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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

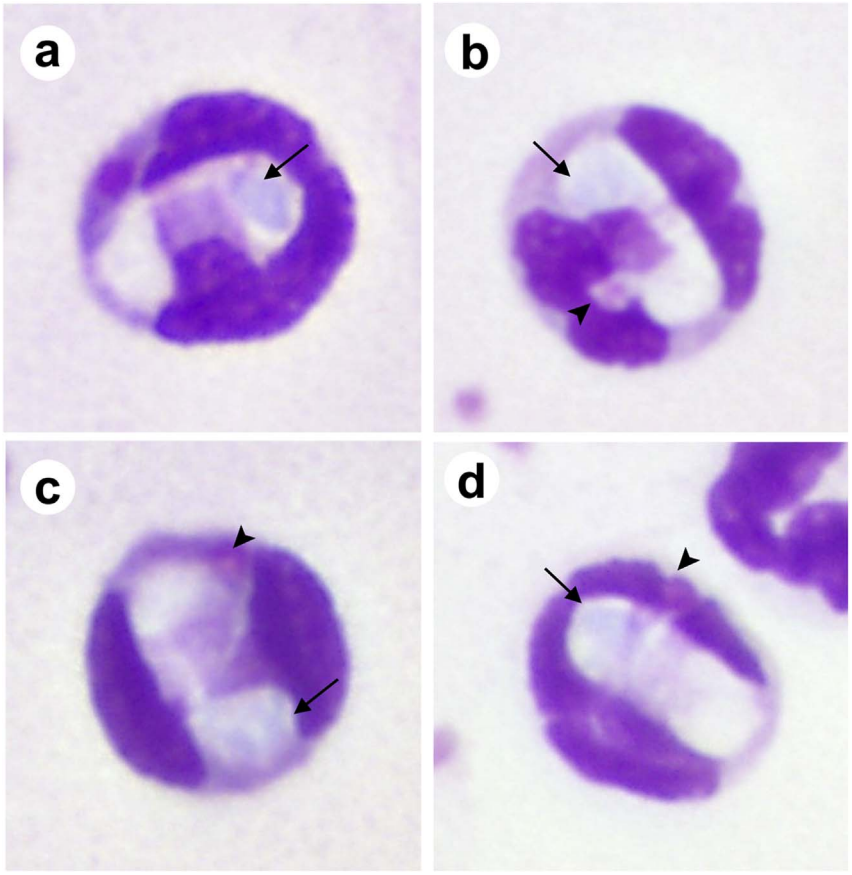


**Fig. 1.** a–b) Gamonts of *Hepatozoon apri* n. sp. in the cytoplasm of neutrophils detected in the blood smear of a boar (ID: 28-11), showing acentric and rounded nuclei (arrows) and a small protrusion containing eosinophilic granules (arrowheads).

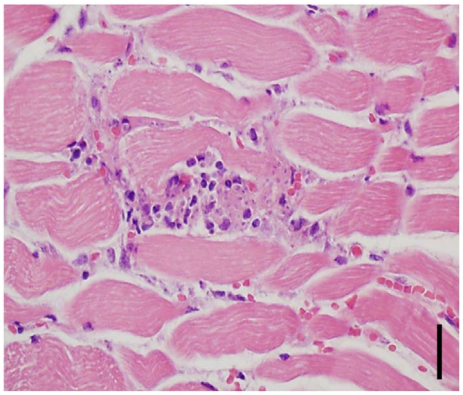

**Fig. 2.** Inflammatory lesion with released merozoites or gamonts in the femoral muscle of Japanese wild boar.

Genus *Hepatozoon* Miller, 1908
*Hepatozoon apri* n. sp.

### 3.2.1. Gamont (Fig. 1a–d)

The gamont was ellipse-shaped, occupied the longitudinal length of the infected leukocytes, and possessed an acentric nucleus (Fig. 1a–d) and one small protrusion containing eosinophilic granules (Fig. 1b–d). The sizes were 11.59 ± 1.4 (9.08–16.52) μm in length and 6.67 ± 1.3 (4.28–11.52) μm in width. Based on morphological appearance, infected leukocytes resembled neutrophils.

### 3.2.2. Trophozoite and meront (Fig. 3a–d)

Trophozoites were found in the parasitophorous vacuoles of the host cells with foamy cytoplasm (Fig. 3a). Two types of developing meronts were observed in the tissues of myocardium muscles. These immature meronts were located in the center of enlarged vacuoles (Fig. 3b and c). One immature meront was wheel spoke-shaped and 45.0 × 27.5 μm in size, with approximately 30 peripherally arranged merozoites as palisades inside a membrane, and foamy cytoplasm (Fig. 3b). The other immature meront was ovoidal, 35.0 × 26.5 μm in size, and with a lumen loosely filled with approximately 50 merozoites with rounded large nuclei (Fig. 3c). One mature meront found in the skeletal muscle tissue was 47.5 × 30.0 μm in size, with a lumen densely filled with numerous small nucleated merozoites (Fig. 3d).

## 3.3. Taxonomic summary

### 3.3.1. Type host (intermediate host)

Japanese wild boar *Sus scrofa leucomystax*.

### 3.3.2. Location in type host

Gamonts were found in leukocytes in the peripheral blood. Trophozoites and meronts were found in the myocardium and skeletal muscle tissues.

### 3.3.3. Prevalence of infection in type hosts

Nested PCR using a specific primer set showed positive results in blood specimens and muscle, heart, liver and spleen tissues from five wild boars (Table 2) whose blood smears were microscopically positive for *Hepatozoon* parasites in leukocytes. Therefore, muscle samples were used to examine the prevalence of *Hepatozoon* infections among 181 Japanese wild boars hunted in Tokushima, Japan. A total of 96 (53.0%) were positive based on nested PCR (Table 3). Twenty positive samples were sequenced; these sequences were 100% identical to each other

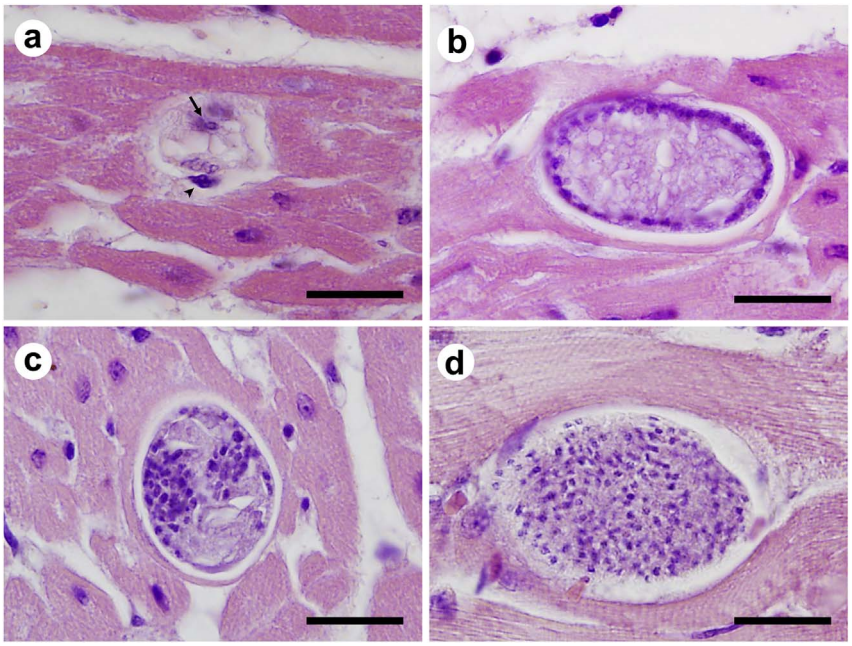


**Fig. 3.** a–d) Various developmental stages of *Hepatozoon apri* n. sp. detected in the muscles. a) A trophozoite (arrow) in the femoral muscles. The outer layer contains a fibroblast-like nucleus (arrowhead). b–c) Immature meronts found in the heart. d) Mature meront in the femoral muscles. H&E stain. Bar = 20 μm.

**Table 2**
Results of nested PCR for detection of *Hepatozoon* 18S in five Japanese wild boars, whose blood smears showed *Hepatozoon* parasites in leukocytes.

| Sample IDs | Blood | Muscle | Heart | Liver | Kidney | Spleen |
|---|---|---|---|---|---|---|
| 28–3 | + | + | ND | ND | ND | ND |
| 28–11 | + | + | + | + | – | + |
| 28–12 | + | + | – | – | – | – |
| 28–18 | + | + | + | – | – | – |
| 28–26 | + | + | ND | ND | ND | ND |

+, PCR amplicon with nested PCR.
-, no amplicon.
ND, not done.

**Table 3**
Prevalence of *Hepatozoon* infection in muscle specimens of 181 Japanese wild boars and 113 sika deer hunted in Tokushima, Japan.

| | Japanese boars ($n$ = 181) | | | | Sika deer ($n$ = 113) |
|---|---|---|---|---|---|
| | Juvenile | | Young or older | | |
| | Male | Female | Male | Female | |
| Positive | 0 (0%) | 1 (0.6%) | 49 (27.1%) | 46 (25.4%) | 0 (0%) |
| Negative | 8 (4.4%) | 2 (1.1%) | 36 (19.9%) | 39 (21.5%) | 113 (100%) |
| Total | 8 | 3 | 85 | 85 | 113 |

and were identified as *H. apri* n. sp.

### 3.3.4. Vector (final host)
Unknown.

### 3.3.5. Other host
Unknown. All muscle specimens collected from 113 sika deer hunted in Tokushima, Japan were negative in a nested PCR analysis (Table 3).

### 3.3.6. Locality
Tokushima and Gifu prefectures, Japan. Based on the high infection rate, it is likely present in other areas of Japan.

### 3.3.7. Type specimens
A buffy coat smear from a Japanese wild boar (ID: 28-11) stained using the Diff-Quik Staining Kit containing intraleukocytic gamonts and muscle tissue slides from a Japanese wild boar (ID: 20B) stained by H&E containing mmature and immature meronts were deposited at the Meguro Parasitological Museum, Meguro, Tokyo, Japan under accession numbers MPM Col. No.21398, MPM Col. No. 21399, and MPM Col. No. 21340, respectively.

### 3.3.8. ZooBank LSID
The ZooBank LSID is urn:lsid:zoobank.org:act:pub:CE832E19-BF49-4A76-AA35-8C205573F308.

### 3.3.9. Representative DNA
One sequence representing a 1007 bp fragment of 18S rRNA has been deposited at DNA Data Bank of Japan (DDBJ) under accession no. LC314791.

### 3.3.10. Etymology
The specific epithet, *apri,* is derived from the genitive of the Latin noun *aper*, meaning a wild boar, the type host for the new species.

### 3.4. Phylogenetic analyses

A 522-bp nucleotide sequence alignment of the short 18S was generated for 41 sequences of *Hepatozoon* species and 4 sequences of other hemogregarines. Phylogenetic analyses using NJ and ML methods generated a similar tree topology with minor variation in bootstrap values. For brevity, we present only the ML tree with bootstrap values obtained from both ML and NJ trees (Fig. 4). *Hepatozoon apri* n. sp. belonged to a clade that contained sequences of *Hepatozoon* sp. detected in wild boar (ID: IB20) in Gihu, Japan (accession no. LC062147) and *Dermacentor* ticks collected from *S. scrofa* in Thailand (accession nos. KF318170 and KF318171). This clade was within a major clade containing *Hepatozoon* isolates from carnivores (dogs, cats, wild cat, bears, and martens). The uncorrected *p*-distances of 18S within the first clade containing *Hepatozoon* from wild boars and from *Dermacentor* ticks was

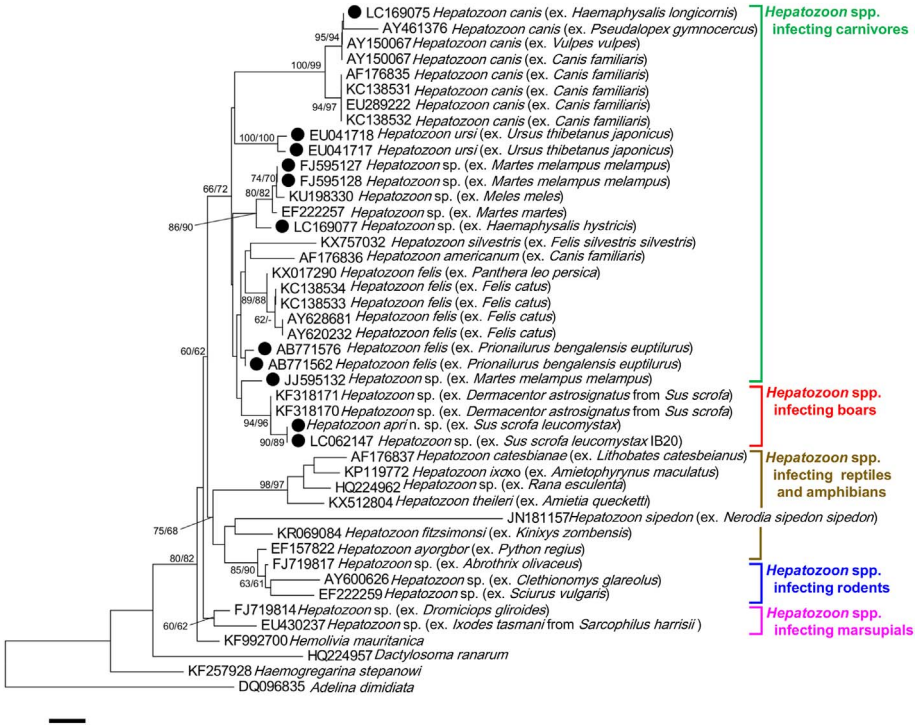


**Fig. 4.** Phylogenetic analysis of *Hepatozoon apri* n. sp. based on 18S rDNA sequences (522-bp). *Adelina dimidiata* (accession no. DQ096835) was chosen as the outgroup to root the phylogeny. Neighbor-joining (NJ) and maximum likelihood (ML) analysis showing the phylogenetic relationships among boar isolates and two *Hepatozoon* spp. (KF318170, KF318171) detected in *Dermacentor* ticks collected from wild boar in Thailand. Sequences included in the comparison were downloaded from the DDBJ/EMBL/GenBank databases. Filled circles indicate *Hepatozoon* species reported from Japan. Nodal support values based on 1000 bootstrap replicates (NJ/ML) are represented on the ML tree. Scale bar represents 0.01 nucleotide substitutions per site.

0.04; between *H. apri* n. sp. and the clade containing *Hepatozoon* species infecting carnivores was 0.032 ± 0.01 and ranged from 0.013 (*H. felis* found in the Iriomote cat *Prionailurus bengalensis iriomotensis*) to 0.055 (*H. canis* found in the dog and pampas fox *Lycalopex gymnocercus*); between *H. apri* n. sp. and other *Hepatozoon* species was 0.048 ± 0.02 and ranged from 0.029 (*Hepatozoon* sp. found in the Chielo opossum *Dromiciops gliroides*) to 0.101 (*Hepatozoon* sp. found in the Northern water snake *Nerodia sipedon sipedon*). The uncorrected *p*-distances between species in the genus *Hepatozoon* and *Hemolivia mauritanica, Haemogregarina stepanowi,* and *Dactylosoma ranarum* were 0.034 ± 0.01 (0.013–0.088), 0.068 ± 0.01 (0.047–0.112), and 0.078 ± 0.01 (0.059–0.118), respectively.

### 3.5. Remarks

Based on morphological characteristics of developmental stages, histological findings, and molecular and phylogenetic analyses, *Hepatozoon* species reported in the present study represent a new species, herein described as *H. apri* n. sp. No *Hepatozoon* species have been described in other ungulates, including those in the family Suidae. *Hepatozoon apri* n. sp. is closely related to *Hepatozoon* spp. isolated from carnivores, but the following features can be used to distinguish these species. (1) Gamonts of *H. apri* n. sp. (11.6 ± 1.4 × 6.7 ± 1.3 μm) were wider than *H. ursi* gamonts (10.9 ± 0.3 × 3.3 ± 0.2 μm) (Kubo et al., 2008), *H. canis* gamonts from the domestic dog *Canis lupus familiaris* (9.7 ± 1.4 × 5.4 ± 0.9 μm) (Baneth et al., 2007), *H. americanum* gamonts from the domestic dog (8.8 ± 0.6 × 3.9 ± 0.5 μm) (Vincent-Johnson et al., 1997), *H. felis* gamonts from the domestic cat *Felis catus* (10.5 ± 0.6 × 4.7 ± 0.8 μm) (Baneth et al., 2013), and *H. procyonis* from the raccoon *Procyon lator* (7.5 ± 0.5 × 3.9 ± 0.4 μm) (Clark et al., 1973). (2) Mature meronts of *H. apri* n. sp. (47.5 × 30.0 μm) are markedly smaller in size than *H. americanum* meronts (85 ± 7 × 79 ± 13 μm) (Vincent-Johnson et al., 1997). (3) Wheel spoke-shaped meronts of *H. apri* n. sp. contain around 30 merozoites, whereas *H. felis* from domestic cat and *H. silvestris* from European wild cat *Felis silvestris silvestris* contain 20–30 and 10–15 merozoites, respectively (Hodžić et al., 2016).

## 4. Discussion

Members of the genus *Hepatozoon* have been recorded from a wide variety of vertebrate hosts, and reptiles are the most commonly infected (Smith, 1996). However, in ungulates, *Hepatozoon* infections are apparently rare and there are no described species. Basson et al. (1967) found organisms in mononuclear leukocytes of the liver tissues of the impala *Aepyceros melampus* presumed to be *H. canis* gamonts on the basis of morphology, including the typical banana shape and slight curvature. These characteristics clearly differ from those of the gamont of *H. apri* n. sp., which have are ellipsoid. Interleukocytic parasites, probably *Hepatozoon* gamonts, were found in the blood smear of the reedbuck *Redunca arundinum* and the giraffe *Giraffa camelopardalis* in Barotseland, located in western Zambia (Fantham, 1922; 1921). Presumed *Hepatozoon* spp. were also observed in the mononuclear leukocytes of tissues of the nyalas *Tragelaphus angasi* and bushbuck *T. scriptus* in South Africa (Basson et al., 1971). Clark et al. (1973) detected *Hepatozoon* gamonts and meronts in the myocardial tissues of a white-tailed deer *Odocoileus virginianus* from Liano country, Texas. Unfortunately, we could not compare *H. apri* n. sp. gamonts and *Hepatozoon*-like organisms detected in other ungulates owing to the paucity of morphological descriptions. Further investigations of the morphological and phylogenetical characteristics of *Hepatozoon* spp. detected in ungulates are needed in the future.

There are few reports of *Hepatozoon* isolates from wild mammals in Japan. *Hepatozoon ursi* has been reported in the Japanese black bear *Ursus thibetanus japonicus* (Uni et al., 2003; Kubo et al., 2008), *H. felis* in the Iriomote cat *Prionailurus bengalensis iriomotensis* (Sakuma et al., 2011), and unidentified *Hepatozoon* spp. in the Hokkaido brown bear *Ursus arctos yesoensis* (Kubo et al., 2010), the Ezo red fox *Vulpes vulpes*

*schrencki* (Maeda et al., 1982), the Tsushima leopard cat *Felis bengalensis euptilura* (Kubo et al., 2006), the Northern red-backed vole *Myodes rutilus* and the large Japanese field mouse *Apodemus speciosus* (Moustafa et al., 2017), the Japanese marten *Martes melampus* (Yanai et al., 1995), and the Iriomote cat (Kubo et al., 2006). Among these species, morphological properties of immature and mature meronts of *H. apri* n. sp. are similar to those of undescribed *Hepatozoon* species found in the heart muscle of the Japanese marten, showing ellipsoidal and ovoidal shapes and sizes of approximately 25–40 μm (Yanai et al., 1995).

The majority of descriptions of *Hepatozoon* species are based mainly on the morphology of the gamont in hematocytes and systematic relationships among host species from which they are isolated. According to Smith (1996), species of *Hepatozoon* that parasitize vertebrate hosts display low host specificity to both their final and intermediate hosts; accordingly, descriptions of new *Hepatozoon* species based merely on new host records should be avoided. However, host specificity of *Hepatozoon* species found in carnivores has been poorly investigated, and each family or suborder level of carnivores has been assigned their own *Hepatozoon* species; *H. canis, H. americanum,* and *H. procyonis* found in canids, *H. felis* and *H. silvestris* in felids, *H. ursi* in ursids (Vincent-Johnson et al., 1997; Ewing and Panciera, 2003; Kubo et al., 2008; Baneth et al., 2013; Hodžić et al., 2016). Although canine *Hepatozoon*-like species were also reported from domestic cats (Jittapalapong et al., 2006; Allen et al., 2011), some researchers speculate that these are opportunists that take advantage of immunocompromised states that are otherwise resistant to infection (Baneth et al., 2007; Allen et al., 2011). Our findings showing high infection rates in Japanese wild boars indicate that the *Hepatozoon* species parasitizing wild boars is a new, dominant, and adapted species.

Sequence analyses using 18S rRNA are used to identify poorly described, rarely isolated, or phenotypically aberrant species, and are routinely used for the identification of *Hepatozoon* species (Criado-Fornelio et al., 2006; Kubo et al., 2008; Panciera et al., 2011; Baneth et al., 2013; Najm et al., 2014; Cook et al., 2016; Hodžić et al., 2016). In the present study, phylogenetic analyses using the same region support the conclusion that *H. apri* n. sp. is a novel species related to carnivore-related *Hepatozoon* species. Further, the genetic distance between the partial 18S sequence of *H. apri* n. sp. of boar isolates and *Hepatozoon* sp. detected in *Dermacentor* ticks from *Sus scrofa* in Thailand is apparently lower than inter-specific distances. This indicates that *H. apri* n. sp. or closely related *Hepatozoon* species are present in wild boars in Asia.

The diagnosis of *Hepatozoon* infection is usually performed by blood cytology, buffy coat smears, and/or PCR methods (Otranto et al., 2011); however, obtaining blood specimens from shot animals is difficult because they are sensitive to handling. Tissue samples are easy to collect in large quantities in the butchering process and can also be used for the direct detection of bacteria, viruses, and parasites. Although we did not find developmental stages of *Hepatozoon* in muscle specimens from four boars via histopathological observations, using nested PCR for the detection of *H. apri* n. sp., we amplified *Hepatozoon* DNA from all muscle specimens from infected boars. Results obtained using other tissues are not consistent with the smear test results, implying the organ specificity of *H. apri* n. sp. in boars. Nested PCR analysis using muscle specimens is useful to investigate the prevalence of *H. apri* n. sp. among a large number of wild boars. The prevalence of *H. apri* n. sp. in wild boars (53.0%) suggests that it is a widely distributed parasite in populations of Japanese wild boars.

In the life cycle of the genus *Hepatozoon,* merogony development takes place in vertebrate hosts, i.e., the intermediate host (Smith, 1996; Graig, 2001). Sporozoites in arthropods, the final host for *Hepatozoon*, are released into the intestinal tract and penetrate the gut wall to enter the circulation, and meronts that form merozoites occur in various internal organs, such as the intestinal wall, liver, lungs, spleen, bone marrow, and muscles. For mammalian *Hepatozoon* species, the free merozoites from mature meronts may enter other endothelial cells or penetrate mononuclear leukocytes and become gamonts in most species. After blood sucking by arthropods, both gametogony and sporogony take place in their bodies. The transmission of *Hepatozoon* to vertebrates occurs by ingestion by the final host, which contains the sporulated oocyst. Species of *Hepatozoon* isolated from the dog have been transmitted to uninfected rats and rabbits by the ingestion of *H. americanum* sporozoites and developed to a persistent cystozoite stage. Moreover, tissues containing those cystozoites are infectious in dogs, indicating that small animals could act as a natural reservoir for *Hepatozoon* species and that predator-prey cycles can occur in the life cycle of *Hepatozoon* (Johnson et al., 2008, 2009). Furthermore, vertical transmission of *H. canis* in puppies has been reported (Murata et al., 1993). The final host for *H. apri* n. sp. is unknown, but molecular evidence indicates that *Dermacentor* ticks are potential vectors for the present species. The pathogenicity of *H. apri* n. sp. is unknown; however, the high prevalence in wild boars in the present study is evidence that the virulence of this parasite might be low and exposure is frequent in the wild populations, as observed in Japanese black bears (Kubo et al., 2008) and Japanese marten (Yanai et al., 1995) in Japan.

In conclusion, the newly described *Hepatozoon* species is widely distributed in wild boars. Future research should focus on the parasitological features, such as the developmental process, host specificity, transmission route, and pathogenicity, as well as the risk of *H. apri* n. sp. infection in domestic pig *Sus scrofa domesticus* and other mammals.

## Conflicts of interest

None.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2017.11.001.

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