First molecular detection of *Hemolivia* and *Hepatozoon* parasites in reptile-associated ticks on Iriomote Island, Japan

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Abstract

Hepatozoon and *Hemolivia* are members of the haemogregarines and are reported in reptiles and reptile-associated ticks. However, no studies have reported on *Hepatozoon* and *Hemolivia* in Japanese reptile-associated ticks. This study aimed to molecularly identify and to characterize *Hepatozoon* and *Hemolivia* in Japanese reptile-associated ticks, *Amblyomma geoemydae* (Cantor, 1847) and *Amblyomma nitidum* (Hirst & Hirst, 1910). A total of 41 and 75 DNA samples from *A. geoemydae* and *A. nitidum* ticks, respectively, were used for screening of *Hepatozoon* and *Hemolivia* with polymerase chain reaction targeting 18S rDNA. As a result, *Hemolivia* and *Hepatozoon* were detected in two *A. geoemydae* and one *A. nitidum*, respectively. The sequences of *Hemolivia* spp. showed a 99.5% (1,050/1,055 bp) identity with

Hemolivia parvula (KR069083), and the *Hemolivia* spp. were located in the same clade as *H. parvula* in the phylogenetic tree. The sequences of *Hepatozoon* sp. showed a 98.4% (1,521/1,545 bp) identity with *Hepatozoon colubri* (MN723844), and the *Hepatozoon* sp. was distinct from validated *Hepatozoon* species. in the tree. Our findings highlight the first molecular record of *Hemolivia* in Japan and present the first detection of *Hepatozoon* in *A. nitidum*. Further investigations on these tick-borne protozoa are required to understand their life cycle and pathogenicity.

Keywords

Hemolivia; *Hepatozoon; Amblyomma geoemydae*; *Amblyomma nitidum*; reptile-associated ticks; Japan

Declarations

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Code availability: Not applicable

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Introduction

The development of a haemogregarine parasite requires invertebrate vectors and vertebrate hosts to complete the life cycle. Invertebrate vectors, such as blood-sucking arthropods and leeches, serve as definitive hosts for parasite development, where the sexual cycle and sporogony of the parasite occur (O'Donoghue 2017; Smith 1996), whereas cyclic merogony and gametogony occur after transmission to the vertebrate hosts, which serve as the intermediate host for the parasite (O'Donoghue 2017). Transmission to the new vertebrate host can occur via ingestion, wherein the sporozoites are transmitted when the infected vector, such as the tick, is eaten by the new vertebrate host. The other is inoculation, wherein parasites enter the new vertebrate host while the vector is sucking blood from the host. There are nine genera in haemogregarines: *Babesiosoma*, *Bartazoon*, *Cyrilia*, *Dactylosoma*, *Desseria*, *Haemogregarina*, *Hemolivia*, *Hepatozoon*, and *Karyolysus* (Cook et al. 2018; O'Donoghue 2017; Lee et al. 2000). Among them, *Hepatozoon* and *Hemolivia* are transmitted through ingestion of infected ticks (O'Donoghue 2017; Smith 1996).

Members of the genus *Hepatozoon* are intraerythrocytic and intraleukocytic parasites found in both homeothermic and poikilothermic animals (Smith 1996). To date, more than 300 *Hepatozoon* species. have been identified worldwide (Allen et al. 2011; Smith et al. 1999). In Japan, *Hepatozoon* spp. have been detected from Japanese black bears (*Ursus thibetanus japonicus*) (Kubo et al. 2008), Japanese martens (*Martes melampus*) (Kubo et al. 2009), Japanese wild cats (*Prionailurus iriomotensis* and *Felis bengalensis euptilura*) (Kubo et al. 2006), Japanese dogs (*Canis lupus familiaris*) (Murata et al. 1991), wild rodents (*Myodes rutilus*) (Moustafa et al. 2017), and wild foxes (*Vulpes vulpes*) (Maede et al. 1982). Moreover, *Hepatozoon* spp. have also been reported in ticks infesting homeothermic animals in Japan (Masatani et al. 2017; Murata et al. 1995). On the other hand, *Hepatozoon* spp. in poikilothermic animals, such as reptiles and their associated ticks, have not been recorded in Japan but have been detected elsewhere globally (Han et al. 2015; Sumrandee et al. 2015; Vilcins et al. 2009).

The genus *Hemolivia* have only four validated species; *Hemolivia mariae*, *Hemolivia mauritanica*, *Hemolivia parvula*, and *Hemolivia stellata*. They infect poikilothermic vertebrates, such as lizards, tortoises, and toads, and they are transmitted by ticks to the vertebrates (Harris et al. 2013; Smallridge et al. 1997; Petit et al. 1990). As reported, the cane toad (*Rhinella marina*) and *Amblyomma rotundatum* are the vertebrate host and vector tick, respectively, for *H. stellata* in Brazil (Petit et al. 1990). For *H. mauritanica*

reported in Algeria, the Greek tortoise (*Testudo graeca*) and *Hyalomma aegyptium* are the vertebrate host and vector tick, respectively (Harris et al. 2013). Finally, the Australian sleepy lizard (*Tiliqua rugosa*) and *Amblyomma limbatum* are the vertebrate host and vector tick, respectively, for *H. mariae* in Australia (Smallridge et al. 1997). However, no *Hemolivia* spp. have been reported in Japan until now.

Asian turtle ticks (*Amblyomma geoemydae*) and sea snake ticks (*Amblyomma nitidum*) are considered major reptile-associated tick species in Japan (Takada et al. 2019). Moreover, some species of *Hepatozoon* and *Hemolivia* have been reported in reptile-associated ticks globally (Han et al. 2015; Sumrandee et al. 2015; Smallridge et al. 1997). Although bacterial microorganisms belonging to the genera *Borrelia* and *Rickettsia* and members of the family Anaplasmataceae have been previously investigated in these tick species (Qiu et al. 2021; Takano et al. 2011), there are no studies on *Hepatozoon* and *Hemolivia* parasites in these ticks. Therefore, we aimed to investigate and to analyze *Hepatozoon* and *Hemolivia* parasites in these reptile-associated ticks previously collected from wild reptiles in Japan.

Materials and Methods

In this study, DNA samples prepared in our previous study (Qiu et al. 2021) were used for the screening of *Hepatozoon* and *Hemolivia*. To briefly explain, 104 *A. geoemydae* and 77 *A. nitidum* ticks were removed from wild yellow-margined box turtles (*Cuora flavomarginata evelynae*) and amphibious sea kraits (55 *Laticauda semifasciata*, 35 *Laticauda colubrina*, and 1 *Laticauda laticaudata*), respectively, in Iriomote Island, Okinawa. DNA was extracted from individual adult and nymph ticks and pools of three to six larvae ticks. This yielded 75 DNA samples (68 individual and 7 larval pool samples) of *A. geoemydae* and 41 DNA samples (31 individual and 10 larval pool samples) of *A. nitidum*.

Hepatozoon spp. were first screened using conventional polymerase chain reaction (PCR) with the primer pair HepF300 (5′-GTTTCTGACCTATCAGCTTTCGACG-3′) and HepR900 (5′-CAAATCTAAGAATTTCACCTCTGAC-3′), which amplified a 660-bp fragment of *Hepatozoon* spp. 18S ribosomal DNA (rDNA) (Ujvari et al. 2004). In a previous study, this primer pair also amplified 18S rDNA of *Hemolivia* spp. (Cook et al., 2015). Briefly, PCR was performed in a 20-μL reaction mixture containing 0.1 μL Ex Taq Hot Start version (Takara Bio Inc., Shiga, Japan), 2 μL 10× Ex Taq buffer, 1.6 μL 2.5 mM dNTP mixture, 200 nM of each primer, and 2 μL of template DNA. The reaction conditions were as follows: 98°C for 1 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and a final

extension at 72°C for 5 min. Distilled water was used as a negative control. DNA sample, which tested positive for *Hepatozoon* spp. in a previous study (Qiu et al. 2018), was included as a positive control for each PCR assay. The PCR products were electrophoresed on a 1.2% agarose gel and stained with Gel-Red™ (Biotium, Hayward, CA, USA). The PCR product size was compared with a 100-bp DNA ladder (Nippon Gene, Tokyo, Japan).

To obtain longer sequences of 18S rDNA (approximately 1,550 bp), BTH primers described previously for *Babesia*, *Theileria*, and *Hepatozoon* (Masatani et al. 2017) were employed in nested-PCR using positive samples from the screening with the primer pair HepF300 and HepR900. To the best of our knowledge, this is the first study using BTH-nested PCR for the amplification of *Hemolivia* 18S rDNA. The BTH-nested PCR products were then purified using a NucleoSpin Gel and PCR Clean-Up kit (Takara Bio Inc.) or ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3130x genetic analyzer (Applied Biosystems) according to the corresponding manufacturers' instructions. The 5′ and 3′ ends of the sequences were analyzed and trimmed using ATGC software version 9.1 (GENETYX Corporation, Tokyo, Japan).

The sequences obtained from BTH-nested PCR were compared with those in the public database using a standard nucleotide basic local alignment search tool (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identity comparison of the sequence between the detected species and other previously described species of *Hemolivia* was performed. Phylogenetic analysis was conducted using MEGA version 10.1 (Kumar et al. 2018). ClustalW in MEGA version 10.1 was used for all sequence alignments. A phylogenetic tree based on 18S rDNA sequences derived from BTH-nested PCR was constructed using the maximum likelihood method with Kimura-2 parameter. The following accession numbers were assigned to the sequences determined in this study: LC603339, LC603340, and LC603342.

Results and Discussion

Molecular screening results of the 75 and 41 samples obtained from *A. geoemydae* and *A. nitidum* ticks, respectively, revealed that 2.7% (2/75) of the samples from *A. geoemydae* and 2.4% (1/41) from *A. nitidum* showed positive results for PCR with Hep300 and Hep900 primers. Additionally, all the positive samples were amplified in subsequent BTH-nested PCR with the expected size. Further analyses of the

144 sequences derived from BTH-nested PCR showed that sequences from *A. geoemydae* (Sample IDs: AG13
145 and AG25) had six nucleotide differences and a 1-bp gap in a 1,568-bp fragment, and both sequences
146 showed 98.7% (1,549/1,568 bp) identity with that of *H. stellata* from *A. rotundatum* (KP881349). The
147 identity comparison results are presented in Table 1. Further, it should be noted that although our sequences
148 from *A. geoemydae* showed the highest identity with *H. parvula*, the comparison sequence length was the
149 shortest, as the sequence in the database was only 1,052 bp for *H. parvula*. In BLASTn analysis, the
150 sequence obtained from *A. nitidum* (Sample ID: AN9) showed a 98.4% (1,521/1,545 bp) identity with that
151 from *Hepatozoon colubri* isolate 9689 (MN723844).

152 A phylogenetic tree was constructed for the sequences derived from sample IDs AG13, AG25, and
153 AN9 to obtain information concerning their genetic relatedness with other *Hemolivia* and *Hepatozoon*
154 species. Based on the phylogenetic inference, the sequences of *Hemolivia* spp. from *A. geoemydae* (Sample
155 IDs: AG13 and AG25) were located in the same clade with *H. parvula* (Fig. 1). In contrast, the sequence
156 of the *Hepatozoon* sp. from *A. nitidum* (Sample ID: AN9) formed a distinct clade from other *Hepatozoon*
157 species and was closely related to that of *He. colubri* (MN723844) and another *Hepatozoon* sp.
158 (MH174343) (Fig. 1).

159 We investigated *Hemolivia* and *Hepatozoon* in the reptile-associated ticks, *A. geoemydae* and *A.*
160 *nitidum*, collected in the Iriomote Island in Japan. We identified *Hemolivia* spp. closely related to *H.*
161 *parvula* and a putative novel *Hepatozoon* species that was distinct from other validated *Hepatozoon* species
162 in the phylogenetic tree. To the best of our knowledge, this is the first report of *Hemolivia* spp. in Japan
163 and the first record of a *Hepatozoon* sp. in *A. nitidum*.

164 *Hemolivia* spp. detected in *A. geoemydae* infesting yellow-margined box turtles (*C. f. evelynae*)
165 had the highest identity with and clustered in the same clade on the phylogenetic tree as *H. parvula* (Table
166 1 and Fig. 1). *H. parvula* has been detected in Bell's hinge-back tortoise (*Kinixys zombensis*) in South Africa
167 (Cook et al. 2015). Furthermore, considering that all validated *Hemolivia* species are tick-transmitted
168 hemogregarines of poikilothermic vertebrates (Harris et al. 2013; Smallridge et al. 1997; Petit et al. 1990),
169 our detected *Hemolivia* spp. might take a poikilothermic vertebrate as a host. Therefore, further
170 investigations of *Hemolivia* parasites in reptiles, especially in the yellow-margined box turtle, which is the
171 main blood meal host of *A. geoemydae* on Iriomote Island, are required to determine the vertebrate host of
172 *Hemolivia* spp. detected in this study.

Previous studies of *H. stellata* in the tick vector and *H. parvula* in host tortoises provided morphological information on these *Hemolivia* spp. at each developmental stage, which was helpful in species identification in addition to the molecular method (Cook et al. 2015; Karadjian et al. 2015). Similarly, the morphological description would be useful to confirm whether the presence of *Hemolivia* parasites in *A. gemoemydae* in the current study is *H. parvula* or a closely related species. Thus, morphological investigations of our detected *Hemolivia* spp. in vertebrate hosts and tick vectors are required to further characterize this protozoan parasite.

Hepatozoon sp. detected in *A. nitidum* infesting sea kraits (*Laticauda* sp.) showed high identity with *H. colubri*. In the phylogenetic tree, this *Hepatozoon* sp. was distinct from other reported *Hepatozoon* species but formed a monophyletic group with *H. colubri*, which was also associated with snakes (Han et al. 2015). This finding suggested that the *Hepatozoon* sp. detected in this study might be a putative novel species or *H. colubri*. In the future, including a morphological investigation of the *Hepatozoon* sp. would be helpful in understanding the parasite species. Furthermore, *Hepatozoon* parasites require vertebrate hosts and invertebrate vectors to complete their life cycle, and *A. nitidum* is a blood-sucking ectoparasite specific to sea kraits (*Laticauda* spp.) (Takada et al. 2019). Therefore, an investigation of *Hepatozoon* parasites in reptiles, especially in sea kraits (*Laticauda* spp.), is needed to clarify the vertebrate host of the detected *Hepatozoon* sp. in the future.

The diversity of *Hepatozoon* species remains unknown, and many novel *Hepatozoon* species associated with reptiles have been recently described (Han et al. 2015; Mansour et al. 2020; Telford 2010). Of the 79 reptile species listed in Japan, 58 (73.4%) are endemic (Ota 2000). Further investigations to elucidate the species diversity of *Hepatozoon* parasites in Japanese reptiles are needed. In addition, infections of *Hepatozoon* spp. resulting in clinical disease in reptiles have been reported in previous laboratory experiments (Wozniak et al. 1996). Heavy and long-term parasitemia of *Hepatozoon* spp. has been associated with negative impacts on growth, body condition, and reproductive output of reptiles (Madsen et al. 2005; Ujvari et al. 2004). Therefore, the pathogenicity evaluation of *Hepatozoon* parasites in Japanese reptiles, especially endemic species, could be important for disease monitoring in the reptile species.

The limitation of this study was that the sampling was conducted in a single location with no repetition; therefore, it does not provide the actual distribution and prevalence of *Hemolivia* and

Hepatozoon parasites in Japan. Nonetheless, this study revealed the presence of *Hemolivia* and *Hepatozoon* species in Japanese reptile-associated ticks for the first time. Although cases of hepatozoonosis in Japanese reptiles have not yet been reported, further studies, such as those employing continuous surveillance of these parasites in reptile-associated ticks and their host reptiles, are warranted to evaluate their potential as pathogenic agents.

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296

297 **Figure Caption**

298 **Fig 1. Phylogenetic tree of *Hemolivia* spp. and *Hepatozoon* sp. based on 18S rDNA sequences**

299 18S rDNA sequences derived from BTH-nested PCR were used for phylogenetic analysis. The accession
300 numbers for nucleotide sequences are shown after the species names. The analysis was performed using
301 the maximum likelihood method with the Kimura-2 parameter. Bootstrap values >70% based on 1,000
302 replications are presented on the interior branch nodes.

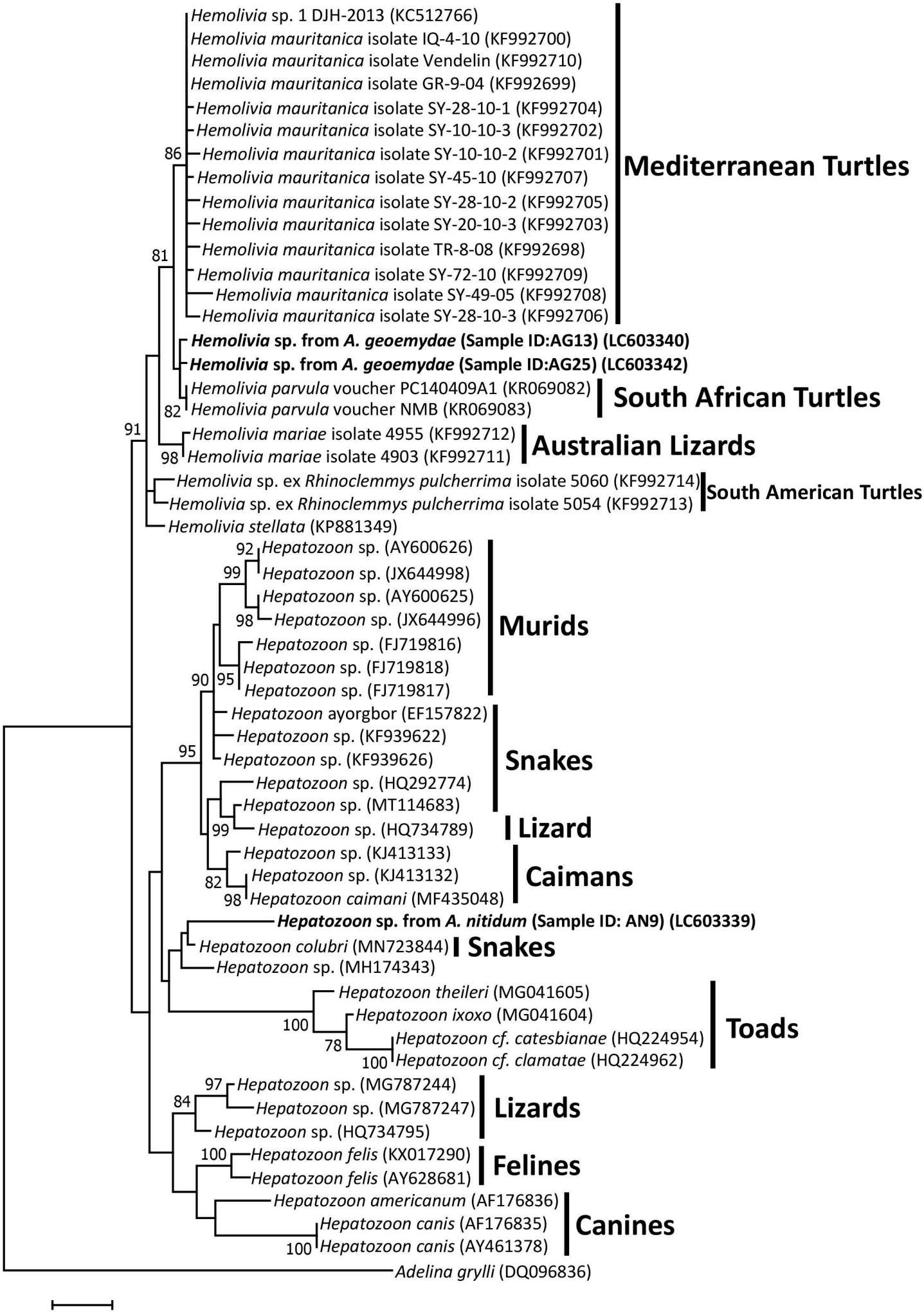
303

304

Table 1. Identity comparison of 18S ribosomal DNA partial sequences between the *Hemolivia* spp. and other validated *Hemolivia* spp.

	Hemolivia sp. from *A. geoemydae* (AG13) (LC603340) 1,568 bp	*Hemolivia* sp. from *A. geoemydae* (AG25) (LC603342) 1,567 bp
Hemolivia parvula (KR069083) 1,052 bp	99.5% (1,050/1,055 bp)	99.5% (1,050/1,055 bp)
Hemolivia mauritanica (KF992706) 1,422 bp	98.9% (1,409/1,424 bp)	99.2% (1,411/1,423 bp)
Hemolivia mariae (KF992711) 1,420 bp	98.0% (1,399/1,427 bp)	98.2% (1,401/1,426 bp)
Hemolivia stellata (KP881349) 1,816 bp	98.7% (1,549/1,568 bp)	98.7% (1,549/1,568 bp)

305


Hemolivia sp. 1 DJH-2013 (KC512766)
Hemolivia mauritanica isolate IQ-4-10 (KF992700)
Hemolivia mauritanica isolate Vendelin (KF992710)
Hemolivia mauritanica isolate GR-9-04 (KF992699)
Hemolivia mauritanica isolate SY-28-10-1 (KF992704)
Hemolivia mauritanica isolate SY-10-10-3 (KF992702)
86
Hemolivia mauritanica isolate SY-10-10-2 (KF992701)
Hemolivia mauritanica isolate SY-45-10 (KF992707)
Hemolivia mauritanica isolate SY-28-10-2 (KF992705)
Hemolivia mauritanica isolate SY-20-10-3 (KF992703)
81
Hemolivia mauritanica isolate TR-8-08 (KF992698)
Hemolivia mauritanica isolate SY-72-10 (KF992709)
Hemolivia mauritanica isolate SY-49-05 (KF992708)
Hemolivia mauritanica isolate SY-28-10-3 (KF992706)
Mediterranean Turtles
Hemolivia sp. from A. geoemydae (Sample ID:AG13) (LC603340)
Hemolivia sp. from A. geoemydae (Sample ID:AG25) (LC603342)
Hemolivia parvula voucher PC140409A1 (KR069082)
82
Hemolivia parvula voucher NMB (KR069083)
South African Turtles
91
Hemolivia mariae isolate 4955 (KF992712)
98
Hemolivia mariae isolate 4903 (KF992711)
Australian Lizards
Hemolivia sp. ex Rhinoclemmys pulcherrima isolate 5060 (KF992714)
Hemolivia sp. ex Rhinoclemmys pulcherrima isolate 5054 (KF992713)
South American Turtles
Hemolivia stellata (KP881349)
92
Hepatozoon sp. (AY600626)
99
Hepatozoon sp. (JX644998)
Hepatozoon sp. (AY600625)
98
Hepatozoon sp. (JX644996)
Hepatozoon sp. (FJ719816)
Hepatozoon sp. (FJ719818)
90
95
Hepatozoon sp. (FJ719817)
Murids
Hepatozoon ayorgbor (EF157822)
Hepatozoon sp. (KF939622)
95
Hepatozoon sp. (KF939626)
Hepatozoon sp. (HQ292774)
Hepatozoon sp. (MT114683)
99
Hepatozoon sp. (HQ734789)
Snakes
Lizard
Hepatozoon sp. (KJ413133)
82
Hepatozoon sp. (KJ413132)
98
Hepatozoon caimani (MF435048)
Caimans
Hepatozoon sp. from A. nitidum (Sample ID: AN9) (LC603339)
Hepatozoon colubri (MN723844)
Hepatozoon sp. (MH174343)
Snakes
Hepatozoon theileri (MG041605)
Hepatozoon ixoxo (MG041604)
100
78
Hepatozoon cf. catesbianae (HQ224954)
100
Hepatozoon cf. clamatae (HQ224962)
Toads
97
Hepatozoon sp. (MG787244)
84
Hepatozoon sp. (MG787247)
Hepatozoon sp. (HQ734795)
Lizards
100
Hepatozoon felis (KX017290)
Hepatozoon felis (AY628681)
Felines
Hepatozoon americanum (AF176836)
Hepatozoon canis (AF176835)
100
Hepatozoon canis (AY461378)
Canines
Adelina grylli (DQ096836)
0.01