasites are considered benign, despite being persistent (Davis and Sterrett 2011). Davis and Sterrett (2011) sug-

Fig. 2 Morphology of *Haemogregarina* sp. trophozoite (a–d) and premeront (d–f). Intraerythrocytic parasites are indicated by white arrows. $\times 1000$, Giemsa stain. Scale bar = 10 μ m

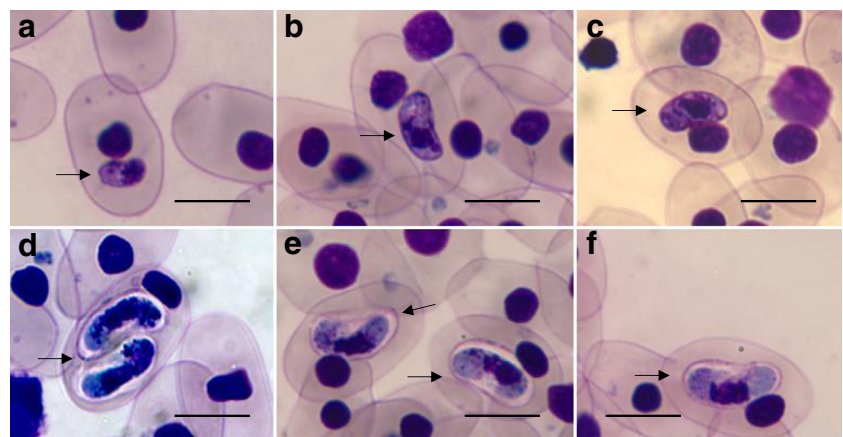
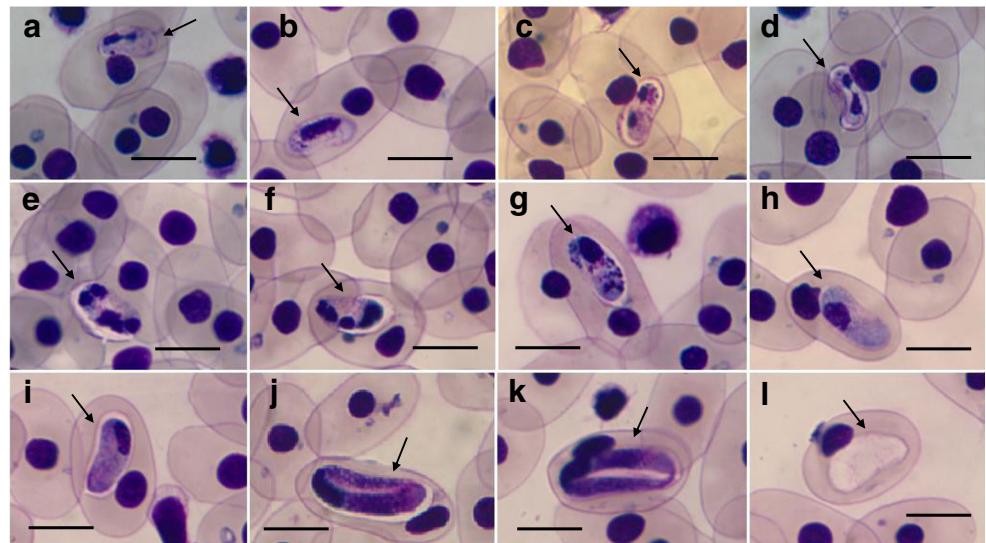


Fig. 3 Morphology of *Haemogregarina* sp. meront with single nucleus (a, b), with 2 nuclei (c, d), and multi-nucleated (e, f). Immature gamont (g–i), gamont (j, k), and encapsulated gamont (l) are shown. Intraerythrocytic parasites are indicated by white arrows. $\times 1000$, Giemsa stain. Scale bar = 10 μm



gest that the high incidence of haemogregarine infections in aquatic turtles, averaging 70 % in North American published reports, is evidence of the benign effect of these parasites on these hosts.

Intracellular haematozoa (Order Eucoccidioida, suborder Adeleorina) were found at a low parasitemia in erythrocytes from the alligator snapping turtle in this study. The morphology and morphometric data for the parasite forms indicate conformity to *Hemogregarina* spp. previously reported in freshwater turtles in the USA (Davis and Sterrett 2011; Telford et al. 2009). The intraerythrocytic premeront and immature gamont in the current report are similar to the haemogregarine small and large forms, respectively, described in *M. temminckii* in Arkansas (McAllister et al. 1995). However, the medium form described as resembling a microgamont by McAllister and others (1995), was not seen in the blood film from the Texas alligator snapping turtle in this study.

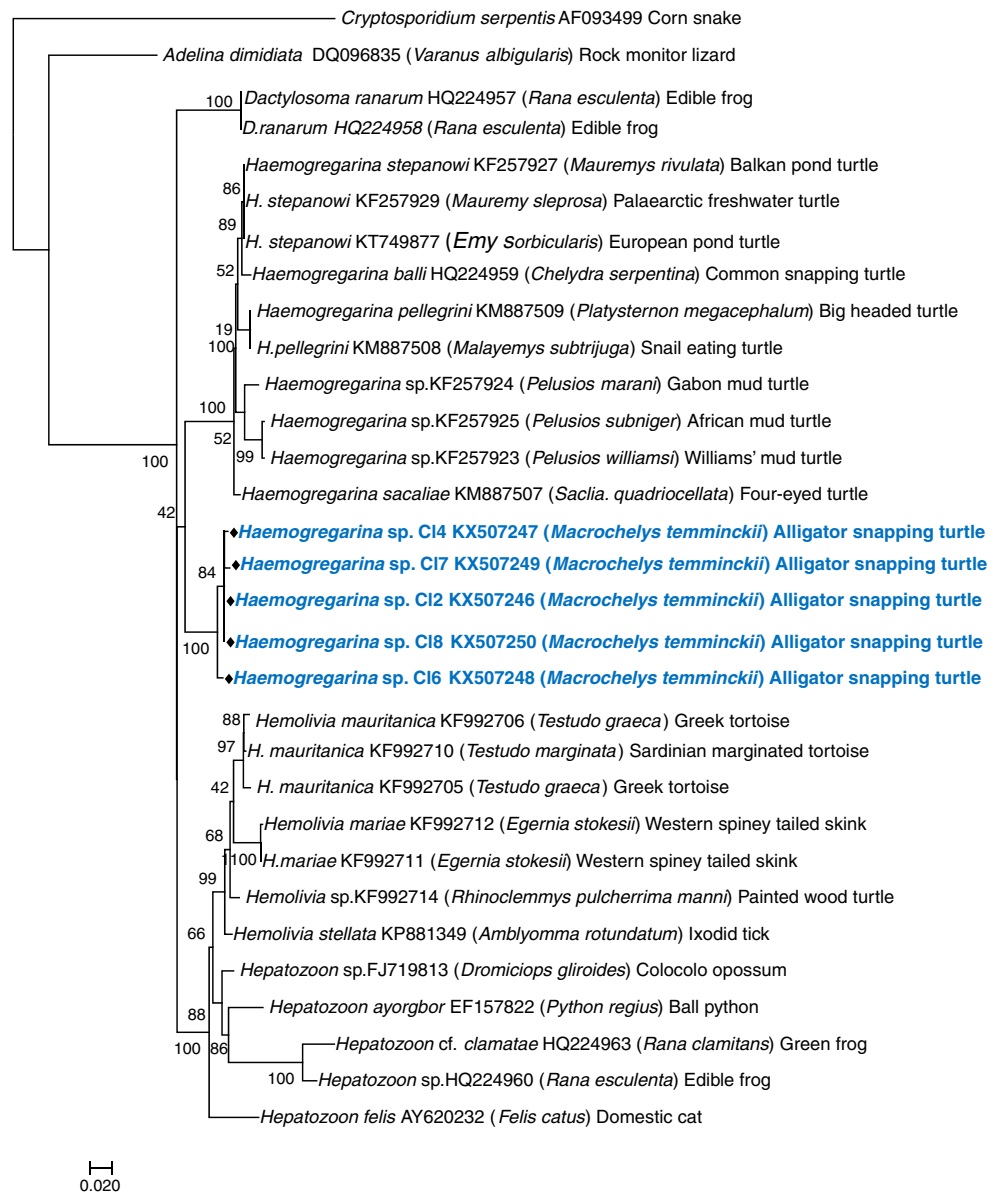
The various trophozoites, premeronts, meronts, and gamonts observed in the current study generally resemble those seen in *Haemogregarina macrochelysi* n. sp. (Apicomplexa: Haemogregarinidae) from alligator snapping turtles in the states of Georgia and Florida (Telford et al. 2009). However, Telford et al. (2009) described *H. macrochelysi* n. sp. as set apart from all other described species by macromeronts that contained 150 or more nuclei in circulating blood. These were not seen in the *Haemogregarina* sp. in this current study; no more than 4 nuclei within a meront was observed. Another distinguishing feature between the two haemogregarines from this host is the appearance of the meront nuclei. Multiple nuclei within a meront were uniform in shape and size in *H. macrochelysi* n. sp., whereas they were irregular in the *Haemogregarina* sp. In the current study, encapsulated gamonts were observed, which were not reported for *H. macrochelysi* n. sp. (Telford et al. 2009). We were unable to determine the invertebrate vector for the

Table 2 Upper matrix (unshaded) shows percent differences in nucleotide sequences, and the lower matrix (shaded) shows the number of base differences between clones of *Haemogregarina* sp. from the alligator snapping turtle

Clone	2	4	6	7	8
	KX507246	KX507247	KX507248	KX507249	KX507250
2	100	99.8	99.4	99.6	99.9
4	1	100	99.3	99.7	99.9
6	9	10	100	99.3	99.5
7	4	5	10	100	99.7
8	1	1	8	3	100

Assigned GenBank accession numbers for each clone are listed

Fig. 4 Maximum likelihood phylogenetic tree based on 18S rRNA gene sequences with bootstrap support at branches. Cloned sequences for the *Haemogregarina* sp. found in the alligator snapping turtle in this study are indicated by *black diamonds*



Haemogregarina sp. in the alligator snapping turtle in this study. Whether the haemogregarine in the alligator snapping turtle in this current study is conspecific with *H. macrochelysi* n. sp. or with the haemogregarine previously described in the alligator snapping turtle in Arkansas remains to be definitively determined. While molecular comparisons would undoubtedly resolve this question, no genetic data are available for either the Arkansas isolate or *H. macrochelysi* n. sp. at this time.

Molecular characterization of the *Haemogregarina* sp. in the current study was based on 18S rRNA gene sequence analysis of five clones. As previously reported in cloned haemogregarine 18S rRNA genes (Perkins and Keller 2011), sequence variation was found among the five

Haemogregarina sp. clones and none was identical to each other. Nonetheless, the clones were more like one another than to other reported 18S rDNA sequences for haemogregarines. Interestingly, the highest sequence identity (98–99 %) was to an unnamed *Haemogregarina* sp. found in the Caspian freshwater turtle *Mauremys caspica* in Fars Province in southern Iran (Rakhshandehroo et al. 2016). Only a short 18S rDNA sequence (774 bp) is available for this parasite (GenBank accession no. KR006985), precluding its incorporation in the phylogenetic analysis. *H. balli* found in the common snapping turtle *Chelydra serpentina serpentina* Linnaeus, 1758, might be expected to be more closely related to a *Haemogregarina* sp. of an alligator snapping turtle, but a comparison of this corresponding 18S rRNA gene fragment shows only 96.3 % identity between

the two. Unfortunately, there are no genetic data from US haemogregarines available for comparison to those obtained in this study for the *Haemogregarina* sp. in the alligator snapping turtle.

This study entails morphological and molecular characterization of a *Haemogregarina* sp. in an alligator snapping turtle in Texas. The findings of this study compare with available morphological data of haemogregarine developmental stages previously described and provide the first genetic data for a *Haemogregarina* sp. in this hemisphere.

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