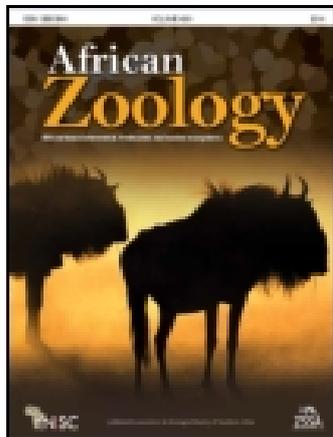


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## African Zoology

Publication details, including instructions for authors and subscription information:  
<http://www.tandfonline.com/loi/tafz20>

### First Hemolivia from southern Africa: reassigning chelonian Haemogregarina parvula Dias, 1953 (Adeleorina: Haemogregarinidae) to Hemolivia (Adeleorina: Karyolysidae)

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Published online: 10 Jun 2015.



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To cite this article: Courtney A Cook, Edward C Netherlands & Nico J Smit (2015): First Hemolivia from southern Africa: reassigning chelonian Haemogregarina parvula Dias, 1953 (Adeleorina: Haemogregarinidae) to Hemolivia (Adeleorina: Karyolysidae), African Zoology

To link to this article: <http://dx.doi.org/10.1080/15627020.2015.1044467>

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# First *Hemolivia* from southern Africa: reassigning chelonian *Haemogregarina parvula* Dias, 1953 (Adeleorina: Haemogregarinidae) to *Hemolivia* (Adeleorina: Karyolysidae)

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To date, only a single species of *Hemolivia*, *Hemolivia mauritanica* (Sergent & Sergent, 1904), has been described from African terrestrial tortoises. Although various haemogregarines have been described from southern African terrapins and tortoises, including species from the genus *Haemogregarina* and one from the genus *Hepatozoon*, no species of *Hemolivia* have been identified previously from southern Africa. Since its morphological redescription, the taxonomic placement of one of these species, *Haemogregarina parvula* Dias, 1953, was in doubt. Hence, research was undertaken to resolve the true taxonomic position of this haemogregarine. Blood smears from nine wild tortoises of two species, *Stigmochelys pardalis* and *Kinixys zombensis*, from South Africa were screened, with the focus on *H. parvula*. Parasite DNA was extracted from ethanol-preserved blood samples, and fragments of the 18S rDNA gene were amplified by PCR using the primer sets HepF300/HepR900 and 4558/2733. Maximum likelihood and maximum parsimony analyses of 18S rDNA sequences showed that *Haemogregarina parvula* formed a monophyletic clade with species of *Hemolivia* within the *Hepatozoon* clade, not with species of *Haemogregarina*. It is thus recommended that this haemogregarine be reassigned to the genus *Hemolivia*, rendering *Hemolivia parvula* (Dias, 1953) the first species of the genus recorded from southern African tortoises.

**Keywords:** 18S rDNA, adeleorid phylogeny, apicomplexan haemoparasite, blood parasite taxonomy, South Africa, tortoise

## Introduction

The genus *Hemolivia* Petit, Landau, Baccam & Lainson, 1990, as compared with other haemogregarine genera of the families Haemogregarinidae Léger, 1911, Hepatozoidae Miller, 1908, and Karyolysidae Labbé, 1894, was only recently erected. Its defining characteristics are the occurrence of merogony and cyst formation in the reticulo-endothelial cells and erythrocytes of their ectothermic vertebrate host, with gamonts in the peripheral blood erythrocytes. Further characteristic features include typical star-shaped oocysts within the intestinal cells of the invertebrate host during sporogony (Davies and Johnston 2000). However, this characteristic has not been demonstrated for the African species, *Hemolivia mauritanica* (Sergent & Sergent, 1904) (Telford 2009). Transmission of parasites of this genus is through ingestion of an infected invertebrate, or possibly an infected vertebrate, by a potential vertebrate host (Davies and Johnston 2000; Telford 2009). The invertebrate vector has for many years been considered to be a tick, particularly because the type species *Hemolivia stellata* Petit, Landau, Baccam & Lainson, 1990 in the toad *Rhinella marina* (syn. *Bufo marinus*) is transmitted by a tick host, *Amblyomma rotundatum* Koch, 1844 (Telford 2009). This is further supported by other studies of *Hemolivia* species in reptiles, such as *Hemolivia mariae* Smallridge & Paperna, 1997, in the scinid lizard *Tiliqua rugosa* (Gray, 1825), transmitted by *Amblyomma limbatum* Neumann, 1899, and *H. mauritanica* in the terrestrial tortoise *Testudo*

*graeca* Linnaeus, 1758, transmitted by *Hyalomma aegyptium* Linnaeus, 1758 (see Michel 1973; Landau and Paperna 1997; Smallridge and Paperna 1997; Široký et al. 2007; Telford 2009). Very recently, a possible new species of *Hemolivia* was discovered from an aquatic terrapin host, *Rhinoclemmys pulcherrima manni* (Dunn, 1930) (see Kvičeroová et al. 2014), but the authors did not name the species.

Only one of the above, *H. mauritanica*, has been described from Africa, originally described from *T. graeca* from Algeria by Sergent and Sergent (1904) and only recently molecularly identified from ticks collected from the tortoise host *T. graeca* from the North African countries Algeria, Morocco and Tunisia (Harris et al. 2013). To the authors' knowledge, no other *Hemolivia* species have been reported or described from herpetofauna in Africa, particularly from chelonians.

Numerous haemogregarines have been described infecting chelonians from Africa, many of which are still assigned to the genus *Haemogregarina* (*sensu stricto*), transmitted strictly by an aquatic leech vector (see Siddall 1995) (Table 1). The assignment of two of these haemogregarines, *Haemogregarina fitzsimonsi* Dias, 1953 and *Haemogregarina parvula* Dias, 1953, infecting purely terrestrial tortoises in South Africa to the genus *Haemogregarina* was questioned by Cook et al. (2009). These authors suggested that these haemogregarines might rather

**Table 1:** Formally described haemogregarines infecting type freshwater terrapin and terrestrial tortoise hosts from Africa. Recorded are the countries, haemogregarine species, gamont descriptions, type host and other hosts, and references (superscript letters indicate the reference from which specific size data were sourced)

Country	Species	Mature gamont shape and size ( $\mu\text{m}$ )	Type host	Other hosts	References
South Africa	<i>Haemogregarina</i> sp.	Globular: (17.6 $\times$ 9.7)	<i>Stigmochelys pardalis</i> (Testudinidae)	None known	Cook et al. (2014b)
Mali	<i>Haemogregarina bruneti</i> Commes, 1919	Slender: (22) $\times$ (8)	<i>Kinixys homeana</i> (syn. <i>Cinixis homeana</i> ) (Testudinidae)	None known	Commes (1919)
Mozambique and South Africa	<i>Hepatozoon fitzsimonsi</i> (Dias, 1953) Cook et al., 2014	Slender: (15.8–18.9) $\times$ (3.6–5.7) <sup>a</sup> ; (17.1–17.7) $\times$ (3.3–4.3) <sup>b</sup> ; (17–17.5) $\times$ (3.4–4.2) <sup>c</sup>	<i>Kinixys zombensis</i> (syn. <i>Kinixys belliana belliana</i> , <i>Kinixys belliana zuluensis</i> ) (Testudinidae)	<i>Chersina angulata</i> , <i>Kinixys zombensis</i> , <i>Kinixys lobatsiana</i> , <i>Kinixys natalensis</i> , <i>Stigmochelys pardalis</i> (Testudinidae)	Dias (1953) <sup>a</sup> , Cook et al. (2009) <sup>b</sup> , 2014a <sup>c</sup> , 2014b)
Mozambique and South Africa	<i>Haemogregarina parvula</i> Dias, 1953	Globular: (9.2–13.2) $\times$ (5.7–6) <sup>a</sup> ; (11–12.1) $\times$ (5.4–7.2) <sup>b</sup>	<i>Kinixys zombensis</i> (syn. <i>Kinixys belliana belliana</i> , <i>Kinixys belliana zuluensis</i> ) (Testudinidae)	<i>Stigmochelys pardalis</i> (Testudinidae)	Dias (1953) <sup>a</sup> , Cook et al. (2009) <sup>b</sup> , 2014a, 2014b)
Mozambique and South Africa	<i>Haemogregarina pelusiensi</i> Pienaar, 1962	Recurved and lentiform, within a translucent, hard capsule: (12.5–24) $\times$ (3.4–9) and (9–17) $\times$ (5–9), respectively <sup>d</sup> ; (13–24) $\times$ (5–9) and (9–17) $\times$ (5–9), respectively <sup>e</sup>	<i>Pelusios sinuatus</i> (syn. <i>Pelusios sinuatus sinuatus</i> ) (Pelomedusidae)	None known	Pienaar (1962) <sup>d</sup> , Paperna (1989) <sup>e</sup> , Telford (2009)
Mozambique	<i>Haemogregarina maputensis</i> Dias, 1950	Unknown	<i>Pelusios sinuatus sinuatus</i> (syn. <i>Pelusios sinuatus zuluensis</i> ) (Pelomedusidae)	Unknown	Dias (1950) <sup>*</sup>
Not reported, but in French West Africa and Portuguese Guinea	<i>Haemogregarina sternotheri</i> Bouet, 1909	Recurved: (12.6) $\times$ (1.8); also reported by França (1911)	<i>Pelusios subniger</i> (syn. <i>Sternotherus derbianus</i> ) (Pelomedusidae)	None known	Bouet (1909), França (1911)
Algeria, Bulgaria, Greece and Turkey	<i>Hemolivia mauritanica</i> (Sergent & Sergent, 1904) Landau & Paperna, 1997	Strongly recurved gamonts in thick capsule: (12–15) $\times$ (6) <sup>f</sup> ; (12.2) $\times$ (6.2) <sup>g</sup> ; (9–12) $\times$ (5–7) <sup>h</sup>	<i>Testudo graeca</i> (syn. <i>Testudo mauritanica</i> ) (Testudinidae)	<i>Testudo marginata</i> (Testudinidae)	Sergent and Sergent (1904) <sup>f</sup> , Michel (1973) <sup>g</sup> , Landau and Paperna (1997), Široký et al. (2007) <sup>h</sup>

\* Unavailable

belong to the genus *Hemolivia* or *Hepatozoon*, and may be transmitted by ticks similar to *H. mauritanica* infecting *T. graeca*. Consequently, based on parasite morphology, life cycle and phylogenetic analysis of 18S rDNA sequence fragments, *Haemogregarina fitzsimonsi* was assigned to the genus *Hepatozoon* (Cook et al. 2014a). Similarly, it can be expected that *H. parvula* does not belong within the genus *Haemogregarina*. The aim of the current study was thus to clarify this parasite's taxonomy and identify the haemogregarine genus to which it should be allocated.

## Methods

### Tortoise blood collection and preparation

Tortoises were collected during the day in the Ndumo Game Reserve (26°54'32.48" S, 32°18'48.85" E), KwaZulu-

Natal, South Africa, during the warmer and rainy months of November 2013 and February, April and December 2014. Tortoises were processed and identified to species level at the site of capture using field guides (Branch 2008; Bates et al. 2014). Any possible haematophage vectors attached to tortoises, such as ticks, were collected, fixed in 70% ethanol and identified, at a later stage, to species level as in Cook et al. (2014a). Blood, 0.2–1 ml depending on the size of the tortoise, was collected from the subcarapacial sinuses using sterile 21G 1½ inch needles fitted to 1 ml syringes (see McArthur et al. 2004). A drop of this blood was used to make duplicate thin blood smears and the remaining volume was fixed in an equal volume of 70% ethanol for further molecular work. Once air-dried in a dustproof container, blood slides were immediately fixed in absolute methanol, and thereafter stained with a

modified solution of Giemsa stain (FLUKA, Sigma-Aldrich, Steinheim, Germany) according to the methods of Cook et al. (2014a).

#### **Screening of blood slides, haemogregarine identification and determination of parasitaemia**

Smears (voucher NMB P: 371) were screened on a Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, The Netherlands) using a 100× immersion oil objective, and images were captured with an attached Nikon digital camera. Haemogregarines were identified to species level by comparing morphometric characteristics to previous data on these parasites (see Cook et al. 2009, 2014a, 2014b). Parasitaemia was calculated per 100 erythrocytes, with c. 10<sup>4</sup> erythrocytes examined per blood smear (see Cook et al. 2009, 2010; Netherlands et al. 2014a).

#### **DNA extraction, PCR amplification and parasite 18S rDNA sequence analysis**

Ethanol-preserved blood samples obtained from the parasitised *K. zombensis* ( $n = 3$ ) were used for molecular work. Genomic DNA of *Hepatozoon* and *Hemolivia* species were extracted from the samples 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).

Identification of *Hepatozoon* and *Hemolivia* species was initially completed using the *Hepatozoon*-specific primer set HepF300 (5'-GTTTCTGACCTATCAGCTTTTCGACG-3') and HepR900 (5'-CAAATCTAAGAATTTACCTCTGAC-3'). The PCR reactions were run targeting a fragment (approximately 600 bp) of the 18S rDNA gene (see Ujvari et al. 2004). 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 60 °C for 30 s with an end extension at 72 °C for 1 min, and following the cycles a final extension of 72 °C for 10 min as detailed according to previous methods (Netherlands et al. 2014a). A second PCR was carried out using the apicomplexan-specific parasite primer set 4558 (5'-GCTAATACATGAGCAAAATCTCAA-3') and 2733 (5'-CGGAATTAACCAGACAAAT-3') (see Mathew et al. 2000), targeting a longer fragment (approximately 1 120 bp) of the 18S rDNA. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 40 cycles, entailing 94 °C denaturation for 1 min, annealing at 55 °C for 2 min with an end extension at 72 °C for 2 min, and following the cycles a final extension of 72 °C for 10 min (see Mathew et al. 2000; Netherlands et al. 2014b).

PCR reactions were performed with volumes of 25 µl, using 12.5 µl Thermo Scientific DreamTaq PCR master mix (2×) (2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM MgCl<sub>2</sub>), 1.25 µl of each primer, 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 visualised 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). Two 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 using Geneious 7.1 (Biomatters; available at <http://www.geneious.com>) and chromatogram-based contigs were generated, trimmed and manually corrected for ambiguous base calls. Sequences were identified using the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/>), and deposited in the NCBI GenBank database (accession numbers KR069082 and KR069083).

Comparative sequences of *Hemolivia*, *Hepatozoon*, *Haemogregarina*, *Dactylosoma* and *Babesiosoma* species parasitising reptiles, amphibians, mammals and ticks were downloaded from GenBank and aligned to the sequences generated in this study. *Adelina dimidiata* (GenBank accession no. DQ096835) was chosen as the outgroup as in Dvořáková et al. (2015) (see Table 2). Sequences were aligned using the MUSCLE alignment tool (Edgar 2004) implemented in Geneious 7.1. The alignment consisted of 39 sequences, with a 994 bp conserved region selected using the Gblocks 0.91b server ([http://molevol.cmima.csic.es/castresana/Gblocks\\_server.html](http://molevol.cmima.csic.es/castresana/Gblocks_server.html)) (Castresana 2000).

Uncorrected pair-wise distances (p-distance), base pair differences as well as parsimony informative sites and the number thereof were identified or determined with the MEGA6 bioinformatics software program (<http://www.megasoftware.net>) for the aligned 18S rDNA sequences between all available species of *Hemolivia*, including a sequence for chelonian *Haemogregarina* (GenBank accession no. KF257925) and *Hepatozoon* (GenBank accession no. KJ702453 and new sequence KR069084), all appearing in the phylogenetic analyses.

To infer phylogenetic relationships, both maximum likelihood (ML) and maximum parsimony (MP) methods were implemented using MEGA6 (Tamura et al. 2013). The best evolutionary model for ML analysis was identified using MEGA6 based on the lowest Bayesian information criterion value. Maximum likelihood trees were constructed using the Tamura three-parameter model with a discrete Gamma distribution (T92+G). Nodal support was assessed by means of a bootstrap analysis with 1 000 replicates. The ML phylogeny with the highest log-likelihood score (ln -3377.2225) was selected as being the most accurate and best supported reconstruction. The MP phylogeny was obtained using the subtree pruning and regrafting (SPR) algorithm with search level 2, in which the initial trees were obtained with the random addition of sequences (100 replicates) and a strict consensus tree was inferred from the 23 most parsimonious trees. In all phylogenetic analyses performed, only nodal support values greater than 50% are shown.

## **Results**

### **Prevalence, parasitaemia and morphology of *H. parvula* in peripheral blood smears**

Nine tortoises (all adults) were collected over the four sampling trips, comprising six *Stigmochelys pardalis* (Bell, 1828) and three *Kinixys zombensis* Hewitt, 1931 (syn. *Kinixys belliana belliana*). Of these tortoises, all

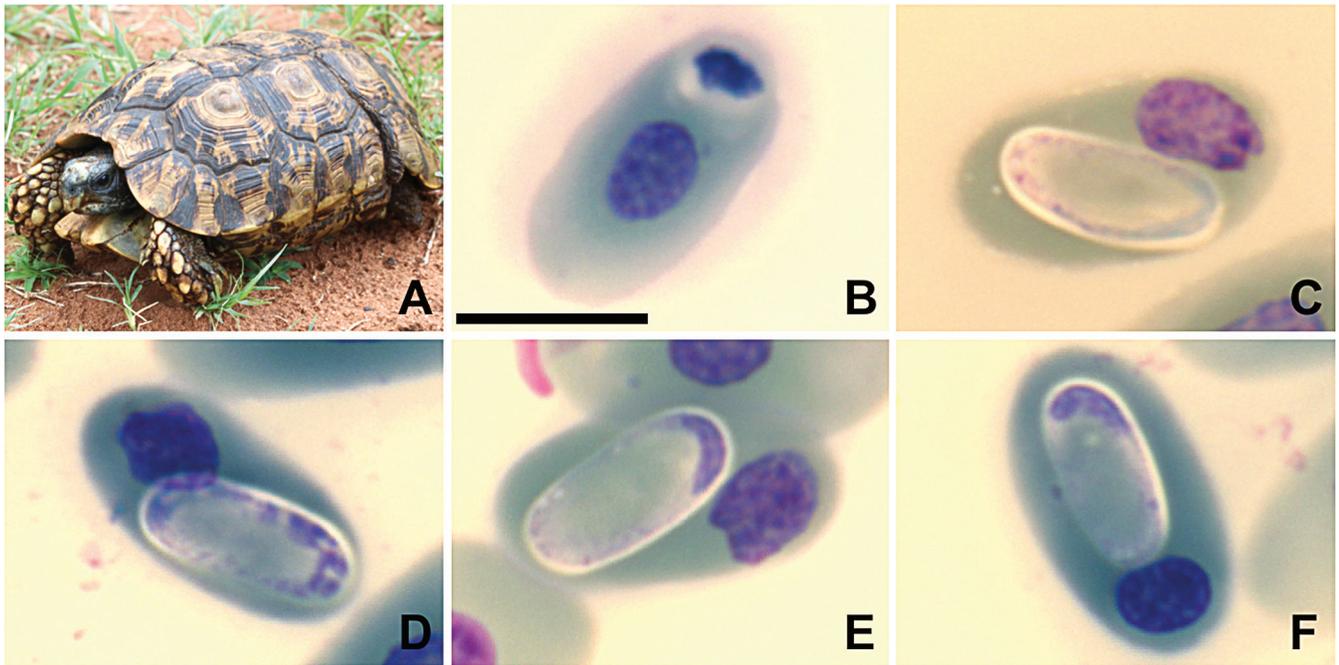
**Table 2:** List of organisms used in this study, with associated GenBank accession numbers as well as references

Organism	Accession number	Reference
<i>Adelina dimidiata</i>	DQ096835	Kopečná et al. (2006)
<i>Babesiosoma stableri</i>	HQ224961	Barta et al. (2012)
<i>Dactylosoma ranarum</i> ex <i>Pelophylax esculentus</i>	HQ224957	Barta et al. (2012)
<i>Haemogregarina balli</i>	HQ224959	Barta et al. (2012)
<i>Haemogregarina</i> sp. ex <i>Pelusios subniger</i>	KF257925	Dvořáková et al. (2014)
<i>Haemogregarina stepanowi</i>	KF257929	Dvořáková et al. (2014)
<i>Hemolivia mariae</i>	KF992711	Kvičerová et al. (2014)
<i>Hemolivia mariae</i>	KF992712	Kvičerová et al. (2014)
<i>Hemolivia mauritanica</i>	KF992700	Kvičerová et al. (2014)
<i>Hemolivia mauritanica</i>	KF992710	Kvičerová et al. (2014)
<i>Hemolivia mauritanica</i>	KF992704	Kvičerová et al. (2014)
<i>Hemolivia mauritanica</i>	KF992709	Kvičerová et al. (2014)
<i>Hemolivia parvula</i> syn. <i>Haemogregarina parvula</i> ex <i>Kinixys zombensis</i>	KR069082	Present study
<i>Hemolivia parvula</i> syn. <i>Haemogregarina parvula</i> ex <i>Kinixys zombensis</i>	KR069083	Present study
<i>Hemolivia</i> sp. ex <i>Rhinoclemmys pulcherrima manni</i>	KF992713	Kvičerová et al. (2014)
<i>Hemolivia</i> sp. ex <i>Rhinoclemmys pulcherrima manni</i>	KF992714	Kvičerová et al. (2014)
<i>Hepatozoon americanum</i>	AF176836	Mathew et al. (2000)
<i>Hepatozoon ayorgbor</i> ex <i>Python regius</i>	EF157822	Sloboda et al. (2007)
<i>Hepatozoon canis</i>	AY461378	Criado-Fornelio et al. (2006)
<i>Hepatozoon canis</i>	EU289222	NCBI GenBank database
<i>Hepatozoon catesbiana</i> ex <i>Lithobates catesbeianus</i>	HQ224954	Barta et al. (2012)
<i>Hepatozoon clamata</i> ex <i>Lithobates clamitans</i>	HQ224962	Barta et al. (2012)
<i>Hepatozoon felis</i> ex <i>Panthera pardus fusca</i>	HQ829444	NCBI GenBank database
<i>Hepatozoon felis</i> ex <i>Prionailurus iriomotensis</i>	AB771548	NCBI GenBank database
<i>Hepatozoon fitzsimonsi</i> ex <i>Chersina angulata</i>	KJ702453	Cook et al. (2014a)
<i>Hepatozoon fitzsimonsi</i> ex <i>Kinixys zombensis</i>	KR069084	Present study
<i>Hepatozoon ixoxo</i> ex <i>Amietophrynus maculatus</i>	KP119772	Netherlands et al. (2014b)
<i>Hepatozoon magna</i> ex <i>Pelophylax esculentus</i>	HQ224960	Barta et al. (2012)
<i>Hepatozoon seychellensis</i> ex <i>Grandisonia alternans</i>	KF246566	Harris et al. (2014)
<i>Hepatozoon sipedon</i> ex <i>Nerodia sipedon sipedon</i>	JN181157	Barta et al. (2012)
<i>Hepatozoon</i> sp. ex <i>Abrothrix olivaceus</i>	FJ719818	Merino et al. (2009)
<i>Hepatozoon</i> sp. ex <i>Amblyomma fimbriatum</i> ex <i>Varanus panoptes</i>	EU430234	Vilcins et al. (2009)
<i>Hepatozoon</i> sp. ex <i>Elaphe carinata</i>	KF939620	NCBI GenBank database
<i>Hepatozoon</i> sp. ex <i>Liasis fuscus</i>	AY252105	Ujvari et al. (2004)
<i>Hepatozoon</i> sp. ex <i>Varanus panoptes</i>	AY252106	Ujvari et al. (2004)
<i>Hepatozoon theileri</i>	KJ599676	Netherlands et al. (2014a)
<i>Hepatozoon theileri</i> ex <i>Amietia quecketti</i>	KP119773	Netherlands et al. (2014b)
<i>Hepatozoon ursi</i>	EU041717	Kubo et al. (2008)
<i>Hepatozoon ursi</i> ex <i>Ursus thibetanus japonicus</i>	EU041718	Kubo et al. (2008)

three *K. zombensis* were found to be infected with haemogregarines. Two of these tortoises were collected and examined in April 2014; both were parasitemic with a concurrent infection of *Hepatozoon fitzsimonsi* and *Haemogregarina parvula*. The third tortoise, collected in December 2014, appeared to be infected only with the latter parasite. One individual had a *H. fitzsimonsi* parasitaemia of 11% and a much lower *H. parvula* parasitaemia of 0.02%. The second individual (Figure 1a) showed the opposite, with a *H. fitzsimonsi* parasitaemia of 0.00001% and a *H. parvula* parasitaemia of 0.1%. The third tortoise showed a low (0.002%) infection of *H. parvula*, and *H. fitzsimonsi* was seemingly absent. Only the first of the above-mentioned *K. zombensis* individuals were infested with the tick *Amblyomma nuttalli* Dönitz, 1909 ( $n = 2$  males). All six *S. pardalis* were infested with adult and juvenile stages of *Amblyomma marmoreum* Koch, 1844.

Stages of *H. parvula* observed in peripheral blood smears from this study were compared morphologically with those observed in previous studies (Cook et al.

2014a, 2014b), but particularly with those stages reported for *H. parvula* by Cook et al. (2009). In size, the *H. parvula* stages observed during the current study conformed to those described by both Dias (1953) and Cook et al. (2009) (see Table 3). Three stages of the parasite were observed: a previously unreported rare trophozoite stage (Figure 1b), and both intra-erythrocytic immature (Figure 1c) and mature gamonts (Figure 1d–f). The most observable characteristic was the non-staining capsule, broadly oval, and surrounding the enclosed gamont (Figure 1c–f). Only the mature gamont stage was visible in the capsules with its nucleus located at one pole of the parasite and staining deep purple to blue (Figure 1d–f). This observation, as mentioned previously by Cook et al. (2009), might suggest that the gamont body may be bent double within the capsule. Both the immature and mature gamont cytoplasm stained very light blue, with no visible granules or vacuolation. Furthermore, in both stages the infected host erythrocyte nucleus was always observably displaced (Figure 1c–f).



**Figure 1:** *Kinixys zombensis* Hewitt, 1931 from Ndumo Game Reserve, South Africa, and light micrographs of Giemsa-stained blood films showing the haemogregarine *Hemolivia parvula* (syn. *Haemogregarina parvula*) (Dias, 1953). (a) *Kinixys zombensis* adult female, identified with the aid of Branch (1998) and Bates et al. (2014). (b–f) Intra-erythrocytic stages of *H. parvula*, voucher specimen (NMB P 371), accession number (KR069083): (b) extremely rare trophozoite stage; (c) immature or mature gamont stage, the nucleus indiscernible; (d–f) mature gamont stages; (d) with darkly stained material around the periphery of the encapsulated gamont; and (e–f) showing the characteristic terminal nucleus of the parasite, suggesting the gamont is folded within the thick capsule. Scale bar = 10  $\mu\text{m}$

**Table 3:** *Hemolivia* species infecting freshwater terrapins and terrestrial tortoises from around the world. Recorded is the realm, *Hemolivia* species, mature gamont dimensions where available, type host and other hosts, and references

Realm/species	Mature gamont and nucleus size ( $\mu\text{m}$ )	Type host	Other hosts	References
<b>Neotropical</b>				
<i>Hemolivia stellata</i> Petit et al., 1990	(9) $\times$ (5.1); –	<i>Rhinella marina</i> (syn. <i>Bufo marinus</i> ) (Bufonidae)	<i>Ameiva ameiva</i> (Teiidae)	Petit et al. (1990), Lainson et al. (2007)
<i>Hemolivia</i> sp.	(7–10) $\times$ (4–6); (2–4) $\times$ (1–3)	<i>Rhinoclemmys pulcherrima manni</i> (Geoemydidae)	None known	Kvičerová et al. (2014)
<b>Palaearctic/Ethiopian</b>				
<i>Hemolivia mauritanica</i> (Sergent & Sergent, 1904) Landau & Paperna, 1997	(9–12) $\times$ (5–7); –	<i>Testudo graeca</i> (syn. <i>Testudo mauritanica</i> ) (Testudinidae)	<i>Testudo marginata</i> (Testudinidae)	Široký et al. (2007)
<b>Ethiopian</b>				
<i>Hemolivia parvula</i> (syn. <i>Haemogregarina parvula</i> ) (Dias, 1953)	12.1 $\pm$ 0.7 (10.3–13.2) $\times$ 5.6 $\pm$ 0.3 (4.7–6.1); 3.3 $\pm$ 0.5 (2.4–4.3) $\times$ 3.8 $\pm$ 0.4 (3.1–4.4)	<i>Kinixys zombensis</i> (syn. <i>Kinixys belliana belliana</i> , <i>Kinixys belliana zuluensis</i> ) (Testudinidae)	<i>Stigmochelys pardalis</i> (Testudinidae)	Present study
<b>Australian</b>				
<i>Hemolivia mariae</i> Smallridge & Paperna, 1997	(18) $\times$ (5); –	<i>Tiliqua rugosa</i> (Scincidae)	<i>Egernia stokesi</i> (Scinidae)	Smallridge and Paperna (1997); Telford (2009); Kvičerová et al. (2014)

### Sequence identification and phylogenetic analysis

Amplicons of 608 bp, 1 032 bp and 1 052 bp for *H. parvula*, and 650 bp and 1 032 bp for *H. fitsimensi*, were obtained using primer sets HepF300 and HepR900, and 4558 and 2733, respectively. For the phylogenetic analysis only sequences from the 4558 and 2733 primer sets were

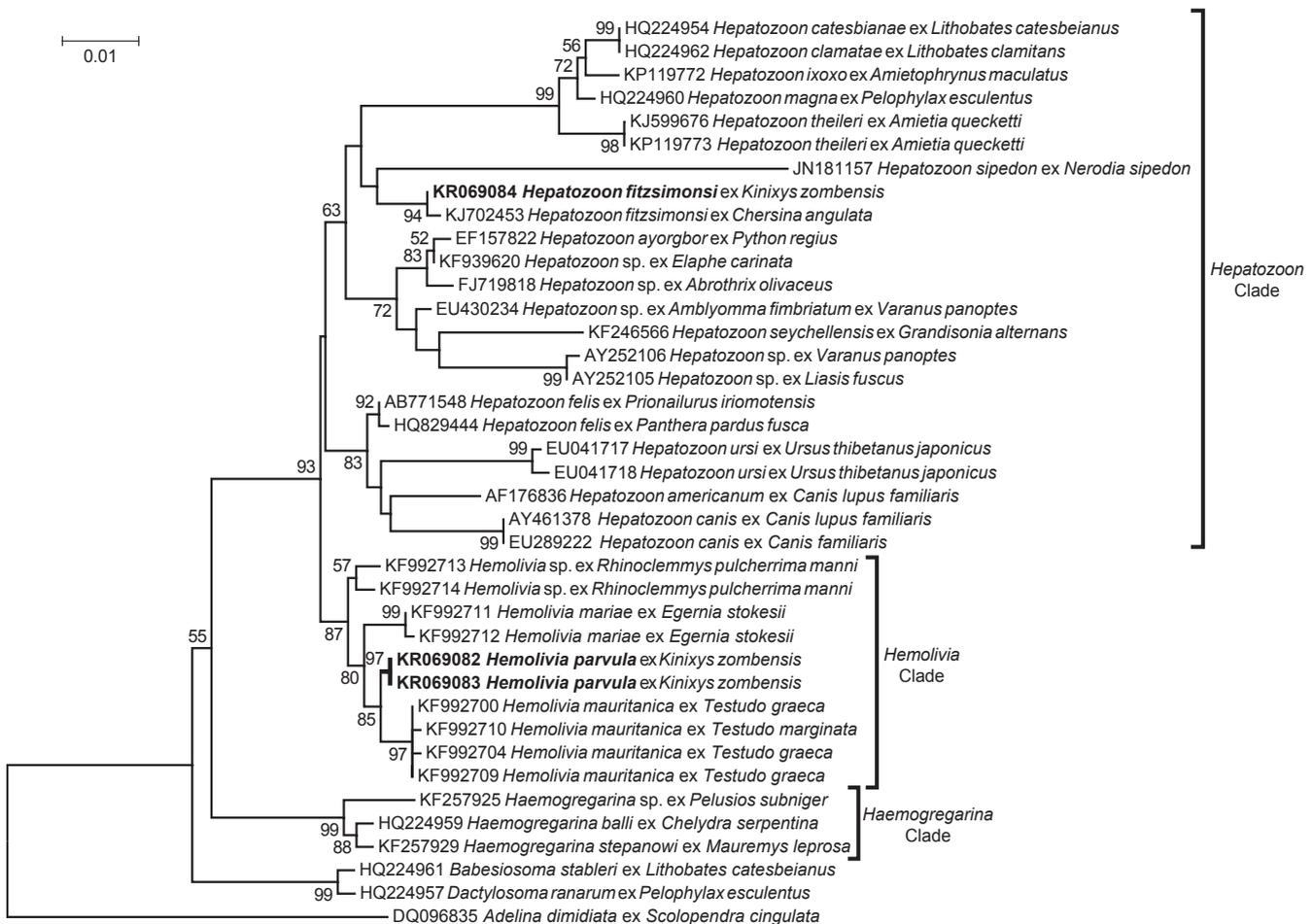
used. In both the ML and MP analyses the overall tree topology was similar, with discrete monophyletic clades of *Hepatozoon* species of amphibians and reptiles, *Hepatozoon* species of mammals, *Hemolivia* species and *Haemogregarina* species resolved. Phylogenetically, *H. parvula* fell within a major monophyletic clade

containing both *Hemolivia* and *Hepatozoon* species, and distinct from the clade containing *Haemogregarina* species. The genetic dissimilarities between the species within these two clades were evident (Table 4). In the ML analysis, although the species of *Hemolivia* and *Hepatozoon* formed two separate subclades, they were

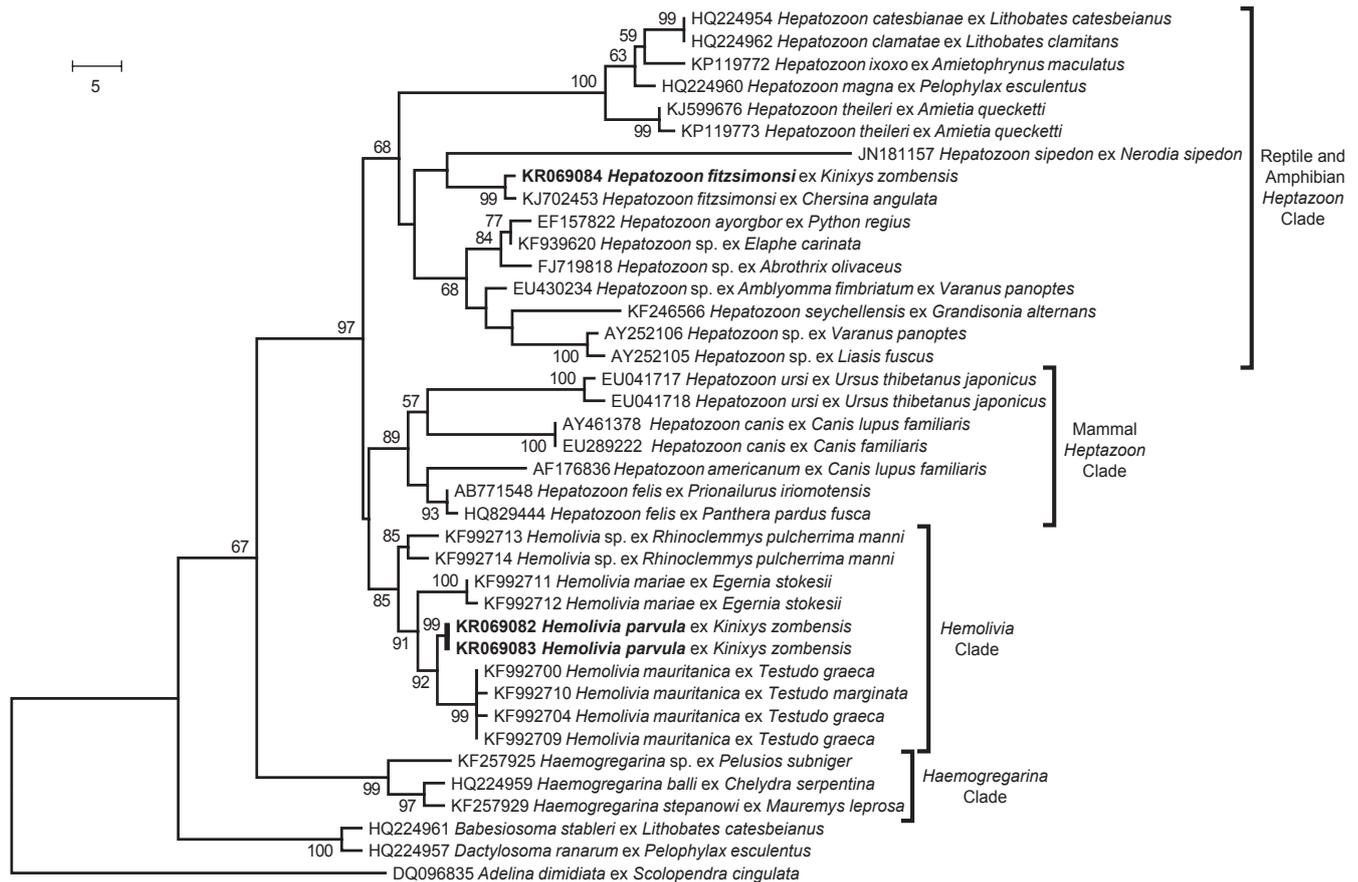
grouped to form a major monophyletic clade (bootstrap support 93%) (see Figure 2). However, in the MP analysis, the monophyly of *Hepatozoon* and *Hemolivia* was not supported, and instead *Hemolivia* was indicated to be sister to *Hepatozoon* species from mammalian hosts (see Figure 3).

**Table 4:** Matrix showing ranges for uncorrected p-distances (below diagonal) and ranges for base pair differences with the percentage of parsimoniously informative sites in square parentheses (above diagonal). The latter are presented only for *Hemolivia* species amplified directly from the vertebrate host. Species included here comprise all *Hemolivia* species, with examples of one *Haemogregarina* and *Hepatozoon* species (both from chelonians), included in the phylogenetic analyses of the present study. Numbers in parentheses represent the number of sequences used

Species	1	2	3	4	5	6	7
1 <i>Haemogregarina</i> sp. ex <i>Pelusios subniger</i> (1)	0	42	66	58–61	42	54–61	63
2 <i>Hepatozoon fitsimensi</i> ex <i>Kinixys zombensis</i> (1)	0.04	0	22–23	18–19	21	23–24	24
3 <i>Hemolivia mariae</i> ex <i>Egernia stokesii</i> (2)	0.05	0.02	0	19–20 [61]	8–9 [89]	18–23 [83]	22 [70]
4 <i>Hemolivia</i> sp. ex <i>Rhinoclemmys pulcherrima manni</i> (2)	0.04	0.02	0.01	0	8 [42]	10–14 [41]	11–13 [33]
5 <i>Hemolivia parvula</i> ex <i>Kinixys zombensis</i> (2)	0.04	0.02	0.01	0.01	0	5–6 [63]	6 [42]
6 <i>Hemolivia mauritanica</i> ex <i>Testudo graeca</i> (3)	0.04	0.02	0.01	0.01	0–0.01	0	1–3 [0]
7 <i>Hemolivia mauritanica</i> ex <i>Testudo marginata</i> (2)	0.04	0.02	0.01	0.01	0.01	0	0



**Figure 2:** Maximum likelihood analysis (ML), implemented in Geneious 7.1, of phylogenetic relationships for *Haemogregarina parvula* Dias, 1953 and *Hepatozoon fitsimensi* (Dias, 1953) (both highlighted in bold) with species of *Hemolivia*, *Hepatozoon* and *Haemogregarina*, as well as *Babesiosoma stableri* and *Dactylosoma ranarum*. *Adelina dimidiata* was the outgroup. Nodal support is provided by bootstrap values with only those >50% shown. ML analysis was performed using the T92+G evolutionary model. The tree with the highest log-likelihood score (ln -3377.2225) is shown



**Figure 3:** Maximum parsimony (MP) analysis, implemented in MEGA6, of phylogenetic relationships for *Haemogregarina parvula* Dias, 1953 and *Hepatozoon fitszimonsi* (Dias, 1953) (both highlighted in bold) with species of *Hemolivia*, *Hepatozoon* and *Haemogregarina*, as well as *Babesiosoma stableri* and *Dactylosoma ranarum*. *Adelina dimidiata* was the outgroup. Nodal support is provided by bootstrap values with only those >50% shown. MP analysis was performed using the subtree pruning and regrafting (SPR) algorithm with search level 2, in which the initial trees were obtained with the random addition of sequences (100 replicates)

Within the major *Hemolivia* clade for both the ML and MP analyses, *H. parvula* was nested within the *Hemolivia* clade, placed between those species from Palearctic/Ethiopian hosts (*Testudo graeca* and *Testudo marginata*) and an Australian host (*Egernia stokesii*), and separate from *Hemolivia* of neotropical hosts (see Figures 2 and 3). Furthermore, *H. parvula* formed a monophyletic clade with the only other terrestrial chelonian *Hemolivia* species, *H. mauritanica* (Table 4).

A longer sequence (1 034 bp) for *H. fitszimonsi* (GenBank accession no. KR069084) from an additional host (*K. zombensis*) was produced during this study, and was identical to the isolate (GenBank accession no. KJ702453) described in Cook et al. (2014a). In addition, *H. parvula* and *H. fitszimonsi*, which are known to occur concurrently, belonged to separate clades. This relationship was further supported by close examination of the dissimilarities between the two species (Table 4).

## Discussion

Since its description by Dias (1953) and redescription by Cook et al. (2009), *Haemogregarina parvula* has almost

always been observed as a concurrent infection with *Hepatozoon fitszimonsi* in *Kinixys zombensis* (syn. *Kinixys belliana zuluensis*, *Kinixys belliana belliana*) (see Dias 1953; Cook et al. 2014a, 2014b). *Haemogregarina parvula* has only once been reported to parasitise an additional host species, an individual of *S. pardalis* (see Cook et al. 2009), whilst *H. fitszimonsi* in comparison has been reported from an additional four tortoise species (see Cook et al. 2009, 2014a). Given this repeatedly concurrent infection Siddall (1995) synonymised this species with *H. fitszimonsi* possibly due to the concern that *H. parvula* may be an encapsulated form or stage of *H. fitszimonsi*. Cook et al. (2009), however, preferred to retain its status as a separate species. It is evident from the present phylogenetic analysis that the latter was the correct option; the two haemogregarine species belong to separate clades and different genera, thus supporting the conclusion that they are distinct species.

It was also previously proposed that *H. parvula* might be a junior synonym of *H. mauritanica* (Cook et al. 2009). Given that both haemogregarine species are parasitic to terrestrial tortoises from Africa and conform morphometrically to each other, this suggestion was plausible. However, *H. mauritanica* parasitises two species of *Testudo*,

*T. graeca* and *T. marginata*, which inhabit temperate to desert regions of northern Africa to the western Palearctic realm (Široký et al. 2009). This is in stark contrast to *H. parvula*, which has only ever been found, during surveys of most of South Africa's tortoise species, including arid to tropical biomes, in tropical KwaZulu-Natal and Mozambique (see Dias 1953; Cook et al. 2009, 2014a, 2014b). In addition, *H. parvula* has only, with one exception as mentioned above, ever been reported to parasitise *K. zombensis*. South Africa is home to four *Kinixys* species (see Bates et al. 2014). None of the other *Kinixys* species, of which one species, *K. natalensis*, is sympatric with *K. zombensis*, have been reported to be parasitised with *H. parvula*, suggesting that this parasite is species-specific (see Cook et al. 2009, 2014a, 2014b). Moreover, in light of the present study's molecular findings, whereby *H. parvula* was resolved as monophyletic but formed a smaller clade than *H. mauritanica*, as well as a pending future molecular analysis using faster-evolving mitochondrial genes that are anticipated to provide a more holistic analysis, the two species can at present be retained as separate species.

In both phylogenetic analyses (ML and MP), the monophyletic clade containing the *Hemolivia* species, *H. parvula* included, was nested within the larger, currently unresolved, *Hepatozoon* clade. The phylogenetic analyses performed here concur with the findings of Barta et al. (2012), Haklová-Kočíková et al. (2014), Kvičerová et al. (2014) and Netherlands et al. (2014a, 2014b), but conflict with those of Harris et al. (2013) in that *Hemolivia* is seen as a sister taxon to *Hepatozoon*. A similar problem is evident for classification of species of *Karyolysus* Labbé, 1894, which also fall within the *Hepatozoon* clade (see Haklová-Kočíková et al. 2014). However, the phylogenetic relationship implicated using only 18S rDNA data should be treated with caution (Haklová-Kočíková et al. 2014; Kvičerová et al. 2014). As suggested by Haklová-Kočíková et al. (2014), in order to resolve these phylogenetic questions a faster-evolving marker may need to be employed.

To conclude, both phylogenetic analyses (ML and MP) presented in this study produced discrete *Hemolivia* and *Haemogregarina* clades and showed that *H. parvula* is a species within the genus *Hemolivia* and not *Haemogregarina* as originally proposed. The following nomenclatural correction is proposed: *Hemolivia parvula* (Dias, 1953) (syn. *Haemogregarina parvula* Dias, 1953) in the terrestrial tortoises *Kinixys zombensis* (syn. *Kinixys belliana belliana* and *Kinixys belliana zuluensis*) (type host), and *Stigmochelys pardalis*. *Hemolivia parvula* is thus the first species from this genus known from southern Africa.

**Acknowledgements** — We would like to thank Microbiology, Unit for Environmental Sciences and Management, North-West University, Potchefstroom campus, for use of their facilities. The financial assistance of the National Research Foundation (NRF) towards this research is also hereby acknowledged (project SFP13090332476). ECN is supported by the NRF Scarce Skills masters scholarship (Grant UID: 89924). Opinions expressed and conclusions arrived at are those of the authors and are not necessarily to be attributed to the NRF. We are also grateful to Ezemvelo KZN Wildlife for research permit OP 839/2014. We would also like to acknowledge our dear friend and colleague, the late Prof. Angela Davies, who was the first person to suggest

that *H. parvula* is a species of *Hemolivia* or *Hepatozoon* and not *Haemogregarina*. It is thus the solemn wish of all the authors to dedicate this paper in her memory.

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