FOLIA PARASITOLOGICA 61 [4]: 293–300, 2014
ISSN 0015-5683 (print), ISSN 1803-6465 (online)
doi: 10.14411/fp.2014.046

http://folia.paru.cas.cz/

Redescription and molecular diagnosis of *Hepatozoon theileri* (Laveran, 1905) (Apicomplexa: Adeleorina: Hepatozoidae), infecting *Amietia quecketti* (Anura: Pyxicephalidae)

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Abstract: Blood smears prepared from the peripheral blood of 20 wild caught *Amietia quecketti* (Boulenger) from the North-West University Botanical Gardens, North West Province, South Africa, were examined for the presence of haemogregarines. A haemogregarine species comparative in morphology, host and geographical locality to that of *Haemogregarina theileri* Laveran, 1905 was detected. The original description of *H. theileri* was based solely on frog peripheral blood gamont stages. Later, further parasite stages, including trophozoites and merogonic liver stages, were recorded in a related *Amietia* sp. from equatorial Africa. This species was originally classified as a member of the genus *Haemogregarina* Danilewsky, 1885, but due to the close life cycle and morphological resemblance to those of *Hepatozoon* species, *H. theileri* was later transferred from *Haemogregarina* to *Hepatozoon* Miller, 1908. In the present study, meront and merozoite stages not described before, along with previously observed trophozoite, immature and mature gamont stages, are described from the peripheral blood of hosts. In addition, comparative phylogenetic analysis of the partial 18S rDNA sequence of *Hepatozoon theileri* to those of other haemogregarine species, including those of species of *Hepatozoon* and a *Haemogregarina*, support the taxonomic transfer of *H. theileri* to *Hepatozoon*, nesting *H. theileri* within a clade comprising species parasitising other amphibians. This is the first molecular and phylogenetic analysis of an African anuran species of *Hepatozoon*.

Keywords: Amphibia, apicomplexan, blood parasite, frog, haematozoan, haemogregarine, phylogenetic analysis, South Africa

In recent years there has been a strong focus on amphibians as the most threatened vertebrate class (Stuart et al. 2004) and as a result the number of studies and publications on amphibians increased drastically. The number of known species nearly doubled since 1992 (see Köhler et al. 2005) to the current figure of 7 198 (Frost 2014). In southern Africa, the amphibian fauna comprises currently 165 known species of frogs, with the Pyxicephalidae Bonaparte, being the most speciouse, with 48 species reported to date (du Preez and Carruthers 2009, Channing and Baptista 2013, Channing et al. 2013). Frogs are known to harbour a great variety of parasites including monogeneans, digenetic trematodes, cestodes, nematodes, acantocephalans, mites, leeches and protists (du Preez and Carruthers 2009). Despite increase of known anuran species, studies on anuran parasites did not follow the same trend and the known parasite diversity is most likely only a fraction of what exists.

Haemogregarines are among the most commonly recorded apicomplexan protozoans to parasitise frogs. Genera recorded from anurans include *Haemogregarina* Danilewsky, 1885, *Hemolivia* Petit, Landau, Baccam et Lainson, 1990, *Hepatozoon* Miller, 1908, *Lankesterella* Labbé, 1894, and *Schellackia* Reichenow, 1919 (see Davies and Johnston 2000). In the past many anuran haemogregarines were placed in *Haemogregarina* (see Smith 1996). However, Smith (1996) listed all 42 of these species as *Hepatozoon*, based on the developmental stages of these parasites being more characteristic with those of *Hepatozoon* than those of the genus *Haemogregarina*. The genus *Hepatozoon* is, in contrast to *Haemogregarina*, characterised by merogony in the vascular endothelial cells of the vertebrate host, typically without merogony in the peripheral blood erythrocytes, only intraerythrocytic or rarely intraleukocytic gamont stages being present.

Transmission of these protists occurs via the ingestion of a parasitised invertebrate host including mites, ticks, insects, and possibly, but doubtfully, leeches, in which, sporogony typically occurs in the haemocoel (Smith 1996, Davies and Johnston 2000, Van As et al. 2013). *Hepatozoon theileri* (Laveran, 1905) described from a South African frog was one such species of *Haemogregarina* transferred by Smith (1996) to *Hepatozoon*. The aim of this paper is to redescribe this haemogregarine on both morphological and molecular grounds, extending this species' distribution area along with the confirmation of its taxonomic and phylogenetic placement within the genus *Hepatozoon*.

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MATERIALS AND METHODS

Frog collection and blood smear preparation

Specimens of *Amietia quecketti* (Boulenger) (n = 20) were collected by hand at night in the North-West University Botanical Gardens, Potchefstroom (26°40'56"S; 27°05'43"E) during spring 2012 and winter 2013. Specimens found to be infected were maintained for over a period of three months to monitor peripheral blood parasite stages on a monthly basis. Frogs were kept in vivaria and fed on crickets. Blood was taken from the femoral artery or vein, using a 1 ml fixed needle insulin syringe. Thin blood smears were prepared, air-dried, fixed in absolute methanol for ~10 min and stained using Giemsa-stain (FLUKA, Sigma-Aldrich, Steinheim, Germany) for ~20 min following the method detailed by Cook et al. (2009; 2010). Frogs (n = 5) were euthanised using a 5% ethyl-4-aminobenzoate (MS-222, Sandoz, Basel, Switzerland) before organs (liver, lung, spleen, kidney, heart and intestine) were removed and impressions smears were then made on glass slides.

Smears were screened using a 100× immersion oil objective on a Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, Netherlands). Measurements (µm) of peripheral blood stages were taken using the Nikon NIS-Elements microscope imaging software program D3.2 (Nikon). Parasitaemia was calculated per 100 erythrocytes, with ~10^4 erythrocytes examined per blood smear, following Cook et al. (2009).

DNA extraction and phylogenetic analysis

Whole peripheral blood was obtained from a highly parasitised specimen of *A. quecketti* with a *H. theileri* parasitaemia of approximately 0.1% and transferred to a sterile 1.5 ml eppendorf tube. DNA was extracted from the sample using the standard protocol for human or animal tissue and cultured cells as detailed in the NucleoSpin®Tissue Genomic DNA Tissue Kit (Macherey-Nagel, Düren, Germany). To amplify apicomplexan parasite 18S rDNA from the total DNA extracted from the frog sample, polymerase chain reaction (PCR) sequence runs were undertaken in a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad, Hemel Hempstead, UK), using the *Hepatozoon* specific SIGMA primer set HepF300 and HepR900 targeting a part of the 18S rDNA gene (see Ujvari et al. 2004). PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, with an annealing temperature of 60 °C for 30 s, and an extension and final extension step of 72 °C for 1 min and 72 °C for 10 min respectively. Resulting amplicons were visualised using a Bio-Rad GelDoc Imaging System (Bio-Rad). Sequencing reactions were undertaken on PCR products directly using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) in an ICycler thermal cycler (Bio-Rad), after purification using the NucleoSpin®Gel and PCR Clean-up kit (Macherey-Nagel).

Sequences were identified as those of *Hepatozoon* using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/) and comparative haemogregarine sequences identified. The 18S rDNA sequences (as listed with accession numbers in Table 1) from 21 *Hepatozoon*, one *Haemogregarina* and *Dactylosoma* Labbé, 1911, one *Hemolivia*, and one *Adelina* Hesse, 1911 (AF494058) (used as an outgroup) were acquired from GenBank. Sequences were aligned using the MUSCLE sequence alignment tool, visualised and checked, and further processed using the phylogenetic tree constructing programme, all in MEGA5 (http://www.megasoftware.net). Maximum Likelihood (ML) trees were constructed using MEGA5 (Tamura et al. 2011) under the conditions of the Tamura 3-parameter + Gamma model (T92+G) (Tamura 1992). The T92+G model was also identified in MEGA (Nei and Kumar 2000; Tamura et al. 2011), based on having the lowest Bayesian information criteria relative to other models. The Gamma model used to infer evolutionary rate differences among all sites. The ML phylogeny with the highest log likelihood was selected as being the most accurate and best supported reconstruction. The preformed phylogenetic analysis and nodal support, based on the 1 000 bootstrap replicates, was used, with only bootstrap values of >50% shown.

RESULTS

General observation

In a sample of 20 wild caught *Amietia quecketti*, the prevalence of *Hepatozoon theileri* was found to be 25%. Mature gamonts were the most abundant stages in the smears, with two forms of these observed, i.e. a slender and elliptical form. Smaller, more evidently immature gamont stages were also seen. Additionally, possible trophozoite, meront and merozoite stages were examined from only two specimens during spring. Haemogregarine prevalence varied over spring and winter, with 3/10 (30%) and 2/10 (20%), of frogs parasitised respectively. The overall parasitaemia of *H. theileri* for both seasons was calculated as ~0.2% ± 0.1 (0.01–0.6%). An insignificant difference, (P = 0.4) when using the Student's t-test, in the parasitaemias of *H. theileri* in frogs was observed between spring (mean = 0.2%) and winter (mean = 0.05%), spring being higher. Throughout the collection of host specimens no possible vectors were noted feeding on the frogs.

Redescription of *Hepatozoon theileri* (Laveran, 1905) Smith, 1996

Syn. *Pseudohaemogregarina ranae* Awerinzew, 1949

Developmental stages within the blood of *Amietia quecketti* (measurements in µm, expressed as range with mean ± standard deviation in parentheses).

Trophozoites: rare (see above); occurring singularly within mature erythrocytes (Fig. 1A), 6.0–6.4 (6.2 ± 0.3) long by 4.7–5.4 (5.0 ± 0.5) wide (n = 2), rounded, with finely vacuolated cytoplasm stained whitish-pink, nucleus 3.5–3.6 (3.6 ± 0.1) long by 4.4–4.5 (4.5 ± 0) wide (n = 2), located at one pole, loosely arranged chromatin, stained pink.

Meronts: one possibly early stage, intraerythrocytic, uninucleate meront (Fig. 1B), 11.2 long by 9.5 wide (n = 1), cytoplasm stained whitish-pink, with a purplish-pink, granular nucleus, measuring 4.0 in length and 4.9 in width (n = 1); meront causing some degree of host cell hypertrophy and dehaemoglobinisation.

Merozoites: observed when possibly entering an erythrocyte (Fig. 1C, arrow); measuring 6.9–9.4 (8.1 ± 1.8) long by 3.1–4.4 (3.7 ± 0.9) wide (n = 2), slightly curved or bean-like in shape, cytoplasm stained pinkish-purple, nu-

Table 1. Apicomplexan species partial 18S rDNA sequences, derived from GenBank, used for comparative phylogenetic analysis of *Hepatozoon theileri* (Laveran 1905), with *Adelina bambarooniae* as the outgroup. Included are the host classes, the GenBank accession number of the *Hepatozoon* spp., and parasitised host or vectors.

Class	GenBank Acc. No.	Name	Host	Vector
Amphibia	HQ224962	*Hepatozoon* cf. *clamatae* (Stebbins, 1905)	*Lithobates clamitans* (Latreille) (syn. *Rana clamitans*)	*Culex territans* (Walker)
	KJ599676	*Hepatozoon theileri* (Laveran, 1905)	*Amietia quecketti* (Boulenger)	
	AF176837	*Hepatozoon catesbianae* (Stebbins, 1903)	*Lithobates catesbeianus* (Shaw) (syn. *Rana catesbeiana*)	*Culex territans* (Walker)
	HQ224960	*Hepatozoon magna* (Grassi et Feletti, 1891)	*Pelophylax esculentus* (Linnaeus) (syn. *Rana esculentus*)	*Culex territans* (Walker)
	HQ224957	*Dactylosoma ranarum* (Lankester, 1882)	*Pelophylax esculentus*	
Amphibia & Reptilia	JN181157	*Hepatozoon sipedon* Smith, Desser et Martin, 1994	*Nerodia sipedon sipedon* (Linnaeus) *Lithobates pipiens* (Schreber) (syn. *Rana pipiens*)	*Culex pipiens* (Linneaus), *Culex territans* (Walker)
Reptilia	AY252104	*Hepatozoon* sp.	*Liasis fuscus* Peters	
	JQ670908	*Hepatozoon* sp.	*Ophiophagus hannah* (Cantor)	*Aponomma varanensis* Santos Dias
	AY252110	*Hepatozoon* sp.	*Stegonotus cucullatus* (Duméril, Duméril et Bibron)	
	EF125058	*Hepatozoon* sp.	*Cerastes cerastes* (Linnaeus)	
	KC696569	*Hepatozoon* sp.	*Psammophis schokari* (Forskal)	
	AY252106	*Hepatozoon* sp.	*Varanus panoptes* Storr	
	AY252108	*Hepatozoon* sp.	*Varanus scalaris* Mertens	
	HQ734807	*Hepatozoon* sp.	*Timon pater tangitana* (Lataste)	
	HQ292771	*Hepatozoo*n sp.	*Mabuya wrightii* Boulenger	
	HQ734790	*Hepatozoon* sp.	*Ptyodactylus oudrii* Lataste	
	HQ734806	*Hepatozoon* sp.	*Tarentola mauritanica* (Linnaeus)	
	KC512766	*Hemolivia mauritanica* (Sergent et Sergent, 1904)	*Testudo marginata* Schoepff	*Hyalomma aegyptium* (Linnaeus)
	HQ224959	*Haemogregarina balli* Paterson et Desser, 1976	*Chelydra serpentina* (Linnaeus) (syn. *Chelydra serpentine serpentine*)	
Mammalia	FJ719816	*Hepatozoon* sp.	*Abrothrix olivaceus* (Waterhouse)	
	AB771554	*Hepatozoon felis* (Patton, 1909)	*Prionailurus bengalensis euptilurus* (Elliot)	
	HQ829438	*Hepatozoon felis*	*Panthera leo persica* (Meyer)	
	HQ829444	*Hepatozoon felis*	*Panthera pardus fusca* (Meyer)	
	JQ751276	*Hepatozoon* sp.	*Sus scrofa* (Linnaeus)	*Dermacentor atrosignatus* Arthur
	FJ719813	*Hepatozoon* sp.	*Dromiciops gliroides* Thomas	
Insecta	AF494058	*Adelina bambarooniae* Lai-Fook et Dall, 2002	*Dermolepida albohirtum* (Waterhouse)	

cleus 3.0–4.8 (3.9 ± 1.3) long by 2.9–4.2 (3.5 ± 0.9) wide (n = 2), located closer to one pole, loosely arranged chromatin, stained dark pinkish-purple.

Immature gamonts: intraerythrocytic, altering host cell form, causing dehaemoglobinisation and displacement of host cell nucleus, and occasionally host nucleus condensation (Fig. 1D,E), oval in shape, tapering toward potential posterior pole, 10.5–14.5 (12.5 ± 1.1) long by 4.8–9.6 (7.3 ± 1.4) wide (n = 12), cytoplasm stained pinkish-purple without vacuolation, nucleus, when centrally placed, variable in shape (round to oval) (Fig. 1E), chromatin condensed, 2.4–6.7 (4.3 ± 1.2) long by 2.8–6.4 (4.4 ± 1.2) wide (n = 10).

Mature or more developed immature gamonts (**slender form**): slender, elongated and oval in shape, found intraerythrocytically, causing frequent disruption of host cell shape and dehaemoglobinisation, with, as in mature elliptical form (see below), pycnotic host cell nucleus, with displacement to one pole of host cell (Fig. 1F,G), sometimes superimposed on the gamont (Fig. 1F); cytoplasm stained light-purple with dark purple granules along body of gamont, 14.9–20.1 (18.1 ± 1.2) long by 5.5–8.5 (6.4 ± 0.8) wide (n = 23), round to oval granulated nucleus stained purplish-pink, 3.3–6.2 (4.5 ± 0.9) long by 2.6–5.4 (3.7 ± 0.7) wide (n = 22), more often located closer to potential anterior end.

Mature gamonts (**elliptical form**): elliptical in shape, found intraerythrocytically, causing frequent hypertrophy of host cell form (Fig. 1H–L) and dehaemoglobinisation (Fig. 1H,J–L), as well as a pycnotic host cell nucleus (Fig. 1H,J,K), with displacement to one side (Fig. 1I,J–L) or pole of host cell (Fig. 1H,K – lower gamont). Parasite seldom causing lysis of host cell nucleus into two fragments (Fig. 1J), gamont 16.5–22.3 (18.4 ± 1.3) long by 7.3–10.6 (9.1 ± 0.7) wide (n = 27), pinkish-stained cytoplasm with minimal granulation in comparison to

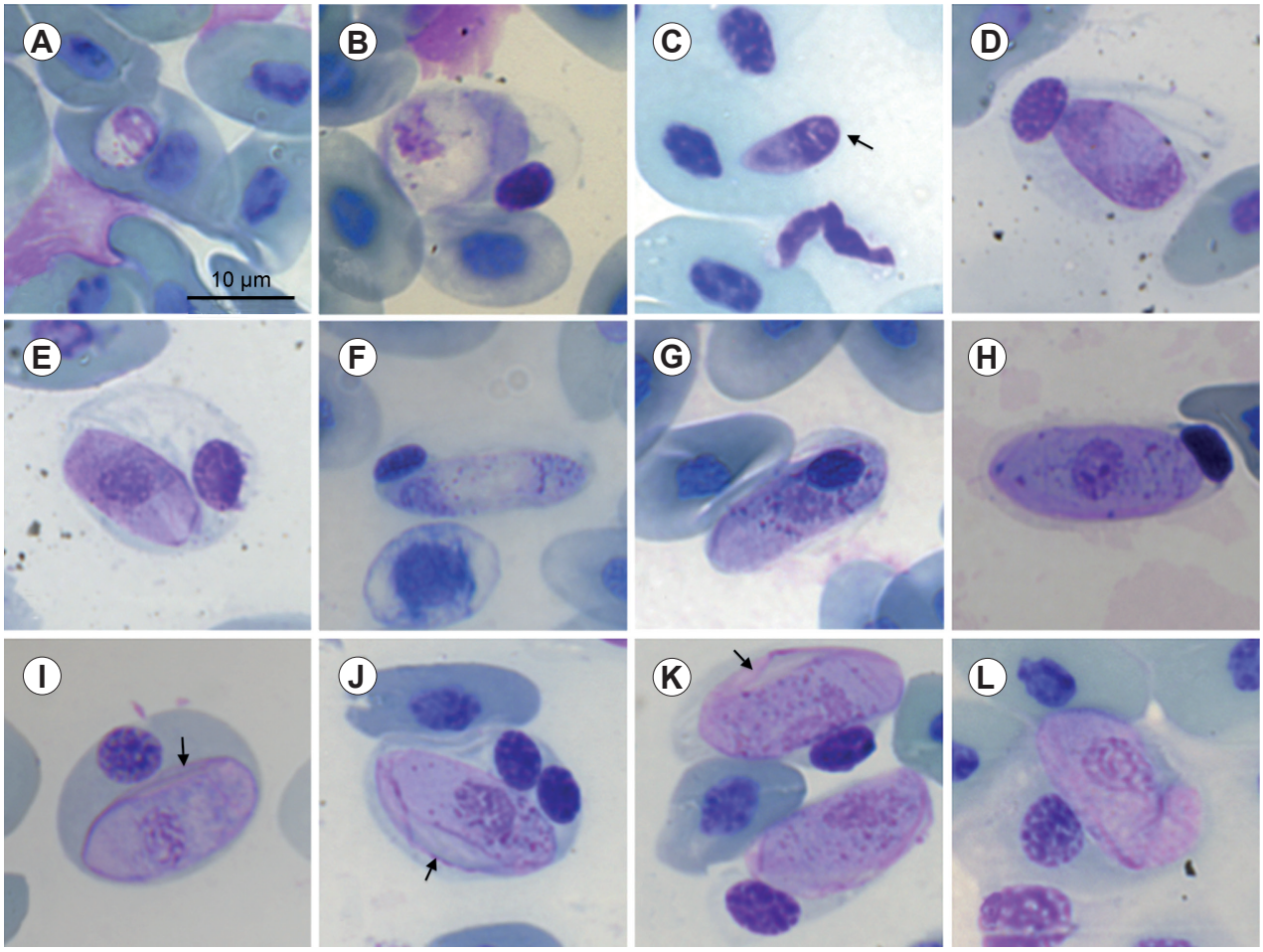

Fig. 1. Micrographs of *Hepatozoon theileri* (Laveran, 1905) in the peripheral blood of the frog *Amietia quecketti* parasitising immature and mature erythrocytes. **A** – trophozoite; **B** – meront; **C** – free merozoite (arrow), seemingly at initial infection of an unparasitised erythrocyte; **D**–**E** immature intraerythrocytic gamonts; **F**, **G** mature slender form or larger immature intraerythrocytic gamonts; **H**–**L** mature elliptical form gamonts; **I**–**K** showing the cystic-pocket or parasitophorous vacuole – arrows.

slender form (Fig. 1H,J,K), light-pink staining parasitophorous vacuole, noticeable often to one side of gamont (Fig. 1I–L, arrows), round to oval nucleus stained pinkish-purple, 3.3–9.6 (4.9 ± 1.3) long by 2.2–6.5 (4.6 ± 1.1) wide (n = 27), more often located closer to the possible anterior pole of parasite; parasite rarely observed with noticeably recurved tail (Fig. 1L).

Molecular analysis: Once edited for phylogenetic analysis, a useable rDNA sequence of 407 bp was produced using HepF300 and HepR900 primer sets targeting part of the 18S rRNA gene, following Ujvari et al. (2004). The sequence has been deposited in GenBank under the accession number of KJ599676. Phylogenetic analysis of the 18S rDNA sequences by the ML method, using *A. bambarooniae* Lai-Fook et Dall, 2002 as the outgroup, supported the classification of *H. theileri* within the *Hepatozoon* and more specifically within the clade of other amphibian *Hepatozoon*, distinctly separate from the species of *Haemogregarina* and *Hemolivia* (Fig. 2).

Type host: *Amietia quecketti* (Boulenger, 1895) (Anura: Pyxicephalidae) (syns. *Amietia angolensis*, *Rana angolensis* and *Rana nutti*).

Type locality: Pretoria, Gauteng province, South Africa.

Other localities: Amani, Tanzania (see Awerinzew 1949); vicinity of Njoro, Kenya (see Ball 1967).

Locality in this study: North-West University Botanical Gardens (26°40'56"S; 27°05'43"E), Potchefstroom, North-West province, South Africa.

Site of infection: Peripheral blood (see Laveran 1905; current study).

Other sites of infection: Liver (see Awerinzew 1949, Ball 1967).

Vector: Unknown.

Deposition of voucher specimens: Protozoan collection of the National Museum, Bloemfontein, South Africa NMB P 255.

Sequence accession number: KJ599676.

Remarks. The gamont stages of *H. theileri* described by Laveran (1905) were elliptical to slender. This author described three gamont forms: (i) an oval form, rounded

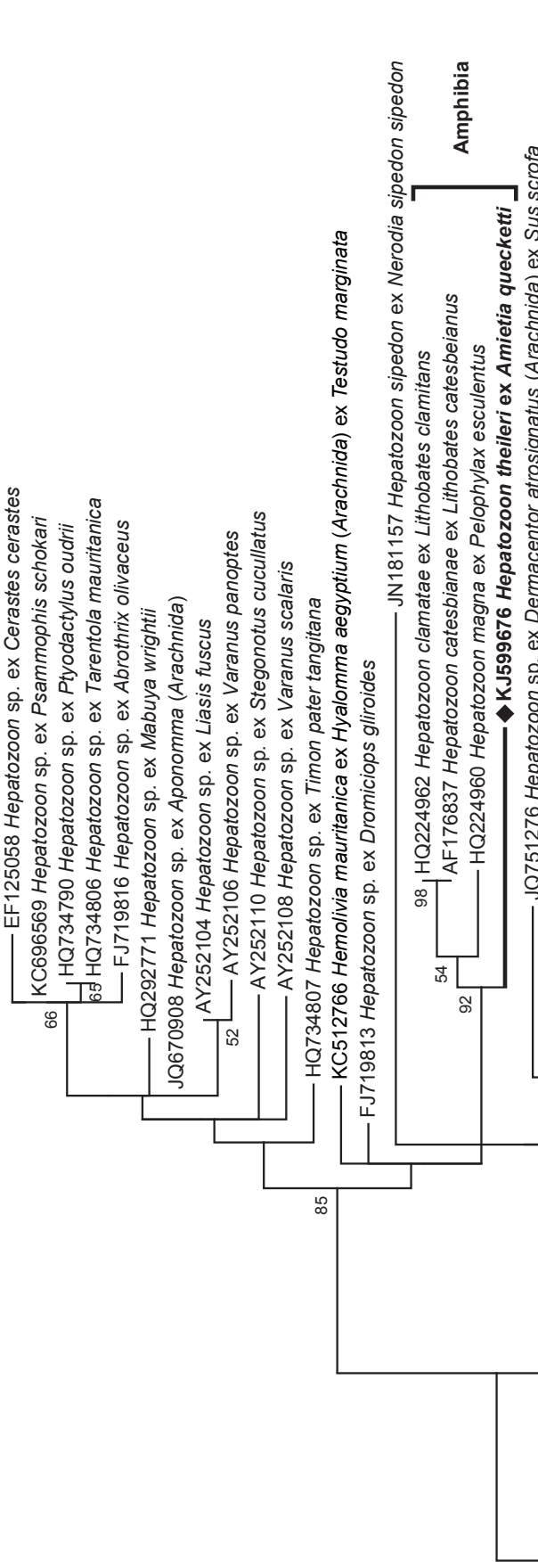

Fig. 2. Maximum Likelihood phylogenetic analysis of *Hepatozoon* species highlighting the position of *Hepatozoon theileri*. Tree was constructed under the conditions of the Tamura 3-parameter model as implemented in MEGA5. The tree with the highest log likelihood (-1858.7884) is shown and nodal support is provided by bootstrap values with only those >50 shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

Table 2. *Hepatozoon* species infecting amphibians from Africa.

Parasite species	Host family	Type host	Locality	References
Hepatozoon aegyptia Mohammed et (Mansour, 1963)	Bufonidae	*Amietophrynus regularis* (Reuss) (syn. *Bufo regularis*)	Egypt, Sudan	Mohammed and Mansour (1963), Smith (1996)
Hepatozoon assiuticus Abdel-Rahman, (El-Naffer, Sakla et Khalifa, 1978)	Bufonidae	*Amietophrynus regularis*	Egypt	Smith (1996)
Hepatozoon boueti (França, 1911) [syn. *Hepatozoon boneti* França, 1925 of Tuzet and Grjebine (1957)]	Bufonidae	*Amietophrynus* spp.	Angola	Smith (1996)
Hepatozoon faiyumensis Mansour et (Mohammed, 1966)	Bufonidae	*Amietophrynus regularis*	Egypt	Mansour and Mohammed (1966), Smith (1996)
Hepatozoon francai (Abdel-Rahman, El-Naffer, Sakla et Khalifa, 1978)	Bufonidae	*Amietophrynus regularis*	Egypt	Smith (1996)
Hepatozoon froilanoi (França, 1925)	Bufonidae	*Amietophrynus regularis*	Angola	Smith (1996)
Hepatozoon lavieri (Tuzet et Grjebine, 1957)	Bufonidae	*Amietophrynus regularis*	Egypt	Smith (1996)
Hepatozoon magni (Hassan, 1992)	Bufonidae	*Amietophrynus regularis*	Egypt	Smith (1996), Kim et al. (1998)
Hepatozoon moloensis (Hoare, 1920)	Bufonidae	*Amietophrynus* spp.	Kenya	Hoare (1920), Mansour and Mohammed (1966), De Sousa and Borriello Filho (1974), Smith (1996)
Hepatozoon pestanae (França, 1911) [syn. *Hepatozoon pistanea* França, 1910 of Tuzet and Grjebine (1957) lapsus calami]	Bufonidae	*Amietophrynus regularis*	Egypt, Guinea-Bissau	Mansour and Mohammed (1966), De Sousa and Borriello Filho (1974), Smith (1996)
Hepatozoon tunisiensis (Nicolle, 1904)	Bufonidae	*Amietophrynus* spp.	Nigeria, Tunisia, Sudan	Mohammed and Mansour (1963), Smith (1996)
Hepatozoon hyperolii (Hoare, 1932)	Hyperolidae	*Hyperolius* spp.	Uganda	Hoare (1932), Levine and Nye (1977), Smith (1996)
Hepatozoon epuluensis (van den Berghe, 1942)	Ptychadenidae	*Ptychadena oxyrhynchus* (Smith)	Democratic Republic of the Congo (D.R.C.)	Levine and Nye (1977), Smith (1996)
Hepatozoon neireti (Laveran, 1905)	Ptychadenidae	*Ptychadena* spp.	Madagascar	Laveran (1905), Levine and Nye (1977), Smith (1996)
Hepatozoon theileri (Laveran, 1905) (syn. *Pseudohaemogregarina ranae* Awerinzew, 1949)	Pyxicephalidae	*Amietia quecketti*	South Africa, Tanzania	Awerinzew (1949), Ball (1967), Laveran (1905), Smith (1996)

at both ends; (ii) rounded at one pole and tapered at the other; and (iii) more slender with a conical and an opposite tapered, folded pole (alike a short recurved tail). Overall all three forms were 15–17 µm long by 5–6 µm wide. The cytoplasm was described as granular with a centrally placed nucleus that had granular chromatin. The parasite appeared to be within what was described by Laveran (1905) as a possible cystic-pocket, staining a pink-purple. In the present study, the gamont stages of the haemogregarine parasitising *A. quecketti* from the NWU Botanical Gardens, resemble in general appearance and size all three gamont forms of *H. theileri* observed by Laveran (1905). As mentioned above, it is uncertain as to whether or not the slender and elliptical forms are an indication of sexual dimorphism. However, pending correction, we have preliminarily described the slender form to be an immature stage to that of the elliptical form, since the incidence of noticeable sexual dimorphism at the gamont stage in *Hepatozoon* species is rare (see Smith et al. 2002). The distinctive possible cystic-pocket (see above) was also observed in this study, but is suggested to be a parasitophorous vacuole (see Fig. 1I–K). Thus, the gamont forms described in this study are identified as the different developmental forms of *H. theileri*. In addition, the current study describes additional stages appearing alongside the gamont forms not reported by Laveran (1905), including a trophozoite and probable meront and merozoite stage.

Molecular analysis places this species within the genus *Hepatozoon*, confirming Smith's (1996) transfer of this species from *Haemogregarina* to *Hepatozoon*, as well as phylogenetically positioning *H. theileri* within a small clade of other amphibian *Hepatozoon* species (see Fig. 2).

DISCUSSION

Members of the genus *Hepatozoon* have been recorded parasitising a range of vertebrate hosts, including mammals, birds, reptiles, crocodilians and amphibians (Smith 1996). Species from amphibians are possibly the least studied, particularly those from Africa. The majority, 11 of 15, i.e. 73%, of species from the Ethiopian Realm are from the Bufonidae. Only two species parasitise anurans of the family Ptychadenidae and one of the families Pyxicephalidae and Hyperolidae, respectively (see Table 2).

Hepatozoon theileri remains currently the only known species of *Hepatozoon* from frog recorded from South Africa. Other records of this parasite include those of Ball

(1967), who described *H. theileri* from Kenyan *R. angolensis*, now known as *Amietia angolensis* (Boulenger), which was until recently a synonym of *A. quecketti* (see Channing and Baptista 2013). This parasite conforms closely in size to *H. theileri*, measuring 18.9 × 6.9 µm, but it does not entirely conform in morphology. Ball (1967) describes a slender tail projection of up to 11 µm for the mature intraerythrocytic gamont stage, as well as a cap-like structure, staining a lilac colour with Giemsa. The latter characteristic was observed at one end of the small and large forms, as well as in the extracellular gamont forms. However, both the above morphological features mentioned by Ball (1967) were not mentioned by Laveran (1905) in his description of *H. theileri* and were not observed during this study. Additionally, Ball (1967) made reference to Awerinzew's (1949) description of *P. ranae* from frogs in Tanzania. This haemogregarine was found parasitising the same frog species, *A. angolensis* from which Ball (1967) described *H. theileri.* On examination of Awerinzew's (1949) description of *Pseudohaemogregarina ranae* (Awerinzen, 1949), Ball (1967) suggested, that it may be a junior synonym of *H. theileri*.

Phylogenetically, *H. theileri* fell in a clade among species of *Hepatozoon*, together with *Hepatozoon sipedon* Smith, Desser et Martin, 1994, a haemogregarine described from the snake *Nerodia sipedon* (Linnaeus), the frog *Lithobates pipiens* and the mosquitos *Culex pipiens* (Linnaeus) and *C. territans* (Walker). Furthermore, quite significantly, it was nested within a smaller monophyletic clade comprising other anuran *Hepatozoon* species, *Hepatozoon catesbianae* (Stebbins, 1903) (GenBank AF176837), *Hepatozoon clamatae* (Stebbins, 1905) (GenBank HQ224962) and *Hepatozoon magna* (Grassi et Feletti, 1891) (GenBank HQ224960). *Hepatozoon catesbianae* and *H. clamatae*, occurring sympatrically, were described from two North American frog species *Lithobates catesbeianus* (Shaw, 1802) and *Lithobates clamitans* (Latreille, 1801) (see Smith et al. 1994, Desser et al. 1995, Kim et al. 1998), while *H. magna* was described from the European frog *Pelophylax esculentus* (Linnaeus) (see Table 1 and Fig. 1).

All three *Hepatozoon* species have a two-host life-cycle without a cystic stage occurring in the intermediate host, and can be experimentally transmitted to culicine mosquito vectors in which sporogonic stages have been described (Smith et al. 1994, 1999, Desser et al. 1995, Kim et al. 1998, Barta et al. 2012). This is in contrast to *H. sipedon*, which has a three-host life cycle as mentioned above and appears, outside the smaller two-host life cycle clade (Fig. 2). This position of *H. sipedon* thus supports the assumption of Barta et al. (2012) on the co-evolution of definitive hosts and their haemogregarine parasites. As no cystic stages were observed in frogs, it may be suggested that *H. theileri* examined in the present study may have a two-host life-cycle. Therefore, the future identification of potential mosquito vectors *in situ* is an important next step in the elucidation of this haemogregarine's life cycle.

During the present study, a difference, although insignificant, in *H. theileri* parasitaemia was observed between frogs captured in winter and spring. Since the vector for *H. theileri* could be any one of a range of biting arthropods such as mosquitos (see Davies and Johnston 2000), with adult diapause prior to overwintering hibernation (Koenraadt and Takken 2013), reduction in parasitaemia may be attributable to a lack or reduction in activity of the vector during winter.

The present redescription of *H. theileri*, using both morphological and molecular methods, the first one of any African species thus represent an initial step in the furture taxonomic and phylogenetic characterisation of African amphibian haemogregarines.

Acknowledgements. Courtney A. Cook is funded by a North-West University postdoctoral fellowship. We are most grateful to Dr. Edward Stanley of the California Academy of Sciences, USA, for obtaining papers by Laveran (1905), Hoare (1920) and Awerinzew (1949); Dr. Patricks Voua Otomo of the North-West University (Potchefstroom campus) (NWU-P), South Africa for translating Laveran's (1905) work; Miss Hermoine Venter, NWU-P, for assistance with molecular work; the late Prof. Angela J. Davies, Kingston University, UK, for providing Hoare's (1932) article, as well as aiding in the identification of haemogregarine stages; her dedication to studies of haemogregarines and deep knowledge will be sorely missed. North-West University Botanical Gardens are thanked for permission to examine frogs in the garden.

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Received 20 December 2013

Accepted 11 March 2014