Made available by Hasselt University Library in https://documentserver.uhasselt.be

Monophyly of the species of Hepatozoon (Adeleorina: Hepatozoidae) parasitising (African) anurans, with the description of three new species from hyperoliid frogs in South Africa

Non Peer-reviewed author version

Netherlands, Edward C.; Cook, Courtney A.; Du Preez, Louis H.; VANHOVE, Maarten; Brendonck, Luc & Smit, Nico J. (2018) Monophyly of the species of Hepatozoon (Adeleorina: Hepatozoidae) parasitising (African) anurans, with the description of three new species from hyperoliid frogs in South Africa. In: PARASITOLOGY, 145(8), p. 1039-1050.

DOI: 10.1017/S003118201700213X
Handle: http://hdl.handle.net/1942/27549

Monophyly of the species of *Hepatozoon* (Adeleorina: Hepatozoidae) parasitising (African) anurans, with the description of three new species from hyperoliid frogs in South Africa

Journal:	*Parasitology*
Manuscript ID	PAR-2017-0291.R1
Manuscript Type:	Research Article - Standard
Date Submitted by the Author:	n/a
Complete List of Authors:	Netherlands, Edward; North-West University, Water Research Group, Unit for Environmental Sciences and Management; North-West University, African Amphibian Conservation Research Group, Unit for Environmental Sciences and Management; University of Leuven, Laboratory of Aquatic Ecology, Evolution and Conservation Cook, Courtney; North West University, Water Research Group, Unit for Environmental Sciences and Management; University of the Free State - Qwaqwa Campus, Department of Zoology and Entomology Du Preez, Louis; North-West University, African Amphibian Conservation Research Group, Unit for Environmental Sciences and Management; South African Institute for Aquatic Biodiversity, Amphibian Collection Facility Vanhove, Maarten M. P. ; Royal Belgian Institute of Natural Sciences, Capacities for Biodiversity and Sustainable Development, Operational Directorate Natural Environment; University of Leuven, Laboratory of Biodiversity and Evolutionary Genomics; Universiteit Hasselt, Centre for Environmental Sciences, Research Group Zoology: Biodiversity & Toxicology; Masaryk University, Department of Botany and Zoology, Faculty of Science Brendonck, Luc; University of Leuven, Laboratory of Aquatic Ecology, Evolution and Conservation; North-West University, Water Research Group, Unit for Environmental Sciences and Management Smit, Nico; North West University, Water Research Group, Unit for Environmental Sciences and Management
Key Words:	Afrixalus, amphibia, apicomplexan, blood parasite, haemogregarine, Hyperoliidae, Hyperolius, morphology, phylogenetic analysis

SCHOLARONE™
Manuscripts

Monophyly of the species of *Hepatozoon* (Adeleorina: Hepatozoidae) parasitising (African) anurans, with the description of three new species from hyperoliid frogs in South Africa

EDWARD C. NETHERLANDS[1,2,3]*, COURTNEY A. COOK[1,4], LOUIS H. DU PREEZ[2,5], MAARTEN P.M. VANHOVE[6,7,8,9], LUC BRENDONCK[1,3] *and* NICO J. SMIT[1,5]

[1] Water Research Group & [2] African Amphibian Conservation Research Group, Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa

[3] Laboratory of Aquatic Ecology, Evolution and Conservation, University of Leuven, Charles Debériotstraat 32, B-3000 Leuven, Belgium

[4] Department of Zoology and Entomology, University of the Free State, QwaQwa campus, Free State, South Africa.

[5] South African Institute for Aquatic Biodiversity, Somerset Street, Grahamstown 6140, South Africa

[6] Capacities for Biodiversity and Sustainable Development, Operational Directorate Natural Environment, Royal Belgian Institute of Natural Sciences, Vautierstraat 29, B-1000 Brussels, Belgium

[7] Laboratory of Biodiversity and Evolutionary Genomics, University of Leuven, Charles Debériotstraat 32, B-3000 Leuven, Belgium

[8] Hasselt University, Centre for Environmental Sciences, Research Group Zoology: Biodiversity & Toxicology, Agoralaan Gebouw D, B-3590 Diepenbeek, Belgium

[9] Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, CZ-611 37 Brno, Czech Republic

Running title: Netherlands *et al.* Three new hyperoliid frog *Hepatozoon* spp.

Corresponding author:

*EC Netherlands, Water Research Group & African Amphibian Conservation Research Group, Unit for Environmental Sciences and Management, Potchefstroom Campus, North-West University, Private Bag X6001, Potchefstroom, 2520, South Africa. Telephone number: +27(0)18 299 2515; Fax: +27(0)18 299 2503; e-mail: ec.netherlands@gmail.com

For Peer Review

SUMMARY

Haemogregarines (Apicomplexa: Adeleiorina) are a diverse group of haemoparasites reported from almost all vertebrate classes. The most commonly recorded haemogregarines to parasitise anurans are species of *Hepatozoon* Miller, 1908. To date 16 *Hepatozoon* species have been described from anurans in Africa, with only a single species, *Hepatozoon hyperolli* (Hoare, 1932), infecting a member of the Hyperoliidae. Furthermore, only two *Hepatozoon* species are known from South African anurans, namely *Hepatozoon theileri* (Laveran, 1905) and *Hepatozoon ixoxo* Netherlands, Cook and Smit, 2014, from *Amietia delalandii* (syn. *Amietia quecketti*) and three *Sclerophrys* species respectively. Blood samples were collected from a total of 225 individuals representing nine hyperoliid species from several localities throughout northern KwaZulu-Natal, South Africa. Twenty frogs from three species were found positive for haemogregarines, namely *Afrixalus fornasinii* (6/14), *Hyperolius argus* (2/39), and *Hyperolius marmoratus* (12/74). Based on morphological characteristics, morphometrics, and molecular findings three new haemogregarine species, *Hepatozoon involucrum* Netherlands, Cook and Smit n. sp., *Hepatozoon tenuis* Netherlands, Cook and Smit n. sp. and *Hepatozoon thori* Netherlands, Cook and Smit n. sp., are described from hyperoliid hosts. Furthermore molecular analyses show anuran *Hepatozoon* species to be a separate monophyletic group, with species isolated from African hosts forming a monophyletic clade within this cluster.

Key words (3-10): *Afrixalus*, amphibia, apicomplexan, blood parasite, haemogregarine, Hyperoliidae, *Hyperolius*, morphology, phylogenetic analysis.

For Peer Review

KEY FINDINGS

1) New diversity of haemogregarines observed in the Hyperoliidae.

2) Based on morphological and molecular findings three new *Hepatozoon* species described.

3) Anuran *Hepatozoon* species separate monophyletic group.

INTRODUCTION

Haemogregarines (Apicomplexa: Adeleiorina) are heteroxenous, intraerythrocytic or intraleucocytic parasites, infecting a broad range of vertebrate intermediate hosts including amphibians, reptiles, fishes, birds and mammals. These parasites are possibly transmitted by an equal diversity of haematophagous invertebrate definitive hosts or vectors, such as dipteran insects, ticks, mites, leeches, and even gnathiid isopods (see Smith 1996; Davies and Johnston 2000; Curtis *et al.* 2013). Haemogregarines are currently divided into four families (Barta *et al.* 2012), namely Dactylosomatidae Jakowska and Nigrelli, 1955, Haemogregarinidae Léger, 1911, Hepatozoidae Miller, 1908, and Karyolysidae Labbé, 1894.

Within the Hepatozoidae, *Hepatozoon* Miller, 1908 is characterised by the presence of gamonts in erythrocytes or leucocytes, with no merogonic division occurring in the peripheral blood of the vertebrate host. Furthermore, *Hepatozoon* species are characterised by the pairing (syzygy) of gamonts in the definitive invertebrate host or vector following a blood meal. These paired gamonts then penetrate the gut wall and enter the haemocoel where sporogonic development and ultimately the formation of large oocysts occur. These thick-walled oocysts (also known as large multisporocystic oocysts) contain sporocysts with sporozoites, the infective stages of the parasite, which emerge upon the ingestion by the intermediate vertebrate host and give rise to merogonic stages in the liver (Desser 1995; Smith 1996; Barta 2000).

Hepatozoon species are the most commonly reported haemogregarines to parasitise anurans. Currently, there are 45 recognised species from anurans globally, with 16 of these described from African hosts (see Smith 1996; Netherlands *et al.* 2014a,b). According to Netherlands 2014a, the majority of these species (12/16) were described from the Bufonidae, namely *H. aegyptia* (Mohammed and Mansour, 1963), *H. assiuticus* (Abdel-Rahman, El-Naffar, Sakla and Khalifa, 1978), *H. boueti* (França, 1925), *H. faiyumensis* (Mansour and Mohammed, 1966), *H. francai* (Abdel-Rahman, El-Naffar, Sakla and Khalifa, 1978), *H. froilanoi* (França, 1925), *H. ixoxo* Netherlands, Cook and Smit, 2014, *H. lavieri* (Tuzet and Grjebine, 1957), *H. magni* (Hassan, 1992), *H. moloensis* (Hoare, 1920), *H. pestanae* (França, 1910), and *H. tunisiensis* (Nicolle, 1904). Two species were described from the Ptychadenidae, namely *H. epuluensis* (van den Berghe, 1942), and *H. neireti* (Laveran, 1905), and only a single species from the Pyxicephalidae and Hyperoliidae, namely *H. theileri*

For Peer Review

(Laveran, 1905), and *H. hyperolli* (Hoare, 1932) respectively. Apart from *H. hyperolli,* which was described from an unidentified *Hyperolius* species in Uganda (Hoare 1932), the only other *Hepatozoon* species reported from the Hyperoliidae are two unnamed species reported in *Hyperolius marmoratus* and *Hyperolius puncticulatus*, from northern KwaZulu-Natal (KZN), South Africa (Netherlands *et al.* 2015) and Amani, Tanzania (Ball 1967), respectively. In South Africa, only two *Hepatozoon* species are known from anurans, namely *H. theileri* and *H. ixoxo*, from the pyxicephalid *Amietia delalandii* (syn. *Amietia quecketti*) and three *Sclerophrys* species (Bufonidae) respectively, namely *Sclerophrys pusilla* (syn. *Amietophrynus maculatus*), *Sclerophrys* (syn. *Amietophrynus*) *garmani* and *Sclerophrys* (syn. *Amietophrynus*) *gutturalis*.

Over the past decade several phylogenetic studies on adeleorinid parasites, using 18S rDNA sequences, have provided useful insight into the evolutionary relationships of this group, as well as better capability to distinguish between species. However, because the 18S rRNA nuclear gene is a relatively conserved marker, it shows certain nodes to be unresolved (Barta 2012; Maia *et al.* 2012; Haklová-Kočíková *et al.* 2014; Cook *et al.* 2016). In an effort to resolve these polytomies, a new genus *Bartazoon* Karadjian, Chavatte and Landau, 2015, was proposed for species previously regarded as belonging to *Hepatozoon* parasitising reptiles, amphibians, marsupials, birds and rodents, and was proposed to be transmitted solely by biting insects (Karadjian *et al.* 2015). However, the suggested life history of certain species within the proposed genus such as *Hepatozoon fitzsimonsi* Dias, 1953 do not conform to the recommended characteristic defining *Bartazoon* (see Cook *et al.* 2014; Karadjian *et al.* 2015). Also as pointed out by Maia *et al.* (2016), it is possible that *Hepatozoon perniciosum* Miller, 1908, the type species of the genus *Hepatozoon*, may in fact form part of the newly proposed genus *Bartazoon*, as most other rodent haemogregarine species do. Furthermore, increased work on the phylogenetic relationships of the haemogregarines continues to identify new genetic lineages, showing that *Bartazoon* is not a well-supported monophyletic group (Tomé *et al.* 2016; Maia *et al.* 2016a). Thus, to revise the deeper taxonomy (family and genus level) of haemogregarines based on their phylogenetic affinities and life histories, more studies using faster-evolving markers such as mitochondrial genes (e.g. Leveille *et al.* 2014), elucidating life cycles, and building larger datasets are necessary. Therefore, as suggested and used by Maia *et al.* (2016b) we will continue to refer to species parasitising anuran hosts as species of *Hepatozoon* and not *Bartazoon*.

Prior to the study of Netherlands *et al.* (2014a) all the African anuran *Hepatozoon* species descriptions, ranging from the early 1900s till the late 1970s, were solely based on the morphology of the peripheral blood gamont stages. Unfortunately many of these descriptions were scantily illustrated and incomplete, with almost 60% of the species described from the same host (*Sclerophrys regularis*) and in more or less the same geographical area (see

For Peer Review

Netherlands *et al.* 2014a,b). Thus many of these species may later need to be synonymised once more advanced and standardised methods are used to characterise these haemogregarines. In South Africa only five studies on amphibian haemogregarines have been carried out (Laveran 1905, Fantham 1942, Netherlands *et al.* 2014a,b, Netherlands *et al.* 2015). From these only a single study was a multispecies haemoparasite survey across different anuran families (Netherlands *et al.* 2015), and although in that study several different haemogregarines were observed in anurans, only one hyperoliid species, *Hyp. marmoratus* (as mentioned above) contained a *Hepatozoon* species, which was not identified to species level.

Thus the objectives of the current study were 1) to establish which hyperoliid frog species in northern KZN, South Africa, contain haemogregarines. 2) to determine the species diversity of the haemogregarine parasites observed. 3) to ascertain if any of the haemogregarines found were previously described or reported species and 4) to compare any parasites characterised in the current study with available molecular data for anuran haemogregarines in order to determine their phylogenetic relationships.

MATERIALS AND METHODS

Frog collection and study area

A total of 225 individuals representing nine hyperoliid species, were collected from several localities throughout northern KwaZulu-Natal, South Africa (Fig. 1), following the collection methods described in Netherlands *et al.* (2015). Frogs were identified using du Preez and Carruthers (2009), and identifications were confirmed by one of the authors of this guide (LdP). After processing all specimens were released at site of capture. This study received the relevant ethical approval from the North-West University's AnimCare ethics committee (ethics number: NWU-00372-16-A5).

Processing of samples and light microscopy screening

Blood (> 0.1 ml) was taken from each frog via cardiac or femoral venipuncture and thin blood smears prepared on clean glass slides, air-dried, fixed and stained using Giemsa-stain (FLUKA, Sigma-Aldrich, Steinheim, Germany). The ~~reaming~~ remaining blood was preserved in 70% ethanol for molecular work (ratio~~n~~ 1:15). Stained blood smears were screened at 1000× and images captured and measured using the imaging software NIS Elements Ver. 4 as described by Netherlands *et al.* (2015). Fifty mature gamonts were measured per *Hepatozoon* species. Measurements comprised the parasite's length (including recurved tail when present) and width within its parasitophorous vacuole (PV), and the parasite's nucleus length and width. Measurements of the PV length and width, and the length from mid nucleus to both

For Peer Review

anterior and posterior end of the parasite were also taken. Parasitaemia was calculated per 100 erythrocytes, with ~10^4 erythrocytes examined per blood smear, following previous methods (see Cook *et al.* 2015a).

DNA extraction, PCR amplification, and phylogenetic analyses

Ethanol-preserved blood samples from parasitised frog specimens (n = 10) were used for molecular work. Two additional blood samples of *A. delalandii* parasitised with *H. theileri* and *S. pusilla* parasitised with *H. ixoxo* from a previous study (Netherlands *et al.* 2014a) were added to obtain longer comparative sequences as compared to the previous study by Netherlands *et al.* (2014a). Genomic DNA of haemogregarine species were extracted from the blood samples using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa). Once extracted, DNA was used for polymerase chain reaction (PCR) amplification. The PCR reactions targeted two fragments of approximately 940 nt and 1400 nt of the 18S rRDNA gene. The 18S rRNA gene sequences were amplified using a combination of two primer sets based on previous studies of haemogregarines belonging to *Karyolysus* Labbé, 1894, *Hemolivia* Petit, Landau, Baccam and Lainson, 1990 and *Hepatozoon* (Ujvari *et al.* 2004; Criado-Fornelio *et al.* 2006; Cook *et al.* 2015b, 2016). The first fragment was amplified using HAM-F (5′-GCCAGTAGTCATATGCTTGTC-3′) and HepR900 (5′-CAAATCTAAGAATTTCACCTCTGAC-3′) (see Ujvari *et al.* 2004; Criado-Fornelio *et al.* 2006), and the second fragment HepF300 (5′-GTTTCTGACCTATCAGCTTTCGACG-3′) and 2868 (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (see Ujvari *et al.* 2004; Medlin *et al.* 1988). Conditions for PCR were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles, entailing a 95 °C denaturation for 30 s, annealing at 61 °C for 30 s with an end extension at 72 °C for 2 min, and following the cycles a final extension of 72 °C for 10 min. PCR reactions were performed with volumes of 25 µl, using 12.5 µl Thermo Scientific DreamTaq PCR master mix (2×) (final concentration: 2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM $MgCl_2$), 1.25 µl (10 µM) of each of the primer sets mentioned above, and at least 25 ng DNA. The final reaction volume was made up with PCR-grade nuclease free water (Thermo Scientific). Reactions were undertaken in a Bio-Rad C1000 Touch™ Thermal Cycler PCR machine (Bio-Rad, Hemel Hempstead, UK). Resulting amplicons were visualized under ultraviolet light on a 1% agarose gel stained with gel red using a Bio-Rad GelDoc™ XR+ imaging system (Bio-Rad, Hemel Hempstead, UK). PCR products from each sample were sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) for purification and sequencing in both directions. Resultant sequences were assembled, and chromatogram-based contigs were generated and trimmed using Geneious R9.1 (http://www.geneious.com, Kearse *et al.* 2012). Sequence and species identity was verified against previously published sequences using the Basic Local Alignment

For Peer Review

Search Tool (BLAST) (Altshul *et al.*~~.~~ 1990). Sequences obtained in the current study were deposited in the NCBI GenBank database under the following accession numbers [GenBank: MG041591–MG041605~~TO BE ADDED~~].

For comparison, all 18S rDNA sequences of anuran haemogregarines, longer than 1500 nt (comprising species of *Hepatozoon*, *Hemolivia, Babesiosoma* and *Dactylosoma*) as well as *Hepatozoon sipedon* Smith, Desser and Martin, 1994, [GenBank: JN181157] from the snake *Nerodia sipedon sipedon*, were downloaded from GenBank and aligned to the sequences generated in the current study. *Hepatozoon sipedon* was selected as it was shown by Barta *et al.* (2012) to be sister to *H. catesbianae* (Stebbins, 1904) and *H. clamatae* (Stebbins, 1905), at that point the only two species of *Hepatozoon* of frogs for which 18S rDNA sequences were available. Furthermore, *H. sipedon* first makes use of a frog intermediate host in which tissue development occurs before transmission to its second intermediate snake host (see Smith *et al.* 1994). Thus all species included in the analysis have an anuran host in their life cycle. Although there are other sequences available from a *Hepatozoon* species characterized from the anurans *Pelophylax perezi* [GenBank: KF733812] and *Leptodactylus chaquensis* [GenBank: JX987775], from the Azores in the North Atlantic Ocean, and Pantanal, Brazil respectively, they were not added to our analysis because these concerned shorter fragments (see Harris *et al.* 2013, Leal *et al.* 2015). *Babesiosoma stableri* Schmittner and McGhee, 1961 [GenBank: HQ224961] and *Dactylosoma ranarum* Lankester, 1871 [GenBank: HQ224957; HQ224958] were chosen as the outgroup, as ~~was~~ they were shown by Barta *et al.* (2012) to belong to a sister group to our current ingroup. Sequences were aligned using the MUSCLE alignment tool (Edgar 2004) under the default settings and implemented in Geneious R9.1. The alignment consisted of 14 sequences with a 1,497 nt conserved region selected using the Gblocks 0.91b server (Castresana 2000). To infer phylogenetic relationships both Bayesian inference (BI) and Maximum likelihood (ML) methods were used. The BI analysis was performed using MrBayes 3.2.2 (Huelsenbeck and Ronquist 2001) and the ML analysis was preformed using RAxML Ver. 7.2.8. (Stamatakis 2014) both implemented from within Geneious R9.1. Prior to the analyses a model test was performed to determine the most suitable nucleotide substitution model, according to the Akaike information criterion using jModelTest 2.1.7 (Guindon and Gascuel 2003, Darriba *et al.* 2012). The model with the best AICc score was the Transitional model (Posada 2003) with estimates of invariable sites and a discrete Gamma distribution (TVM+I+ Γ). However, this model was substituted by the General Time Reversible (Tavaré 1986) model (GTR+I+ Γ) in MrBayes and in RAxML, as this was the next model available with the best AICc score. For the BI analysis the Markov Chain Monte Carlo (MCMC) algorithm was run for 10 million generations, sampling every 100 generations, and using the default parameters. The first 25% of the trees were discarded

For Peer Review

as 'burn-in' with no 'burn-in' samples being retained. Results were visualised in Trace (implemented from within Geneious R9.1), to assess convergence and the burn-in period. For the ML analysis nodal support was assessed using 1000 rapid bootstrap inferences. Model-corrected (TVM+I+ Γ) genetic distances were calculated in PAUP version 4.0a152 (Swofford 2002), with the assumed proportion of invariable sites = 0.5988 and the gamma shape parameter = 0.775.

RESULTS

A total of 225 individuals representing nine species from the family Hyperoliidae, namely *Afrixalus aureus* (*n* = 18), *Afrixalus delicatus* (*n* = 13), *Afrixalus fornasinii* (*n* = 14), *Hyperolius argus* (*n* = 39), *Hyperolius marmoratus* (*n* = 74), *Hyperolius tuberlinguis* (*n* = 38), *Hyperolius pusillus* (*n* = 14), *Kassina senegalensis* (*n* = 9), and *Phylctimantis* (syn. *Kassina*) *maculatus* (*n* = 6) were collected and screened for haemogregarines. Twenty frogs (8.9%) from three species were found positive for haemogregarines, specifically *A. fornasinii* (6/14), *Hyp. argus* (2/39), and *Hyp. marmoratus* (12/74) (see Fig. 2A–C). Based on peripheral blood stages, the haemogregarines of the current study conform to the genus *Hepatozoon*. Although possible meront stages were observed in the peripheral blood for one species, these were rare and no merogonic division was detected. Furthermore, these haemogregarines did not compare to the closely related genus *Hemolivia*, as no schizogony or cyst formation in the erythrocytes of the hosts were observed.

Species descriptions

Phylum: Apicomplexa Levine, 1970
Class: Conoidasida Levine, 1988
Order: Eucoccidiorida Léger & Duboscq, 1910
Suborder: Adeleorina Léger, 1911
Family: Hepatozoidae Wenyon, 1926
Genus: *Hepatozoon* Miller, 1908

***Hepatozoon involucrum* Netherlands, Cook and Smit n. sp.**

Type-host: Hyperolius marmoratus Rapp, 1842 (Anura: Hyperoliidae).

Vector: Unknown.

Type-locality: The specimens were collected in the Kwa Nyamazane Conservancy (KNC), KwaZulu-Natal, South Africa (27°23′35″S, 32°08′41″E).

Other localities: St. Lucia on Monzi Farm, KwaZulu-Natal, South Africa (28°26′56″S 32°17′18″E).

For Peer Review

Type-material: Hapantotype, 1 × blood smear from *Hyp. marmoratus* deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number NMB P 467; parahapantotype, 1 × blood smear from *Hyp. marmoratus*; deposited in the Protozoan Collection of the National Museum, Bloemfontein (NMB), South Africa, under accession number NMB P ~~NMB P TO BE ADDED~~468.

Representative DNA sequences: The 18S rRNA gene sequences have been submitted in the GenBank database under the accession numbers MG041591–MG041594~~TO BE ADDED~~.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:~~-~~F73407D7-1E08-4C3C-B066-889058B77C4C~~TO BE ADDED~~. ~~-~~ The LSID for the new name *H.~~-~~epatozoon involucrum* Netherlands, Cook and Smit ~~n. sp.~~ is urn:lsid:zoobank.org:act:~~-~~A43D46E8-5C9F-4405-8907-94D7B02EAEA7~~TO BE ADDED~~.

Etymology: The species epithet is derived from the Latin word *involucrum* meaning envelope or sheath, and is based on the prominent parasitophorous vacuole encircling the gamont.

Description:

Trophozoites: rare, occurring singularly within erythrocytes, oval to rounded, measuring 12.2–12.5 (12.3 ± 0.2) µm long by 4.8–5.7 (4.2 ± 0.6) µm wide (n = 2) with finely vacuolated cytoplasm staining whitish-pink (Fig. 3A–B), note lysis of the host cell nucleus (Fig. 3B). Nucleus containing loosely arranged chromatin, staining pink, measuring 3.7–5.2 (4.5 ± 1.0) µm long by 3.2–4.9 (4.0 ± 1.2) µm wide (n = 2). Mid nucleus position measuring 5.8–7.4 (6.6 ± 1.2) µm to anterior, and 5.4–5.6 (5.5 ± 0.1) µm to posterior.

Meronts: rare, irregular in shape, often with a foamy cytoplasm, staining whitish-blue to purple (Fig. 3C–D), and measuring 9.5 µm long by 8.8 µm wide (n = 1). Nucleus containing loosely arranged chromatin, staining pink to purple, measuring 6.8 µm long by 3.7 µm wide (n = 1).

Immature gamonts: elongated with small-recurved tail, within a vaguely visible parasitophorous vacuole (PV), cytoplasm staining whitish-purple, causing displacement of the host cell nucleus (Fig. 3E). Parasite (including recurved tail) measuring 16.4–23.0 (19.8 ± 1.8) µm long by 4.4–5.7 (5.1 ± 0.4) µm wide (n = 10), PV measuring 14.2–18.4 (15.6 ± 1.3) µm long by 5.2–9.1 (6.5 ± 1.5) µm wide (n = 10). Nucleus rounded, usually situated in the posterior half of the parasite, loosely arranged chromatin, staining purple, and measuring 3.0–7.0 (5.4 ± 1.4) µm long by 2.6–5.6 (3.8 ± 0.9) µm wide (n = 10). Mid nucleus position measuring 10.0–13.7 (11.7 ± 1.4) µm to anterior side, and 6.6–11.1 (8.6 ± 1.6) µm to posterior side (n = 10).

Mature gamonts: elongated and oval, encased in a large PV (Fig. 3F–I); often recurved at both the anterior and posterior poles, and in some cases a clear recurved tail is visible (Fig. 3G arrowhead); infrequent extracellular or free moving gamont (Fig. 3F), as well as single erythrocytes parasitised by two gamonts (Fig. 3I); gamonts cause noticeable

For Peer Review

displacement of the host cell nucleus. Parasite (including recurved tail) measuring 18.7–25.9 (21.8 ± 1.5) µm long by 4.0–6.3 (5.1 ± 0.5) µm wide (n = 50), PV measuring 16.5–20.9 (18.3 ± 1.0) µm long by 6.3–10.8 (8.3 ± 1.1) µm wide (n = 50). Nucleus elongated or loosely arranged, usually situated in the posterior half of the parasite, loose chromatin strands often visible, staining purely-pink, and measuring 4.8–8.9 (6.4 ± 0.9) µm long by 2.2–4.2 (3.2 ± 0.4) µm wide (n = 50). Mid nucleus position measuring 8.4–19.9 (13.8 ± 1.8) µm to anterior side, and 5.4–11.6 (8.2 ± 1.4) µm to posterior side (n = 50). Parasitaemia of all infected individuals (n = 7) in percentage (%) was 1.0–30.0 (8.0 ± 2.0).

Remarks

Based on the morphology and morphometrics of peripheral blood stages in *Hyp. marmoratus*, *H. involucrum* n. sp. does not conform morphologically to any of the 16 currently recognised *Hepatozoon* species in African anurans. The only other named species infecting a member of the Hyperoliidae, is *H. hyperolii*, and can be distinguished from *H. involucrum* n. sp. based on the shape of the former parasite's gamont. The gamont of *H. hyperolii* is cylindrical with rounded ends and a long recurved tail folded onto itself in the absence of a prominent PV (see Fig. 6A–C). In contrast the gamont of *H. involucrum* n. sp. has an elongated and encased gamont, which is often recurved at both the anterior and posterior poles. The mean length and width of *H. involucrum* n. sp., which includes the parasite's PV, is 18.3 µm long by 8.3 µm wide. Although these mean length measurements do overlap with several species namely, *H. faiyumensis*, *H. francai*, *H. moloensis* and *H. neireti*, the mean width in combination with the length of these species do not conform. Overall the gamont measurements of *H. involucrum* n. sp. compare closest to those of *H. moloensis* (18.8 µm long by 7.8 µm wide), which was described from an unidentified *Sclerophrys* species in Molo, Kenya (see Hoare 1920). However, the oval shape, recurved tail and absence of a PV in *H. moloensis* are distinctive and distinguishable from *H. involucrum* n. sp. as described above. Similarly, these distinctive characteristics of *H. involucrum* n. sp, which differentiate it from *H. moloensis*, also differentiate it from other African anuran species of *Hepatozoon*.

In South Africa a *Hepatozoon* species corresponding morphologically to *H. involucrum* n. sp. was reported from the same host and area in an anuran biodiversity blood parasite survey by Netherlands *et al.* (2015), however this parasite was not formally described or named (see Netherlands *et al.* 2015, Fig. 2D).

Globally the species that conforms most closely to *H. involucrum* n. sp. is *Hepatozoon nucleobisecans* (Shortt, 1916) described from the Indian toad *Duttaphrynus melanostictus* (syn. *Bufo melanostictus*). Although the reported gamont length (18.3 µm long) of *H. nucleobisecans*, including the PV, equals the mean length of *H. involucrum* n. sp., the

For Peer Review

width (4.8 µm wide) is almost half. Furthermore the gamont of *H. nucleobisecans* is not recurved at both the anterior and posterior poles within the PV (see Shortt~~,~~ 1916).

***Hepatozoon tenuis* Netherlands, Cook and Smit n. sp.**

Type-host: Afrixalus fornasinii (Bianconi, 1849) (Anura: Hyperoliidae).

Other hosts: Hyperolius argus; *Hyperolius marmoratus.*

Vector: Unknown.

Type-locality: The specimens were collected in St. Lucia on Monzi Farm, KwaZulu-Natal, South Africa (28°26′56″S 32°17′18″E).

Other localities: Kwambonambi/Langepan , KwaZulu-Natal, South Africa (28°39′43″S 32°10′06″E).

Type-material: Hapantotype, 1 × blood smear from *A. fornasinii* deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number NMB P ~~TO BE ADDED~~469; parahapantotypes~~Other voucher material~~, 1 × blood smear from *A. fornasinii*, and ~~*Hyperolius argus* and~~ *Hyperolius marmoratus*; deposited in the Protozoan Collection of the National Museum, Bloemfontein (NMB), South Africa, under accession numbers NMB P ~~TO BE ADDED~~470 and NMB P ~~TO BE ADDED~~471, respectively.

Representative DNA sequences: The 18S rRNA gene sequences have been submitted in the GenBank database under the accession numbers MG041595–MG041599~~TO BE ADDED~~.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:F73407D7-1E08-4C3C-B066-889058B77C4C ~~TO BE ADDED~~. The LSID for the new name *Hepatozoon~~.~~ tenuis* Netherlands, Cook and Smit ~~n. sp.~~ is urn:lsid:zoobank.org:act:AD607D8B-D43D-49C6-8139-2782306FE2F5 ~~TO BE ADDED~~.

Etymology: The species epithet is derived from the Latin word *tenuis*, which means thin or slender. This refers to the long slender shape of the gamont.

Description:

Mature gamonts: slender and elongated, with a pinkish-white staining cytoplasm, within a close-fitting parasitophorous vacuole visible on the concave side of the gamont (Fig. 4A–C); in some cases a recurved tail is visible (Fig. 4A and D arrowhead); also an occasional extracellular or free moving gamont, (Fig. 3E arrow), as well as a single erythrocyte parasitised by two gamonts (Fig. 4F); gamonts cause obvious displacement of the host cell nucleus. Parasites (including recurved tail when visible) measuring 11.2–16.8 (13.9 ± 1.6) µm long by 3.7– 6.7 (4.8 ± 0.6) µm wide (n = 50), PV measuring 17.8–20.7 (19.4 ± 0.8) µm long by 5.0–7.5 (6.7 ± 0.4) µm wide (n = 50). Nucleus elongated and neatly arranged, usually situated in the posterior half of the parasite, loose chromatin staining purely-pink, and measuring 2.1–5.2 (3.9 ± 0.6) µm long by 1.6–4.9 (10.8 ± 0.9) µm wide (n =

For Peer Review

50). Mid nucleus position measuring 4.8–9.4 (6.7 ± 1.1) µm to anterior, and 4.6–10.1 (7.2 ± 1.2) µm to posterior (n = 50). Parasitaemia of all infected individuals (n = 9) calculated in percentage (%) was 1.0–35.0 (6.0 ± 2.0), two (*Hyp. argus* and *Hyp. marmoratus*) of the nine infected individuals contained mixed infections the parasite described below.

Remarks

Hepatozoon tenuis n. sp. parasitising *A. fornasinii*, *Hyp. argus* and, *Hyp. marmoratus*, can be distinguished from *H. involucrum* n. sp., based on the difference in gamont morphometrics. Morphologically, gamonts have an overall similar appearance to *H. involucrum* n. sp., however, gamonts of *H. involucrum* n. sp. measure a mean of 21.8 µm long by 5.1 µm wide (n = 50) (PV not included) and a mean of 18.3 µm long by 8.3 µm wide (n = 50) (PV included), as compared to gamonts of *H. tenuis* n. sp. measuring a mean of 13.9 µm long by 4.8 µm wide (n = 50) (PV not included) and a mean of 19.4 µm long by 6.7 µm wide (n = 50) (PV included). This slender looking parasite can be distinguished from other anuran *Hepatozoon* species based on the marginally visible PV, as well as often being recurved at both the anterior and posterior poles within the PV.

***Hepatozoon thori* Netherlands, Cook and Smit n. sp.**

Type-host: Hyperolius marmoratus Rapp, 1842 (Anura: Hyperoliidae).

Other hosts: Hyperolius argus; *Hyperolius puncticulatus*.

Vector: Unknown.

Type-locality: The specimens were collected in the Kwa Nyamazane Conservancy (KNC), KwaZulu-Natal, South Africa (27°23′35″S, 32°08′41″E).

Other localities: Kwambonambi/Langepan , KwaZulu-Natal, South Africa (28°39′43″S 32°10′06″E); Amani, Tanzania.

Type-material: Hapantotype, 1 × blood smear from *Hyp. marmoratus* deposited in the protozoan collection of the National Museum, Bloemfontein, South Africa under accession number NMB P ~~TO BE ADDED~~472; parahapantotype, 1 × blood smear from *Hyp. marmoratus*; deposited in the Protozoan Collection of the National Museum, Bloemfontein ~~(NMB)~~, South Africa, under accession number NMB P ~~TO BE ADDED~~473.

Representative DNA sequences: The 18S rRNA gene sequences have been submitted in the GenBank database under the accession numbers MG041600–MG041603~~TO BE ADDED~~.

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:F73407D7-1E08-4C3C-B066-889058B77C4C~~TO BE ADDED~~.

The LSID for the new name *Hepatozoon~~.~~ thori* Netherlands, Cook and Smit ~~n. sp.~~ is urn:lsid:zoobank.org:act:00CD84D9-D6A8-4B41-A048-DFD0DBF4B045 ~~TO BE ADDED~~.

For Peer Review

Etymology: The species epithet is derived from Norse mythology after the hammer-wielding god Thor. This is based on the hammer-like shape of the gamont.

Description:

Immature gamonts: rare, elongated without a visible parasitophorous vacuole (PV), cytoplasm staining whitish-purple, measured 18.7 µm long by 5.5 µm wide (n = 1), causing displacement of the host cell nucleus and found parasitising a single erythrocyte together with a mature gamont (Fig. 5A arrow). Nucleus rounded, situated in the posterior half of the parasite, loosely arranged chromatin, staining purple, and measuring 8.1 µm long by 2.7 µm wide (n = 1). Mid nucleus position measured 8.9 µm to anterior side, and 9.8 µm to posterior side (n = 1).

Mature gamonts: elongated, causing displacement of the host cell nucleus. Encased in a prominent hammer-like or boot-shaped PV, with a pseudopodial-like projection (Fig. 5A−F); occasionally a short recurved tail is visible (Fig. 5C−D arrow); mature gamonts cause the host cell nucleus to lyse (Fig. 5E); extracellular or free moving gamont, possibly probing to enter new host cell (Fig. 5F). Parasite measuring 11.2–16.8 (13.9 ± 1.6) µm long by 3.7–6.7 (4.8 ± 0.6) µm wide (n = 50), with the PV measuring 17.8–20.7 (19.4 ± 0.8) µm long by 5.0–7.5 (6.7 ± 0.4) µm wide (n = 50). Parasites, including the recurved tail (see Fig. 5C−D arrow), measuring 19.1–21.7 (20,4 ± 1.1) µm long (n = 5). Nucleus elongated or loosely arranged, usually situated in the posterior half of the parasite, loose chromatin strands often visible, staining purely-pink, and measuring 2.1–5.2 (3.9 ± 0.6) µm long by 1.6–4.9 (10.8 ± 0.9) µm wide (n = 50). Mid nucleus position measured 4.8–9.4 (6.7 ± 1.1) µm to anterior, and 4.6–10.1 (7.2 ± 1.2) µm to posterior (n = 50). Parasitaemia of all infected individuals (n = 6) in percentage (%) was 1.0–21.0 (3.0 ± 2.0), two (*Hyp. argus* and *Hyp. marmoratus*) of the six infected individuals contained mixed infections with *H. tenuis* n. sp.

Remarks

Hepatozoon thori n. sp. parasitising *Hyp. argus* and *Hyp. marmoratus* can be distinguished from *H. involucrum* n. sp., *H. tenuis* n. sp., and other anuran *Hepatozoon* species based on the distinctive shape of the hammer-like or boot-shaped PV that has a pseudopodial-like projection. The mean length and width of the parasite measures 13.9 µm long by 4.8 µm wide (PV not included) and 19.4 µm long by 6.7 µm wide (n = 50) (PV included). Based on the size and shape, the only other haemogregarine *H. thori* n. sp. conforms closest to is an unnamed *Hepatozoon* species (see Fig 6D–E), measuring a mean of 14.1 µm long by 4.8 µm wide (PV not included) and 20.8 µm long by 6.7 µm wide (PV included). This unnamed species was reported in *Hyperolius puncticulatus*, from Amani, Tanzania (see Ball 1967) (see below).

For Peer Review

Phylogenetic analysis

Amplicons of between 1640 and 1701 nt were derived from *H. involucrum* n. sp., *H. tenuis* n. sp., and *H. thori* n. sp. from the blood of *A. fornasinii, Hyp. argus* and *Hyp. marmoratus*. Additionally, sequences of *H. ixoxo* and *H. theileri*, were amplified from the blood collected in a previous study (Netherlands *et al.* 2014a) from *S. pusilla* and *A. delalandii,* respectively. The details of sequences used in the analyses are presented in Table 1.

Based on 1,497 nt sequence comparisons of the 18S rRNA gene (see Table 2), the interspecific divergence (model-corrected genetic distance) between *H. involucrum* n. sp. and its closest relative *H. tenuis* n. sp. was 1.0 %. *Hepatozoon involucrum* n. sp. and *H. thori* n. sp. had an interspecific divergence of 2.0 %, and *H. tenuis* n. sp. and *H. thori* n. sp. differed by 1.8 %. The interspecific divergence between the *Hepatozoon* species parasitising anuran hosts and *Hepatozoon sipedon* Smith, Desser and Martin, 1994 [GenBank: JN181157] was between 7.7–10.6 %. The intergeneric divergence between the *Hepatozoon* species parasitising anuran hosts, and *Hemolivia stellata* Petit, Landau, Baccam and Lainson, 1989 [GenBank: KP881349], *B. stableri* [GenBank: HQ224961] and *D. ranarum* [GenBank: HQ224957; HQ224958] were between 4.9–5.8 %, 8.8–9.6 % and 8.5–9.7 %, respectively (Table 2).

For the phylogenetic analyses the topologies of both the BI and ML trees were similar. The analyses showed *Hemolivia stellata* [GenBank: KP881349] as a well-supported sister taxon to the *Hepatozoon* species cluster, with *H. sipedon* [GenBank: JN181157] shown to be a sister species to a well-supported monophyletic clade comprising *Hepatozoon* species isolated from anuran hosts. The *Hepatozoon* species isolated from African and North American anurans formed two well-supported monophyletic clades, respectively, and were separate from the European species *H. magna* [GenBank: HQ224960]. The African *Hepatozoon* clade represents a polytomy with *H. involucrum* n. sp. and *H. tenuis* n. sp., forming a well-supported monophyletic clade and *H. ixoxo* and *H. theileri*, forming a poorly-supported clade, nested within this polytomy and separate to *H. thori* n. sp.

DISCUSSION

In the present study, we screened the peripheral blood of 225 individual frogs from nine species within the Hyperoliidae. Six species (*A. aureus*, *A. delicates*, *Hyp. tuberlinguis*, *Hyp. pusillus*, *K. senegalensis* and *P. maculatus*), totalling 205 specimens were found negative for haemogregarine parasites. Only 20 frogs from three species were found positive, namely *A. fornasinii* (6/14), *Hyp. argus* (2/39), and *Hyp. marmoratus* (12/74).

For Peer Review

Morphological and molecular data indicate that the haemogregarines parasitising these hosts represent three distinct species of *Hepatozoon*, herein described as *H. involucrum* n. sp. parasitising *Hyp. marmoratus*; *H. tenuis* n. sp., parasitising *A. fornasinii*, *Hyp. argus* and *Hyp. marmoratus*; and *H. thori* n. sp. parasitising *Hyp. argus* and *Hyp. marmoratus*. Mature gamonts of *H. involucrum* n. sp. are characterised by the prominent parasitophorous vacuole (PV) encircling the large gamont, as well as the recurved ends of both poles of the gamont. When compared to *H. tenuis* n. sp., the overall appearance and characteristics are similar, except for a difference in size of the gamont and PV. The interspecific divergence between these two species is 1.0 %. This has been shown in several studies to correspond to species-level differences in haemogregarines and for the slow evolving 18S rRNA marker (see Barta *et al.* 2012; Cook *et al.* 2015b; Borges-Nojosa *et al.* 2017). *Hepatozoon thori* n. sp. can be distinguished from both *H. involucrum* n. sp. and *H. tenuis* n. sp. based on the distinctive hammer-like shape of the gamont's PV. The interspecific divergence between *H. thori* n. sp., *H. involucrum* n. sp. and *H. tenuis* n. sp. was 2.0 % and 1.8 % respectively.

The only other named species of *Hepatozoon* infecting a member of the Hyperoliidae is *H. hyperolii* described in an unidentified *Hyperolius* species by Hoare (1932), this parasite being vermicular in shape and folding over on itself within its host erythrocyte (see Fig 6A–C) and therefore does not conform to any of the *Hepatozoon* species of the present study. However, Ball (1967) reported a second, but unnamed species in *Hyperolius puncticulatus* from Amani, Tanzania, and this species conforms both in size and shape to *H. thori* n. sp. (see Fig 6D–E). In the current study, we propose that these two species are the same, despite parasitising different hosts and possibly being geographically isolated. However, to confirm this, molecular data for this species from Amani, Tanzania is required.

In our phylogenetic analysis, *Hepatozoon* species isolated from anuran hosts formed a well-supported monophyly, separate to other closely related species of *Hepatozoon*. Furthermore, the African clade formed a monophyly, with *H. thori* n. sp. separate from the other species within this clade. *Hepatozoon involucrum* n. sp. and *H. tenuis* n. sp. form a well-supported monophyletic clade nested within the larger African clade. With an interspecific divergence of 1.0 % (model-corrected distance), these two species are closely related, which concurs with their close morphological resemblance. *Hepatozoon ixoxo* and *H. theileri* form a less well supported (0.80/75) monophyletic group. The BI statistical information for the bipartitions of this group showed that apart from the 80 % probability support, only 13 % included *H. thori* n. sp. as part of this clade and 9 % showed *H. theileri* formed a clade with *H. involucrum* n. sp. and *H. tenuis* n. sp., thus explaining the low support of this group. Furthermore, *H. ixoxo* and *H. theileri* differ considerably in morphological structure (see Conradie *et al.* 2017), and if compared to the phylogenetic and morphological relationship of *H. involucrum* n. sp. and *H. tenuis* n. sp. (as mentioned above), the former two species are not

For Peer Review

expected to be sister species. This underlines the importance of increased taxon sampling for these parasites, as the addition of more species to this dataset could result in better-supported clades and the polotomy of the African clade could be resolved. Additionally, faster-evolving markers (e.g. mtDNA) may further explain the biogeography and evolutionary history of these species globally. However, to date, only one haemogregarine, *H. catesbianae* isolated from the frog ~~*Lithobates*~~ *Rana catesbeian~~us~~* has mtDNA sequence data available (see Leveille *et al.* 2014). Although these markers (mtDNA) may be complementary in providing an evolutionary perspective among these parasite groups, a lot more data is required if we want to use similar sized datasets such as those available for 18S rDNA sequences for haemogregarines, especially in terms of vertebrate host diversity (amphibians, reptiles, fishes, birds and mammals) and geographical distribution.

This study highlights the importance of screening anurans from different families and genera in an effort to increase the known biodiversity of these parasites and types of hosts they infect. This study also shows the significance of providing detailed descriptions or reports of species, localities and host records, as we were able to link a species reported by Ball (1967) with *H. thori* n. sp. in the current study based on the morphological details he provided. However, although morphological details are important, the use of them in combination with molecular tools provides a richer dataset with which to work, allowing us to infer historical relationships. Furthermore, if molecular data was available for all the currently recognised species of *Hepatozoon*, those with close morphological characteristics could be correctly distinguished. This stresses the importance of using both of these techniques in combination when describing species, and where possible to provide molecular data for already described species. Future research should, when possible, include faster evolving genes, identification of possible definitive hosts or vectors and life cycle studies.

For Peer Review

ACKNOWLEDGEMENTS

We are grateful to Ndumo Game Reserve, the Kwa Nyamazane Conservancy and Pongola River Company, Bonamanzi Game Reserve, KwaMbonambi/Langepan, and St. Lucia Monzi Farm (Charles and Alisa Wally) for permission to collect samples and do fieldwork. Mr. Nico Wolmarans, Dr. Wynand Vlok, Dr. Leon Meyer and Dr. Donnavan Kurger are thanked for their assistance during field work. Ezemvelo KZN Wildlife is thanked for research permits OP 526/2014, OP 839/2014 and OP 4374/2015. Dr. Wynand Malherbe, NWU, is thanked for designing the study area map. This is contribution number XXX from the NWU-Water Research Group

FINANCIAL SUPPORT

This paper forms part of a VLIR-OUS TEAM funded project (ZEIN21013PR396), co-funded by a Water Research Commission of South Africa funded study (Project K5-2185, NJ Smit). The financial assistance of the National Research Foundation (NRF) towards CAC is also hereby acknowledged (project SFP13090332476), and ECN who is supported by the DAAD-NRF doctoral scholarship (Grant UID: 108803), and the VLIR-OUS university scholarship (ID 0620854 / Contract 000000076310). MPMV is partly supported by the Czech Science Foundation (GBP505/12/G112 – ECIP). Opinions expressed and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the funding bodies.

For Peer Review

583 REFERENCES

584 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local
585 alignment search tool. *Journal of Molecular Biology* 215, 403–410.

586 Barta, J. R. (2000). Suborder Adeleorina Leger, 1911. In An illustrated guide to the protozoa,
587 (ed. Lee, J. J., Leedale, G. F. and Bradbury P. C.), pp. 305–318. Society of Protozoologists,
588 USA.

589 Barta, J. R., Ogedengbe, J. D., Martin, D. S. and Smith, T. G. (2012). Phylogenetic position
590 of the adeleorinid Coccidia (Myzozoa, Apicomplexa, Coccidia, Eucoccidiorida, Adeleorina)
591 inferred using 18S rDNA sequences. *Journal of Eukaryotic Microbiology* 59, 171–180.

592 Ball, G. H. (1967). Blood sporozoans from east African amphibia. *Journal of Eukaryotic*
593 *Microbiology* 14, 521–527.

594 Borges-Nojosa, D. M., Borges-Leite, M. J., Maia, J. P., Zanchi-Silva, D., da Rocha Braga, R.
595 and Harris, D. J. (2017). A new species of *Hepatozoon* Miller, 1908 (Apicomplexa:
596 Adelerina) from the snake *Philodryas nattereri* Steindachner (Squamata: Dipsadidae) in
597 northeastern Brazil. *Systematic Parasitology* 94, 65–72.

598 Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in
599 phylogenetic analysis. *Molecular Biology and Evolution* 17, 540–552.

600 Criado-Fornelio, A., Ruas, J. L., Casado, N., Farias, N. A. R., Soares, M. P., Müller, G.,
601 Brum, J. G. W., Berne, M. E. A., Buling-Saraña, A. and Barba-Carretero, J. C. (2006). New
602 molecular data on mammalian *Hepatozoon* species (Apicomplexa: Adeleorina) from Brazil
603 and Spain. *Journal of Parasitology* 92, 93–99.

604 Conradie, R., Cook, C. A., Preez, L. H., Jordaan, A. and Netherlands, E. C. (2017).
605 Ultrastructural comparison of *Hepatozoon ixoxo* and *Hepatozoon theileri* (Adeleorina:
606 Hepatozoidae), parasitising South African anurans. *Journal of Eukaryotic Microbiology* 64,
607 193–203.

For Peer Review

608 Cook, C. A., Lawton, S. P., Davies, A. J. and Smit, N. J. (2014). Reassignment of the land
609 tortoise haemogregarine *Haemogregarina fitzsimonsi* Dias 1953 (Adeleorina:
610 Haemogregarinidae) to the genus *Hepatozoon* Miller 1908 (Adeleorina: Hepatozoidae) based
611 on parasite morphology, life cycle and phylogenetic analysis of 18S rDNA sequence
612 fragments. *Parasitology* 141, 1611–1620.

613 Cook, C. A., Sikkel, P. C. Renoux, L. P. and Smit, N. J. (2015a). Blood parasite biodiversity
614 of reef-associated fishes of the eastern Caribbean. *Marine Ecology Progress Series* 533, 1–13.

615 Cook, C. A., Netherlands, E. C. and Smit, N. J. (2015b). First *Hemolivia* from southern
616 Africa: reassigning chelonian *Haemogregarina parvula* Dias, 1953 (Adeleorina:
617 Haemogregarinidae) to *Hemolivia* (Adeleorina: Karyolysidae). *African Zoology* 50, 165–173.

618 Cook, C. A., Netherlands, E. C. and Smit, N. J. (2016). Redescription, molecular
619 characterisation and taxonomic re-evaluation of a unique African monitor lizard
620 haemogregarine *Karyolysus paradoxa* (Dias, 1954) n. comb. (Karyolysidae). *Parasites &*
621 *Vectors* 9, 347.

622 Curtis, L. M., Grutter, A. S., Smit, N. J. and Davies, A. J. (2013). *Gnathia aureamaculosa*, a
623 likely definitive host of *Haemogregarina balistapi* and potential vector for *Haemogregarina*
624 *bigemina* between fishes of the Great Barrier Reef, Australia. *International Journal for*
625 *Parasitology* 43, 361–370.

626 Davies, A. J. and Johnston, M. R. L. (2000). The biology of some intraerythrocytic parasites
627 of fishes, amphibians and reptiles. *Advances in Parasitology* 45, 1–107.

628 Darriba, D., Taboada, G. L., Doallo, R. and Posada, D. (2012). jModelTest 2: more models,
629 new heuristics and parallel computing. *Nature Methods* 9, 772.

630 Desser, S. S., Hong, H. and Martin, D. S. (1995). The life history, ultrastructure, and
631 experimental transmission of *Hepatozoon catesbianae* n. comb., an apicomplexan parasite of
632 the bullfrog, *Rana catesbeiana* and the mosquito, *Culex territans* in Algonquin Park, Ontario.
633 *Journal of Parasitology* 81, 212–222.

For Peer Review

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32, 1792–1797.

Guindon, S. and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52, 696–704.

Haklová-Kočíková, B., Hižňanová, A., Majláth, I., Račka, K., Harris, D. J., Földvári, G., Tryjanowski, P., Kokošová, N., Malčeková, B. and Majláthová, V. (2014). Morphological and molecular characterization of *Karyolysus* - a neglected but common parasite infecting some European lizards. *Parasites & Vectors* 7, 555.

Hassan, I. M. (1992). On haemogregarine parasites of the toad *Bufo regularis* in Qena Governorate, upper Egypt with description of a new species. *Haemogregarina magni*. *Journal of the Egyptian-German Society of Zoology* 9, 379–385.

Hoare, C. A. (1920). On some new haemogregarines from British East Africa. *Parasitology* 12, 315–327.

Hoare, C. A. (1932). On protozoal blood parasites collected in Uganda. *Parasitology* 24, 210–224.

Huelsenbeck, J. P. and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755.

Karadjian, G., Chavatte, J. M. and Landau, I. (2015). Systematic revision of the adeleid haemogregarines, with creation of *Bartazoon* n. g., reassignment of *Hepatozoon argantis* Garnham, 1954 to *Hemolivia*, and molecular data on *Hemolivia stellata*. *Parasite* 22, 31.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P. and Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649.

For Peer Review

Laveran, A. (1905). Contribution a l'étude des grandes hémogrégarines des grenouilles. *Comptes Rendus Hebdomadaires des Séances et Memoires de la Société de Biologie* 59, 172–175.

Leal, D. D. M., Dreyer, C. S., da Silva, R. J., Ribolla, P. E. M., dos Santos Paduan, K., Bianchi, I. and O'Dwyer, L. H. (2015). Characterization of *Hepatozoon* spp. in *Leptodactylus chaquensis* and *Leptodactylus podicipinus* from two regions of the Pantanal, state of Mato Grosso do Sul, Brazil. *Parasitology Research* 114, 1541–1549.

Leveille, A. N., Ogedengbe, M. E., Hafeez, M. A., Tu, H. H. and Barta, J. R. (2014). The complete mitochondrial genome sequence of *Hepatozoon catesbianae* (Apicomplexa: Coccidia: Adeleorina), a blood parasite of the green frog, *Lithobates* (formerly *Rana*) *clamitans*. *Journal of Parasitology*, 100, 651–656.

Maia, J. P. M. C., Perera, A. and Harris, D. J. (2012). Molecular survey and microscopic examination of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) in lacertid lizards from the western Mediterranean. *Folia Parasitologica* 59, 241–248.

Maia, J. P., HARRIS, D. J., Carranza, S. and Gomez-Diaz, E. (2016a). Assessing the diversity, host-specificity and infection patterns of apicomplexan parasites in reptiles from Oman, Arabia. *Parasitology* 143, 1730–1747.

Maia, J. P., Carranza, S. and Harris, D. J. (2016b). Comments on the systematic revision of adeleid haemogregarines: Are more data needed? *Journal of Parasitology* 102, 549–552.

Medlin, L., Elwood, H. J., Stickel, S. and Sogin, M. L. (1988). The characterization of enzymatically amplified eukaryotic 16S-Like rRNA coding regions. *Gene* 71, 491–499.

Netherlands, E. C., Cook, C. A. and Smit, N. J. (2014a). *Hepatozoon* species (Adeleorina: Hepatozoidae) of African bufonids, with morphological description and molecular diagnosis of *Hepatozoon ixoxo* sp. nov. parasitising three *Amietophrynus* species (Anura: Bufonidae). *Parasites & Vectors* 7, 552.

For Peer Review

683 Netherlands, E. C., Cook, C. A., Smit, N. J. & du Preez, L. H. (2014b). Redescription and
684 molecular diagnosis of *Hepatozoon theileri* (Laveran, 1905) (Apicomplexa: Adeleorina:
685 Hepatozoidae), infecting *Amietia quecketti* (Anura: Pyxicephalidae). *Folia Parasitologica* 61,
686 239–300.

687 Netherlands, E. C., Cook, C. A., du Kruger, D. J. D., du Preez, L. H. and Smit, N. J. (2015).
688 Biodiversity of frog haemoparasites from sub-tropical northern KwaZulu-Natal, South Africa.
689 *International Journal for Parasitology: Parasites and Wildlife* 4, 135–141.

690 Posada, D. (2003). Using MODELTEST and PAUP* to select a model of nucleotide
691 substitution. *Current Protocols in Bioinformatics*, 6–5.

692 Shortt, H. E. (1917). Notes on two haemogregarines of cold-blooded vertebrates. *Indian*
693 *Journal of Medical Research* 4, 402–413.

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

696 Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis
697 of large phylogenies. *Bioinformatics* 30, 1312–1313.

698 Swofford, D. L. (2002). PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other*
699 *Methods)*, Ver. 4. Sinauer Associates, Sunderland, Massachusetts.

700 Tavaré, S. (1986). Some probabilistic and statistical problems in the analysis of DNA
701 sequences. *Lectures on Mathematics in the Life Sciences* 17, 57–86.

702 Tomé, B., Rato, C., Harris, D. J. and Perera, A. (2016). High diversity of *Hepatozoon* spp. in
703 geckos of the genus *Tarentola*. *Journal of Parasitology* 102, 476–480.
704 doi:http://dx.doi.org/10.1645/15-908

705 Ujvari, B., Madsen, T. and Olsson, M. (2004). High prevalence of *Hepatozoon* spp.
706 (Apicomplexa, Hepatozoidae) infection in water pythons (*Liasis fuscus*) from tropical
707 Australia. *Journal of Parasitology* 90, 670–672.

For Peer Review

Van den Berghe, L. (1942). Parasites du sang des vertébrés. In Exploration du Parc National Albert et du Parc National de la Kagera; Mission L. van den Berghe (1936). volume 1 pp. 1–15. Enquête parasitologique, Brussel, Belgium.

For Peer Review

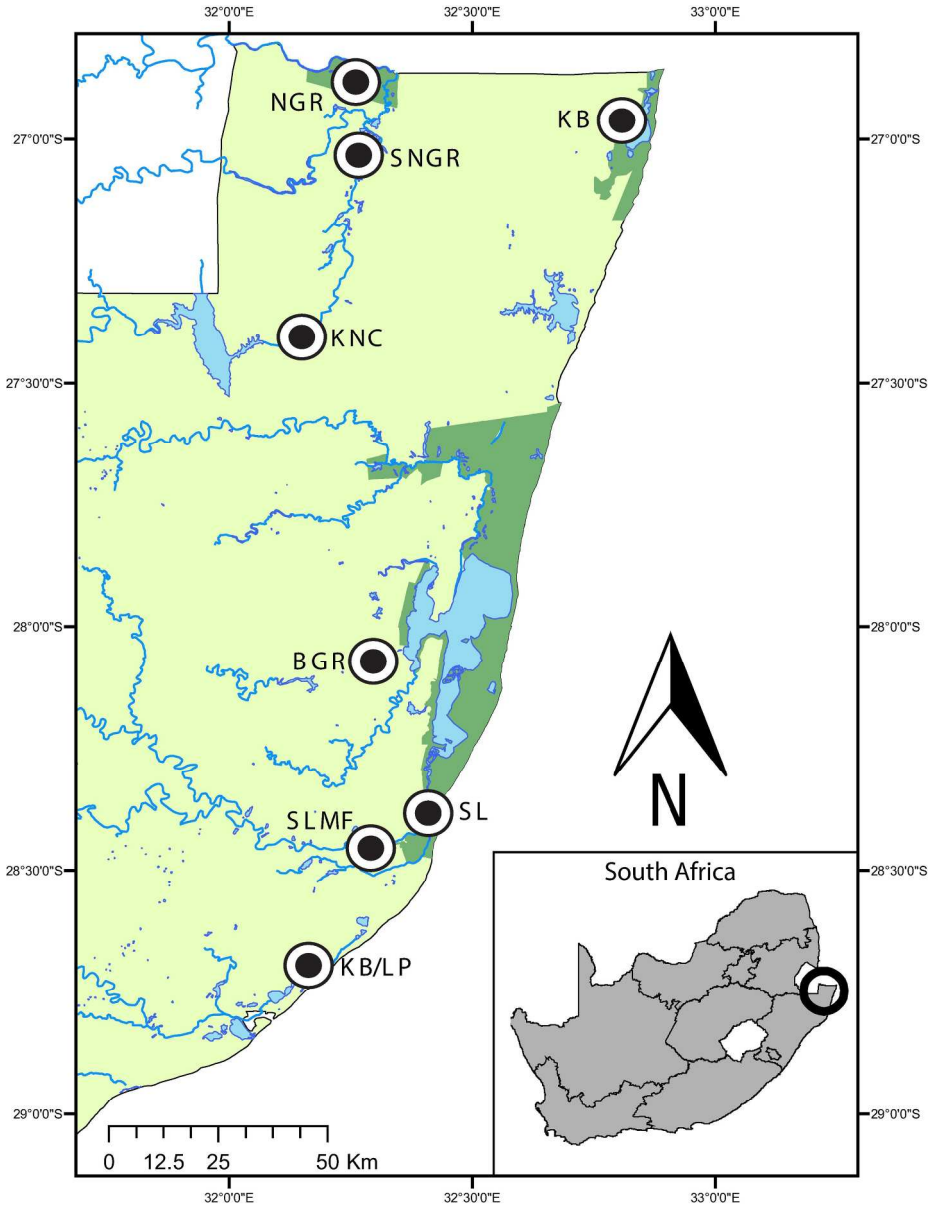

Fig. 1. Map of the sampling localities in northern KwaZulu-Natal, South Africa. Ndumo Game Reserve (NGR) 26°52′00″S, 32°15′00″E, the area directly surrounding the NGR (SNGR) 27°00′13″S, 32°16′50″E, Kwa Nyamazane Conservancy (KNC) 27°23′35″S, 32°08′41″E, Bonamanzi Game Reserve (BGR) 28°03′25″S 32°17′42″E, Kosi Bay (KB) 26°57′16″S 32°48′07″E, KwaMbonambi/Langepan (KB/LP) 28°39′43″S 32°10′06″E, St. Lucia (SL) 28°23′10″S 32°24′29″E and St. Lucia Monzi Farm (SLMF) 28°26′56″S 32°17′18″E.

209x272mm (300 x 300 DPI)

Fig. 2. Three frog species found positive for haemogregarines. (A) Afrixalus fornasinii, (B) Hyperolius argus, and (C) Hyperolius marmoratus.

40x10mm (300 x 300 DPI)

For Peer Review

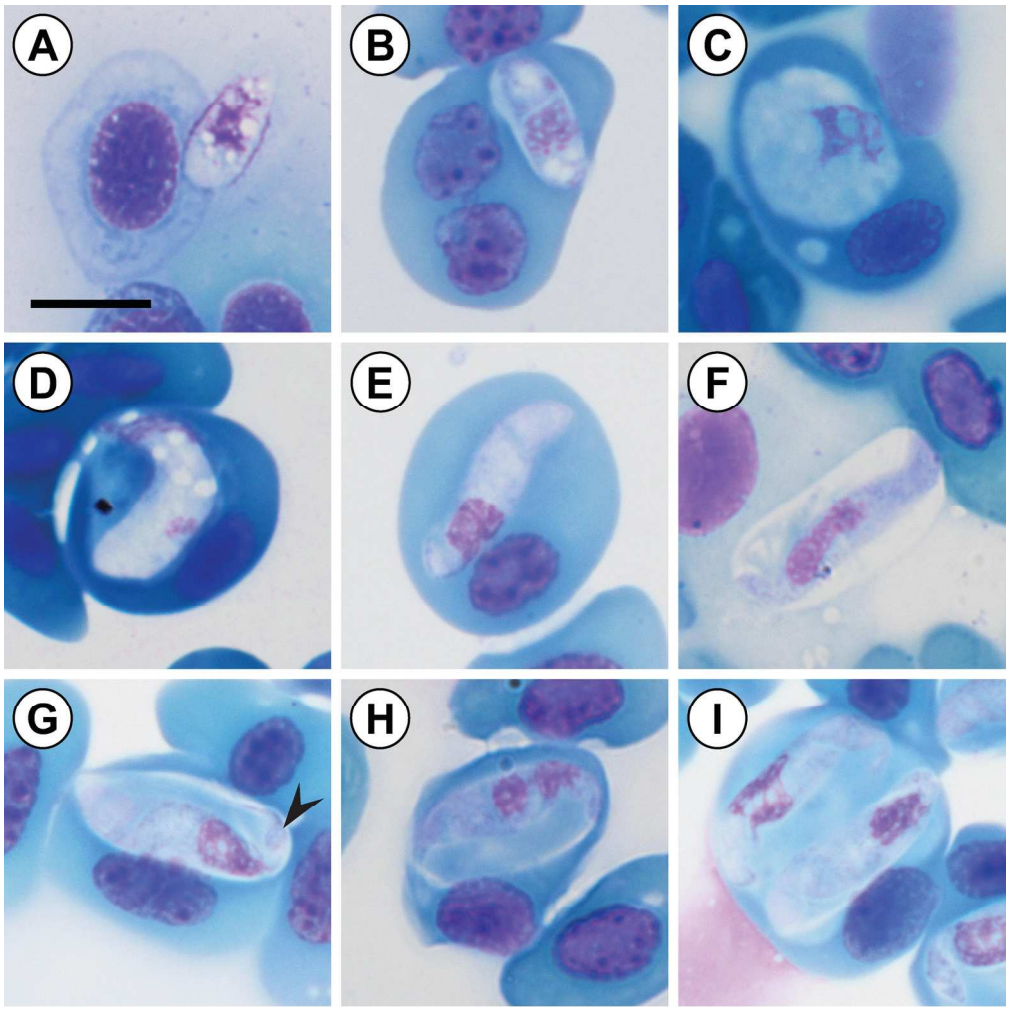

Fig. 3. Hepatozoon involucrum n. sp. in the reed frog Hyperolius marmoratus. (A–B) Trophozoite. (C) Possible meront stage. (D) Possible vacuolated meront stage. (E) Immature gamont stage. (F) Extracellular or free gamont. (G, arrowhead) Mature gamont displaying a recurved tail. (H) Mature gamont, note the expanding parasite nucleus and large parasitophorous vacuole. (I) Double infection of a single erythrocyte. All images captured from the deposited slides (NMB P 467 & 468). Scale bar: 10μm.

165x165mm (300 x 300 DPI)

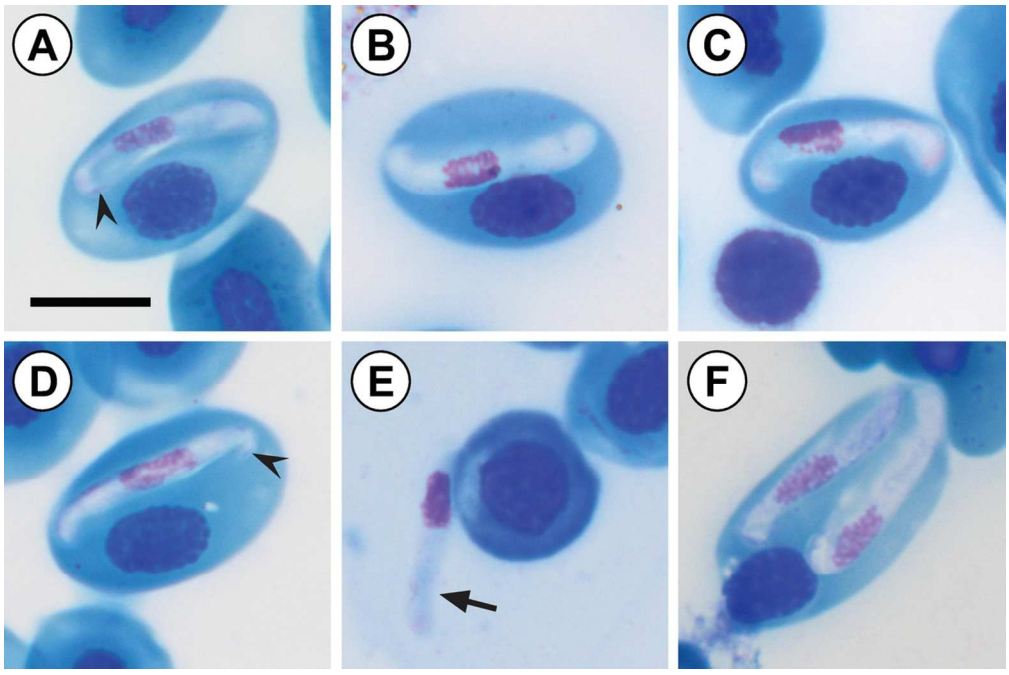

Fig. 4. Hepatozoon tenuis n. sp. mature gamonts parasitising erythrocytes in the folding leaf frog Afrixalus fornasinii (A–C) and the reed frogs Hyperolius marmoratus (D) and Hyperolius argus (E–F). (A–C) Close-fitting parasitophorous vacuole, visible on the concave side of the gamont. (A and D, arrowhead) Gamont with a recurved tail. (E, arrow) Extracellular or free gamont. (F) Double infection of a single erythrocyte. All images captured from the deposited slides (NMB P 469–471). Scale bar: 10μm.

110x72mm (300 x 300 DPI)

Review

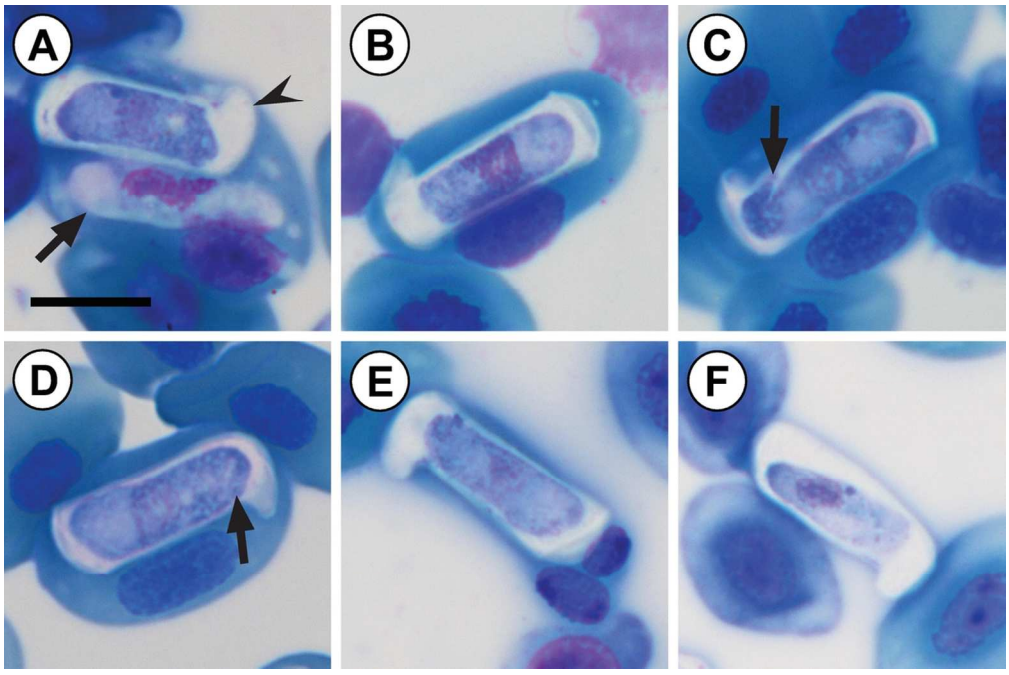

Fig. 5. Hepatozoon thori n. sp. gamonts parasitising erythrocytes in the reed frogs Hyperolius marmoratus (A–C) and Hyperolius argus (D–F). (A) Double infection of a single erythrocyte, with an immature (arrow) and mature (arrowhead) gamont. (B–F) Prominent hammer-like or boot-shaped parasitophorous vacuole, allowing only a certain portion of the gamont to be visible. (C and D, arrow) Gamont displaying a short recurved tail. (E) Gamont causing the host cell nucleus to lyse. (F) Extracellular or free gamont. All images captured from the deposited slides (NMB P 472 & 473). Scale bar: 10μm.

110x72mm (300 x 300 DPI)

view

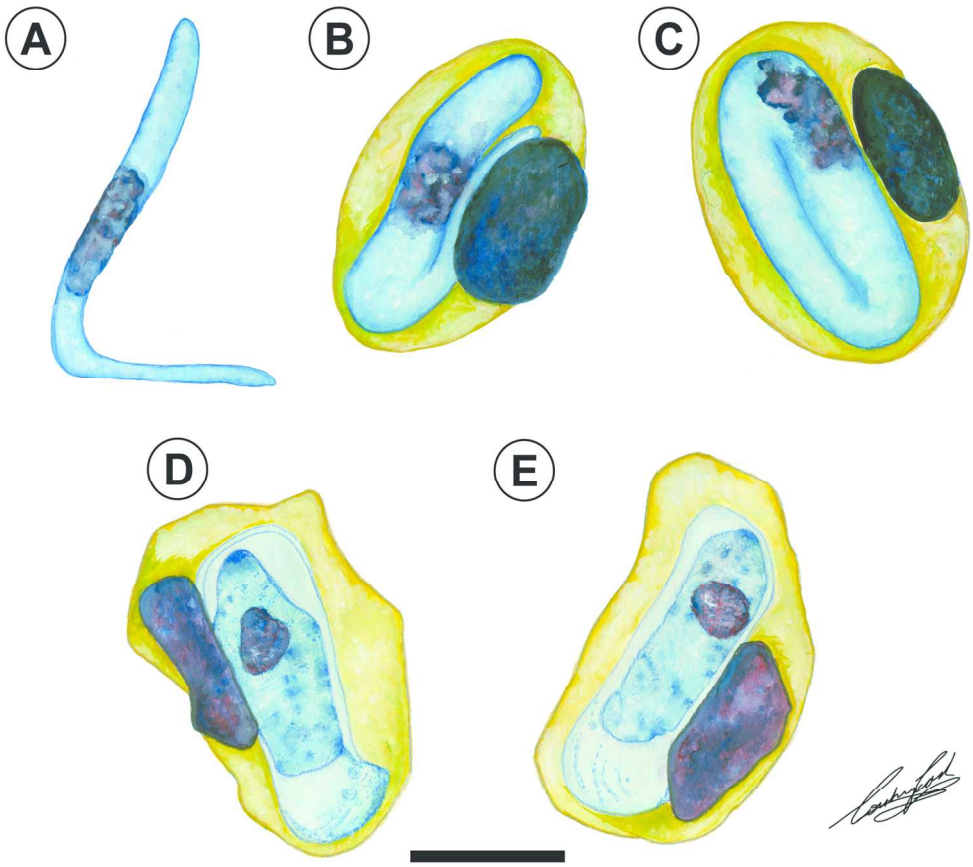

Fig. 6. Illustrations of haemogregarine blood parasites in African hyperoliids. (A–C) Hepatozoon hyperolii Hoare 1932, described from an unidentified Hyperolius species in Uganda. Redrawn and adapted from Hoare (1932). (D–E) Unnamed Hepatozoon species reported in Hyperolius puncticulatus, from Amani, Tanzania. Redrawn and adapted from Ball (1967). Scale bar: 10μm.

153x141mm (300 x 300 DPI)

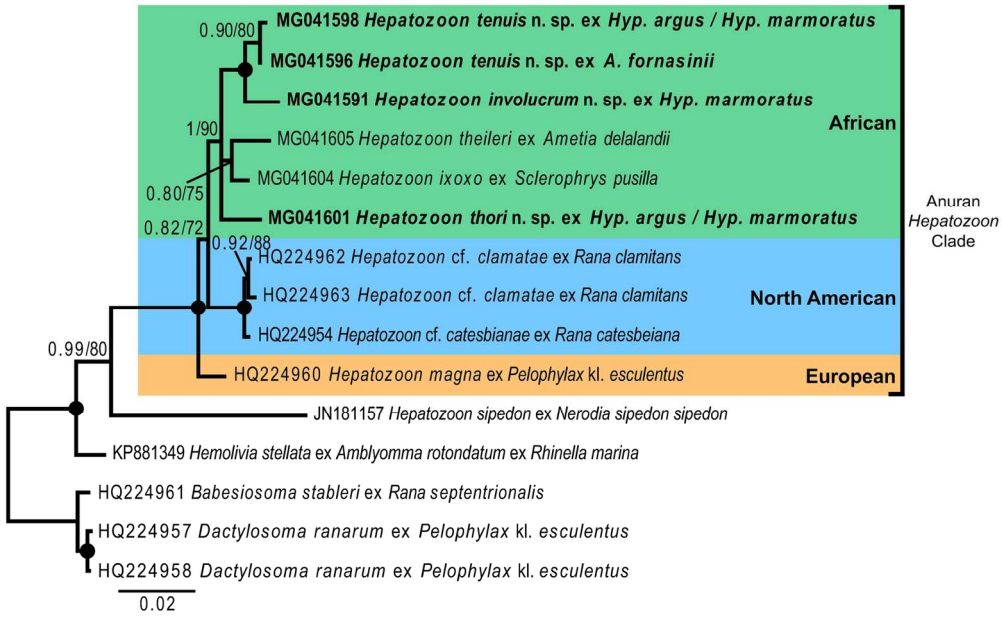

Fig. 7. Consensus phylogram of anuran haemogregarines based on 18S rDNA sequences. Tree topologies for both Bayesian inference (BI) and Maximum Likelihood (ML) analyses were similar (represented on the BI tree), showing the phylogenetic relationships for H. involucrum n. sp., H. tenuis n. sp., and H. thori n. sp. (represented in bold), compared to other species of anuran Hepatozoon (with the exception of Hepatozoon sipedon), Hemolivia, and three species from the Dactylosomatidae as outgroup. Clades that neither produced 0.80 posterior probability (BI) or 70 bootstrap (ML) nodal support values were collapsed. Black circles represent 100% support for both BI/ML. The scale bar represents 0.02 nucleotide substitutions pre site.

156x96mm (300 x 300 DPI)

view

Table 1: List of the sequence (18S rDNA) information used in the current study. The table includes the GenBank accession number, species, host species and the reference study

Accession ~~number~~No.	Species	Host	Reference
MG041591~~XXXXXX~~	*Hepatozoon involucrum* n. sp.	*Hyperolius marmoratus*	Current study
MG041596~~XXXXX~~	*Hepatozoon tenuis* n. sp.	*Afrixlus fornasinii*	Current study
MG041598~~XXXXX~~	*Hepatozoon tenuis* n. sp.	*Hyperolius argus*	Current study
MG041599~~XXXXX~~	*Hepatozoon tenuis* n. sp.	*Hyperolius marmoratus*	Current study
MG041600~~XXXXX~~	*Hepatozoon thori* n. sp.	*Hyperolius argus*	Current study
MG041601~~XXXXX~~	*Hepatozoon thori* n. sp.	*Hyperolius marmoratus*	Current study
MG041605~~XXXXX~~	*Hepatozoon theileri*	*Ametia delalandii*	Current study
MG041604~~XXXXX~~	*Hepatozoon ixoxo*	*Sclerophrys pusilla*	Current study
HQ224962	*Hepatozoon* cf. *clamatae*	~~*Lithobates* ~~*Rana clamitans*	Barta *et al.* (2012)
HQ224963	*Hepatozoon* cf. *clamatae*	~~*Lithobates* ~~*Rana clamitans*	Barta *et al.* (2012)
HQ224954	*Hepatozoon* cf. *catesbianae*	~~*Lithobates* ~~*Rana* ~~*catesbeianus*~~*catesbeiana*	Barta *et al.* (2012)
HQ224960	*Hepatozoon magna*	*Pelophylax* kl. *esculentus*	Barta *et al.* (2012)

For Peer Review

JN181157	*Hepatozoon sipedon*	*Nerodia sipedon sipedon*	Barta *et al.* (2012)
KP881349	*Hemolivia stellata*	*Amblyomma rotondatum* ex *Rhinella marina*	Karadjian *et al.* (2015)
HQ224961	*Babesiosoma stableri*	~~*Lithobates*~~ *Rana septentrionalis*	Barta *et al.* (2012)
HQ224957	*Dactylosoma ranarum*	*Pelophylax* kl. *esculentus*	Barta *et al.* (2012)
HQ224958	*Dactylosoma ranarum*	*Pelophylax* kl. *esculentus*	Barta *et al.* (2012)

For Peer Review

Table 2. Estimates of divergence between partial 18S rDNA sequences from the haemogregarine species used in the current study. Distance matrix showing ranges for the model-corrected genetic distances between the sequences. Alignment length 1,497 nt. Genetic distances shown as percentage (%)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. MG041591 *H. involucrum* n. sp.															
2. MG041596 *H. tenuis* n. sp.	1,0														
3. MG041598 *H. tenuis* n. sp	1,0	0,2													
4. MG041601 *H. thori* n. sp.	2,0	1,8	1,9												
5. MG041605 *H. theileri*	2,1	1,8	1,9	2,1											
6. MG041604 *H. ixoxo*	1,7	1,4	1,4	1,5	1,4										
7. HQ224962 *H.* cf. *clamatae*	2,5	2,2	2,3	2,2	2,4	1,6									
8. HQ224963 *H.* cf. *clamatae*	2,6	2,4	2,5	2,4	2,6	1,7	0,1								
9. HQ224954 *H.* cf. *catesbianae*	2,5	2,2	2,3	2,2	2,4	1,6	0,1	0,3							
10. HQ224960 *H. magna*	2,2	1,7	1,8	1,9	2,1	1,6	1,8	2,0	1,8						
11. JN181157 *H. sipedon*	10,1	9,9	10,0	10,4	10,1	9,6	10,2	10,5	10,2	9,3					
12. KP881349 *Hemolivia stellata*	5,7	5,3	5,2	5,6	5,2	5,5	5,6	5,8	5,6	4,9	7,7				
13. HQ224961 *B. stableri*	9,0	9,0	9,2	9,4	8,9	9,6	9,1	9,3	9,3	9,0	10,6	5,0			
14. HQ224957 *D. ranarum*	9,1	9,1	9,3	9,5	9,0	9,7	9,2	9,4	9,4	9,1	10,7	5,1	0,6		
15. HQ224958 *D. ranarum*	8,7	8,6	8,8	9,0	8,5	9,3	8,8	9,0	9,0	8,6	10,3	4,7	0,3	0,0	

For Peer Review