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Hepatozoon apri n. sp. (Adeleorina: Hepatozoidae) from the Japanese wild boar *Sus scrofa leucomystax* (Mammalia: Cetartiodactyla)

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ABSTRACT

Hepatozoon apri n. sp. is described from Japanese wild boars *Sus scrofa leucomystax* in Japan. The gamonts in the peripheral blood leukocytes were 11.6 ± 1.4 × 6.7 ± 1.3 μm in size. The meronts in the muscle tissues were 35.0–47.5 μm in length and 26.5–30 μm in width. A high rate (53.0%) of infection was found by nested PCR using muscle specimens from 181 wild boars captured in Tokushima, Japan. A phylogenetic analysis based on 18S rRNA gene sequences revealed that *H. apri* n. sp. detected in wild boars is closely related to *Hepatozoon* spp. isolated from carnivores. This is the first description of a species belonging to the genus *Hepatozoon* detected in ungulates.

1. Introduction

Species of the genus *Hepatozoon* Miller, 1908 are apicomplexan parasites that infect terrestrial vertebrates as intermediate hosts and hematophagous arthropods as final hosts. Among mammalian hosts, *Hepatozoon* infections have mainly been reported in rodents, lagomorphs, insectivores, marsupials, and carnivores, and have rarely been reported in ungulates (Clark et al., 1973; McCully et al., 1975; Smith, 1996; Graig, 2001). However, *Hepatozoon* sp. have recently been detected in muscle tissues of the Japanese wild boar *Sus scrofa leucomystax* (Mammalia: Cetartiodactyla) in Gihu Prefecture, Japan (Matsuo et al., 2016) and *Hepatozoon* DNA has been detected in wood ticks in the genus *Dermacentor* (Arthropoda: Ixodidae) collected from wild boars in Thailand (Sumrandee et al., 2015). These reports suggest that unknown *Hepatozoon* species are found in wild boars in Asia.

Population sizes of ungulates, such as wild boars and the sika deer *Cervus nippon,* have increased dramatically throughout Japan in recent decades, resulting in significant agricultural damage (Honda and Sugita, 2007; Honda et al., 2010). These animals play an important ecological role in the dispersal of zoonotic parasites, including *Toxoplasma, Sarcocystis*, *Paragonimus, Onchocerca*, and *Gnathostoma* (Takaoka et al., 2004; Meng et al., 2009; Sugiyama et al., 2015). Further, the risk of diseases caused by these parasites is an increasing concern owing to the frequent contact between humans and domestic animals. In the present study, we detected *Hepatozoon* species in a survey of zoonotic parasites in Japanese wild boars caught in the mountain area of Tokushima Prefecture located on Shikoku Island, Japan. The aims of this study were to determine the morphological and molecular characteristics of *Hepatozoon* species in wild boars, and to evaluate the prevalence of the species in wild populations in Tokushima, Japan.

2. Materials and methods

2.1. Sample collection

Between May 2014 and January 2017, 181 Japanese wild boars (93 males and 88 females), including 11 juveniles (with body striping) and 170 young or older individuals, and 113 sika deer (*Cervus nippon centralis*) were legally hunted by licensed hunters in Tokushima, Japan in accordance with the Protection and Control of Wild Birds and Mammals and Hunting Management Law. This study did not require approval by

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an animal ethics committee, since animals were not killed specifically for the study. Muscle tissue samples were obtained from all individuals. Heart, liver, kidney, and spleen tissues and EDTA-anticoagulated blood were obtained from five boars (sample IDs: 28-3, 28-11, 28-12, 28-18, and 28-26) caught between September 2016 and January 2017.

2.2. Hematological and histopathological examinations

Thin blood or buffy coat smears were prepared, air-dried, fixed, and stained using the Diff-Quik Staining Kit (Sysmex, Hyogo, Japan). Parasitemia was estimated by counting parasitized leukocytes among 3000–3100 leukocytes in these smears. Gamonts and meronts were measured using the cellSense software (Olympus, Tokyo, Japan). For histopathological examinations, the muscle, heart, liver, kidney, and spleen tissues from five boars were fixed in 10% formalin. These specimens were processed routinely, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

In a previous study, *Hepatozoon* sp. was found in muscle tissues from a Japanese wild boar (ID: IB20) captured in Gifu prefecture, Japan (Matsuo et al., 2016). Sequence analyses revealed that *Hepatozoon* sp. from wild boar (ID: IB20) was identical to the *Hepatozoon* species found in the wild boars in the present study. To identify developmental stages of *Hepatozoon* sp. parasitizing wild boar, formalin-fixed paraffin-embedded myocardium and skeletal muscle tissues from IB20 were newly sectioned, stained with H&E, and examined microscopically.

2.3. DNA extraction, DNA amplification, and sequencing

Muscle, heart, liver, kidney, and spleen tissue samples (500 mg) were homogenized separately, supplemented with 700 μL of TE buffer (Nippon Gene, Toyama, Japan), and mixed vigorously for 30 s. After centrifugation at 6000 × *g*, genomic DNA was extracted from 200 μL of supernatants using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For blood specimens, DNA was extracted from buffy coat samples using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions.

PCR was performed to amplify the partial 18S rRNA gene (18S) from five blood specimens using the primer set 18S1F/18S11R to detect apicomplexan parasites (Pritt et al., 2008). Nested PCR of the same region was performed using the blood and tissues of the heart, liver, kidney, and spleen from five boars and muscle samples from all 181 boars and 113 deer. For nested-PCR, the primers Hap1F, designed based on the 18S sequence of *Hepatozoon* species from wild boars in this study, and 18S11R were used. Expected amplicon size for 1st and 2nd PCR was 1108 bp and 557 bp, respectively. Detailed primer information is provided in Table 1.

The PCR mixture contained 2.5 μL of 10 × Ex Taq buffer (Takara Bio Inc., Otsu, Japan), 0.2 mM dNTP (Takara), 0.2 μM each primer, 1 U of Ex Taq polymerase (Takara) and 1 μL of DNA extract or 1st PCR products in a total volume of 20 μL. PCR conditions consisted of initial denaturation at 94 °C for 5 min, followed by 40 (1st PCR) or 25 cycles (2nd PCR) at 94 °C for 30 s, 60 (1st PCR) or 52.5 °C (2nd PCR) for 1 min, and 72 °C for 1 min, and then a final extension step at 72 °C for 5 min. All amplifications were performed using the Gene Atlas thermal cycler (Astec, Chattanooga, TN, USA). The amplified DNA was applied to 2% agarose gels, electrophoresed, and visualized under an LED transilluminator. PCR products were directly sequenced in both directions with the primers used for the 1st or 2nd PCR using the GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman, Brea, CA, USA) and CEQ8000 (Beckman).

Table 1
Primers used in this study.

Name	Direction	Sequence	Reference
18S1F	F	5′- GGATAACCGTGGTAATTCTATG -3′	Pritt et al., 2008
18S11R	R	5′- TCCTATGTCTGGACCTGGTGAG -3′	Pritt et al., 2008
Hap1F	F	5′- GCTTTTAATAAAAGTAGTATCTTGG -3′	Present study

2.4. DNA sequence analyses

The 18S sequences were used to establish the phylogenetic position of the present *Hepatozoon* species. Sequence similarity was determined using a BLASTN search against the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/Blast.cg).

The obtained sequence and 18S sequences of *Hepatozoon* spp. and related genera (*Hemolivia, Haemogregarina, Dactylosoma,* and *Adelina*) available in the DDBJ/EMBL/GenBank databases were aligned using a web-based version of a multiple alignment program (MAFFT, version 7) (Katoh and Standley, 2013) with the Q-INS-i setting, followed by a manual check. Uncorrected *p*-distances between all sequence pairs were calculated using MEGA version 7.0 (Kumar et al., 2016). A phylogenetic analysis was performed using neighbor-joining (NJ) and maximum likelihood (ML) methods implemented in MEGA. All positions containing gaps and missing data were eliminated and both trees were constructed using the Tamura–Nei model and gamma-distributed rates (AICc score, 4313.7). A bootstrap analysis was performed using 1000 replicates.

3. Results

3.1. General findings

All blood smears from five different boar specimens had gamonts in leukocytes (Fig. 1a–d). The average parasitemia of *Hepatozoon* was 0.20%, ranging from 0.03% (ID: 28-12, 1/3060 leucocytes) to 0.39% (ID: 28-11, 12/3081 leucocytes). Evident anemia and leukocytosis were not observed in these specimens. Histological examinations demonstrated that there were a few focal legions in the skeletal muscle tissues of the boar (ID: 28-11). A lesion was comparable to a ruptured meront, characterized by an accumulation of phagocytes, neutrophils, and merozoites- or gamont-like cells (Fig. 2). We did not detect histologic lesions in any of the other tissues of wild boars captured in Tokushima, Japan. On the other hand, histological examinations of the myocardium and skeletal muscles of Japanese wild boar from Gifu prefecture (IB20) demonstrated the presence of trophozoite (Fig. 3a), immature (Fig. 3b and c) and mature meronts (Fig. 3d). These were located in the center of parasitophorous vacuoles and no inflammatory response was found in the surrounding region (Fig. 3a–d).

In a PCR assay of five blood specimens with intraleukocytic parasites, the primer set 18S1F/18S11R yielded positive results, and the PCR products were approximately 1100 bp for all specimens. Partial 18S sequences (1007 bp) of 5 specimens were 100% identical. BLASTN analyses of partial 18S sequences obtained in this study and the top three hits in a search against the GenBank/DDBJ/EMBL databases indicated an identity of 100% (query cover: 100%) with *Hepatozoon* sp. (accession no. LC062147) from a Japanese wild boar (ID: IB20) in Gifu, Japan, 97.6% (query cover: 99.4%) with *Hepatozoon felis* (accession nos. AY620232, AY628688, and KX017290) from the domestic cat *Felis catus*, and 97.5% (query cover 100%) with *Hepatozoon* sp. (accession no. EF222257) from the European pine marten *Martes martes*. Based on the genetic similarities between the present species and *Hepatozoon* sp. from Japanese wild boar (ID: IB20), both were considered to be the same new species, and are described as follows.

3.2. Description

Phylum Apicomplexa Levine, 1970
Family Hepatozoidae Wenyon, 1926