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

30	Hemolivia parvula (KR069083), and the Hemolivia spp. were located in the same clade as H. parvula in
31	the phylogenetic tree. The sequences of Hepatozoon sp. showed a 98.4% (1,521/1,545 bp) identity with
32	Hepatozoon colubri (MN723844), and the Hepatozoon sp. was distinct from validated Hepatozoon species.
33	in the tree. Our findings highlight the first molecular record of Hemolivia in Japan and present the first
34	detection of Hepatozoon in A. nitidum. Further investigations on these tick-borne protozoa are required to
35	understand their life cycle and pathogenicity.
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37	Keywords
38	Hemolivia; Hepatozoon; Amblyomma geoemydae; Amblyomma nitidum; reptile-associated ticks; Japan
39	
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#### 57 Introduction

58 The development of a haemogregarine parasite requires invertebrate vectors and vertebrate hosts to 59 complete the life cycle. Invertebrate vectors, such as blood-sucking arthropods and leeches, serve as 60 definitive hosts for parasite development, where the sexual cycle and sporogony of the parasite occur 61 (O'Donoghue 2017; Smith 1996), whereas cyclic merogony and gametogony occur after transmission to 62 the vertebrate hosts, which serve as the intermediate host for the parasite (O'Donoghue 2017). Transmission 63 to the new vertebrate host can occur via ingestion, wherein the sporozoites are transmitted when the infected 64 vector, such as the tick, is eaten by the new vertebrate host. The other is inoculation, wherein parasites enter 65 the new vertebrate host while the vector is sucking blood from the host. There are nine genera in 66 haemogregarines: Babesiosoma, Bartazoon, Cyrilia, Dactylosoma, Desseria, Haemogregarina, Hemolivia, 67 Hepatozoon, and Karyolysus (Cook et al. 2018; O'Donoghue 2017; Lee et al. 2000). Among them, 68 Hepatozoon and Hemolivia are transmitted through ingestion of infected ticks (O'Donoghue 2017; Smith 69 1996).

70 Members of the genus Hepatozoon are intraerythrocytic and intraleukocytic parasites found in both 71 homeothermic and poikilothermic animals (Smith 1996). To date, more than 300 Hepatozoon species. have 72 been identified worldwide (Allen et al. 2011; Smith et al. 1999). In Japan, Hepatozoon spp. have been 73 detected from Japanese black bears (Ursus thibetanus japonicus) (Kubo et al. 2008), Japanese martens 74 (Martes melampus) (Kubo et al. 2009), Japanese wild cats (Prionailurus iriomotensis and Felis bengalensis 75 euptilura) (Kubo et al. 2006), Japanese dogs (Canis lupus familiaris) (Murata et al. 1991), wild rodents 76 (Myodes rutilus) (Moustafa et al. 2017), and wild foxes (Vulpes vulpes) (Maede et al. 1982). Moreover, 77 Hepatozoon spp. have also been reported in ticks infesting homeothermic animals in Japan (Masatani et al. 78 2017; Murata et al. 1995). On the other hand, Hepatozoon spp. in poikilothermic animals, such as reptiles 79 and their associated ticks, have not been recorded in Japan but have been detected elsewhere globally (Han 80 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.

90 Asian turtle ticks (Amblyomma geoemydae) and sea snake ticks (Amblyomma nitidum) are 91 considered major reptile-associated tick species in Japan (Takada et al. 2019). Moreover, some species of 92 Hepatozoon and Hemolivia have been reported in reptile-associated ticks globally (Han et al. 2015; 93 Sumrandee et al. 2015; Smallridge et al. 1997). Although bacterial microorganisms belonging to the genera 94 Borrelia and Rickettsia and members of the family Anaplasmataceae have been previously investigated in 95 these tick species (Qiu et al. 2021; Takano et al. 2011), there are no studies on Hepatozoon and Hemolivia 96 parasites in these ticks. Therefore, we aimed to investigate and to analyze Hepatozoon and Hemolivia 97 parasites in these reptile-associated ticks previously collected from wild reptiles in Japan.

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### 99 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*.

107 Hepatozoon spp. were first screened using conventional polymerase chain reaction (PCR) with the 108 primer (5'-GTTTCTGACCTATCAGCTTTCGACG-3') and HepR900 (5'pair HepF300 109 CAAATCTAAGAATTTCACCTCTGAC-3'), which amplified a 660-bp fragment of Hepatozoon spp. 18S 110 ribosomal DNA (rDNA) (Ujvari et al. 2004). In a previous study, this primer pair also amplified 18S rDNA 111 of Hemolivia spp. (Cook et al., 2015). Briefly, PCR was performed in a 20-µL reaction mixture containing 112 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 113 dNTP mixture, 200 nM of each primer, and 2 µL of template DNA. The reaction conditions were as follows: 114 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<sup>TM</sup> (Biotium, Hayward, CA, USA). The PCR product size was compared with a 100-bp DNA ladder (Nippon Gene, Tokyo, Japan).

120 To obtain longer sequences of 18S rDNA (approximately 1,550 bp), BTH primers described 121 previously for Babesia, Theileria, and Hepatozoon (Masatani et al. 2017) were employed in nested-PCR 122 using positive samples from the screening with the primer pair HepF300 and HepR900. To the best of our 123 knowledge, this is the first study using BTH-nested PCR for the amplification of Hemolivia 18S rDNA. 124 The BTH-nested PCR products were then purified using a NucleoSpin Gel and PCR Clean-Up kit (Takara 125 Bio Inc.) or ExoSAP-IT<sup>TM</sup> Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, 126 MA, USA). Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied 127 Biosystems, Foster City, CA, USA) and an ABI Prism 3130x genetic analyzer (Applied Biosystems) 128 according to the corresponding manufacturers' instructions. The 5' and 3' ends of the sequences were 129 analyzed and trimmed using ATGC software version 9.1 (GENETYX Corporation, Tokyo, Japan).

130 The sequences obtained from BTH-nested PCR were compared with those in the public database 131 using standard nucleotide basic local alignment search tool (BLASTn) а 132 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identity comparison of the sequence between the detected species 133 and other previously described species of Hemolivia was performed. Phylogenetic analysis was conducted 134 using MEGA version 10.1 (Kumar et al. 2018). ClustalW in MEGA version 10.1 was used for all sequence 135 alignments. A phylogenetic tree based on 18S rDNA sequences derived from BTH-nested PCR was 136 constructed using the maximum likelihood method with Kimura-2 parameter. The following accession 137 numbers were assigned to the sequences determined in this study: LC603339, LC603340, and LC603342.

138

### 139 **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).

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

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

180 Hepatozoon sp. detected in A. nitidum infesting sea kraits (Laticauda sp.) showed high identity 181 with H. colubri. In the phylogenetic tree, this Hepatozoon sp. was distinct from other reported Hepatozoon 182 species but formed a monophyletic group with H. colubri, which was also associated with snakes (Han et 183 al. 2015). This finding suggested that the *Hepatozoon* sp. detected in this study might be a putative novel 184 species or *H. colubri*. In the future, including a morphological investigation of the *Hepatozoon* sp. would 185 be helpful in understanding the parasite species. Furthermore, Hepatozoon parasites require vertebrate hosts 186 and invertebrate vectors to complete their life cycle, and A. nitidum is a blood-sucking ectoparasite specific 187 to sea kraits (Laticauda spp.) (Takada et al. 2019). Therefore, an investigation of Hepatozoon parasites in 188 reptiles, especially in sea kraits (Laticauda spp.), is needed to clarify the vertebrate host of the detected 189 Hepatozoon sp. in the future.

190 The diversity of Hepatozoon species remains unknown, and many novel Hepatozoon species 191 associated with reptiles have been recently described (Han et al. 2015; Mansour et al. 2020; Telford 2010). 192 Of the 79 reptile species listed in Japan, 58 (73.4%) are endemic (Ota 2000). Further investigations to 193 elucidate the species diversity of Hepatozoon parasites in Japanese reptiles are needed. In addition, 194 infections of *Hepatozoon* spp. resulting in clinical disease in reptiles have been reported in previous 195 laboratory experiments (Wozniak et al. 1996). Heavy and long-term parasitemia of Hepatozoon spp. has 196 been associated with negative impacts on growth, body condition, and reproductive output of reptiles 197 (Madsen et al. 2005; Ujvari et al. 2004). Therefore, the pathogenicity evaluation of Hepatozoon parasites 198 in Japanese reptiles, especially endemic species, could be important for disease monitoring in the reptile 199 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

- 202 *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
- 206 pathogenic agents.
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### 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
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	Hemolivia sp. from A. geoemydae (AG13)	<i>Hemolivia</i> sp. from <i>A. geoemydae</i> (AG25)	
	(LC603340) 1,568 bp	(LC603342) 1,567 bp	
Hemolivia parvula	99.5% (1.050/1.055 hp)	99.5% (1,050/1,055 bp)	
(KR069083) 1,052 bp	99.5% (1,050/1,055 bp)		
Hemolivia mauritanica	08.0% (1.400/1.424 hp)	00.20/(1.411/1.422 hr)	
(KF992706) 1,422 bp	76.7% (1,407/1,424 bp)	99.2% (1,411/1,425 bp)	
Hemolivia mariae	98.0% (1.399/1.427 hp)	98.2% (1,401/1,426 bp)	
(KF992711) 1,420 bp	96.070 (1,999/1,427 op)		
Hemolivia stellata	09.70((1.540/1.569 hr))	98.7% (1,549/1,568 bp)	
(KP881349) 1,816 bp	70.170 (1,549/1,500 up)		

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

