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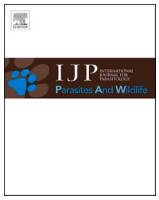

Molecular detection of *Babesia* and *Hepatozoon* species and morphological characteristics of *Babesia* species in Japanese wild boars

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ABSTRACT

We investigated intraerythrocytic *Babesia* parasites in 21 Japanese wild boars, *Sus scrofa leucomystax*, captured in Wakayama Prefecture on the mainland from 2008 to 2009 and in 31 Japanese wild boars from 2011 to 2013 in Kochi Prefecture on Shikoku Island, Japan. We detected small subunit ribosomal RNA (18S rRNA) gene (SSUrDNA) fragments of a *Babesia* species in 17 boars from Wakayama and 18 boars from Kochi. The nearly full SSUrDNA sequence (1669 bps) of this species was determined. A FASTA search revealed that the SSUrDNA sequence of the *Babesia* sp. in Japanese wild boars was the most homologous to those of several *Babesia* isolates reported as *Babesia gibsoni*. Phylogenetic analysis showed that the *Babesia* sp. found in Japanese wild boars was the closest relative to *B. gibsoni* but made a different clade from *B. gibsoni*. The *Babesia* sp. in Japanese wild boars was completely different from *Babesia* sp. Suis found in a European domestic pig, *Sus scrofa domesticus*. By microscopic examination, ring-shaped, oval and pear-shaped small sized intraerythrocytic parasites were observed on blood smears of 12 of 18 Japanese wild boars whose blood smears could be examined in Wakayama. We also detected SSUrDNA fragments of a *Hepatozoon* species in 6 of the 21 wild boars from Wakayama. The nearly full SSUrDNA sequence (1774 bps) of the *Hepatozoon* sp. was shown to be identical to that of *Hepatozoon apri*.

1. Introduction

Organisms of the genus *Babesia* in the family Piroplasmida are tick-borne, intraerythrocytic protozoan parasites confirmed to parasitize a wide spectrum of mammals worldwide (Gorenflot et al., 1998; Telford III and Spielman, 1998; Homer et al., 2000). Basically, *Babesia* species have been considered highly host-specific and classified mainly based on the host species. It has been believed that the more closely related the host species are, the more closely related *Babesia* species parasitize them (Schnittger et al., 2012). However, *Babesia* protozoa genetically closely related to *Babesia divergens*, a bovine babesia, have been found in rabbits, while *Babesia* protozoa genetically closely related to *Babesia microti*, a rodent babesia, have been found in dogs and racoons (Schnittger et al., 2012; Jinnai et al., 2009). Thus, it is advocated that the classification system should be reconsidered.

Since we found the first human babesiosis case caused by indigenous *B. microti* parasitizing field rodents in Japan (Saito-Ito et al., 1999, 2000, 2004), we have surveyed *Babesia* spp. in wild animals with the concern that these species could potentially cause new zoonotic babesiosis in humans. With this in mind, we conducted surveys of *Babesia*-infection in medium-sized wild animals in Wakayama Prefecture on the mainland of Japan from 2008 to 2009 and in Kochi Prefecture on Shikoku Island of Japan, from 2011 to 2013. In this article, we report the molecular and morphological characteristics of a *Babesia* species detected in Japanese

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wild boars, *Sus scrofa leucomystax*, in these areas. We also discovered a *Hepatozoon* species coincidentally in Japanese wild boars.

2. Materials and methods

2.1. Collection of field samples and sample preparation

2.1.1. Samples from Wakayama Prefecture, Japan

Twenty-one Japanese wild boars (*Sus scrofa leucomystax*) were captured for hunting purposes in Tanabe and surrounding areas of Wakayama Prefecture, Japan from November 2008 to October 2009. Blood was collected by cardiac puncture and an adequate amount of EDTA-2Na (Nacalai, Kyoto, Japan) or heparin (Mochida, Tokyo, Japan) was added to the final concentration of 1–1.5 mg/ml and 1–10 U/ml, respectively. Three of the 21 blood samples were frozen in Wakayama and sent to our lab and stored at −20 °C. The remaining 18 blood samples were kept at approximately 4 °C and transported to our lab. Thin blood smears were made using a drop of blood, fixed with methanol and Giemsa-stained. The remaining blood was kept at −20 °C until DNA extraction. DNA was extracted using the MagExtractor Genome (TOYOBO, Osaka, Japan) according to the manufacturer's instruction. DNA aliquots were subjected to polymerase chain reaction (PCR).

2.1.2. Samples from Kochi Prefecture, Japan

Thirty-one Japanese wild boars (*S. scrofa leucomystax*) were captured for hunting purposes in Kochi Prefecture, Japan from 2011 to 2013. About 120 μl of blood was spotted on FTA® Cards (Indicating FTA® Classic Card, Cat. No. WB120206, Whatman® BioScience, MA, USA). After air drying at RT, each disc (a 3 mm diameter) was punched out using a Harris Micro-Punch (Whatman™), placed into an empty Eppendorf tube (200 μl) and stored at RT until PCR analysis.

2.2. Preparation of FTA discs before polymerase chain reaction (PCR)

FTA discs were prepared for PCR as follows according to the manufactural instruction with slight modifications. Briefly, each disc was washed with in total 1–3 ml FTA™ purification reagent (806806019, Whatman™) until the hemoglobin color was gone, centrifuged at 15,000 rpm for 15 min, then washed with at least 200 μl TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) and centrifuged again. Finally, each FTA disc was rinsed with at least 200 μl sterilized water and dried at RT for approximately 1 h in the Eppendorf tube, spent sterilized water being removed. Fifty μl of PCR reaction mixture was added to each Eppendorf tube containing a dried disc, which was directly used as a template.

2.3. PCR targeting SSUrDNA and sequencing analysis

The primers and conditions of PCR analyses employed in this study were summarized in Table 1 and Fig. 1.

FH-PCR and LH-PCR were performed using CF1-CR2 and CF2-CR1 to amplify the former half and the latter half of small subunit ribosomal RNA (18S rRNA) gene (SSUrDNA) of the piroplasm or *Babesia*, respectively. In order to determine the nearly full SSUrDNA sequence, PCR was performed using two pairs of primers: Anl-CR2 and CF2-Bnl, as previously reported (Saito-Ito et al., 2007). The PCR products were directly sequenced as previously described (Saito-Ito et al., 2007). The sequence (1669 bp) obtained was suggested to be the SSUrDNA sequence of a new *Babesia* species (see results). Hereafter, the sequence is designated as InoBabSSUrDNASQ in this paper. Then, PCR primers, InoF-InoR, which are specific to InoBabSSUrDNASQ, were designed. Any sequences of *Babesia* or piroplasm protozoa, which were homologous to InoF and InoR, were not listed up by a BLAST search.

For FTA disc samples from Kochi Prefecture, LH-PCR was first performed. For LH-PCR-positive samples, semi-nested PCR 1 (1st: Anl-InoR; 2nd: Anl-CR2) and semi-nested PCR2 (1st: CF2-CR3; 2nd: NF-CR3) were performed in order to determine the nearly full SSUrDNA sequence of the parasites. The two sequences of products obtained by semi-nested PCR 1 and 2 were directly determined and were shown to be the same as InoBabSSUrDNASQ (see results). Subsequently, nested PCR with CF1-CR1 and InoF-InoR was performed on all 31 samples from Kochi Prefecture. These nested or semi-nested PCR reactions were performed because sufficient products were not obtained by simple PCR, probably due to the use of FTA disc samples.

2.4. Phylogenetic analysis

A homology search was performed by FASTA provided by the European Molecular Biology Laboratory (EMBL) for InoBabSSUrDNASQ, the nearly full SSUrDNA sequence of the *Babesia* species (1669 bp) found in Japanese wild boars, as mentioned above.

Sequences were selected from the top 100 SSUrDNA sequences listed by FASTA in order to construct a phylogenetic tree. Sequences shorter than 1500 bp were excluded. For identical sequences, only the longest one was selected. Fifty-seven SSUrDNA sequences were selected for phylogenetic analysis. Additional reference sequences, *B. divergens* (**AJ439713**, **U16370**), *Babesia bovis* (**L19077**), *Babesia bigemina* (**X59604**), *Babesia odocoilei* **(U16369**, **AY237638)**, and *Babesia* sp. Suis (**HQ437690**) that was found in a European domestic pig (*Sus scrofa domesticus*) were also included. *B. microti* (**U09833**) was used as an outgroup. Sixty-six SSUrDNA sequences, in total, including InoBabSSUrDNASQ, were finally selected for construction of a tree.

The 66 selected sequences were aligned using ClustalW in MEGA software and edited to eliminate or remove all gaps. A phylogenetic tree

Table 1
PCR conditions and primer sequences.

Primer set used for PCR employed	PCR condition
FH-PCR [CF1-CR2], LH-PCR [CF2-CR1], Anl-CR2, CF2-Bnl, 1st PCR [Anl-InoR] & 2nd PCR [Anl-CR2] of Semi-nested PCR1, 2nd PCR [NF-CR3] of Semi-nested PCR2	*TaKaRa Ex Taq® HS* DNA polymerase (1unit/reaction of 50 ml of the supplied buffer with 2 mM Mg^{2+}), 95 °C 5 min, 40 cycles of 94 °C 20 s, 55 °C 40 s, 72 °C 60 s
2nd PCR [InoF-InoR] of nested PCR	AmpliTaq Gold™ DNA polymerase (1unit/reaction of 50 ml of the supplied buffer with 2.5 mM Mg^{2+}), 95 °C 9 min, 45 cycles of 95 °C 60 s, 60 °C 40 s, 72 °C 40 s, and 72 °C for 5 min
1st PCR [CF2-CR3] of Semi-nested PCR2, 1st PCR [CF1-CR1] of Nested PCR	MightyAmp® for Card (1.25 units/reaction) (1unit/reaction of 50 ml of the supplied buffer with 2 mM Mg^{2+}), 95 °C 1 min, 40 cycles of denaturing at 94 °C 30 s, 55 °C 40 s, 72 °C 60 s, and 72 °C for 5 min

CF1: 5′-GACGGTAGGGTATTGGCCT-3'; CR2: 5′-TCTGATCGTCTTCGATCCCCTA-3'; CF2: 5′-TCAGAGGTGAAATTCTTAGATTTGT-3'; CR1: 5′-TCCTTTAAGTGATAAGGTTCAC-3'; CR3: 5′-CGTCCTTCATCGTTGTGTGAGC-3' (Saito-Ito et al., 2007), Anl: 5′-AACCTGGTTGATCCTGCCAGT-3'; Bnl: 5′-TGATCCTTCTGCAGGTTCACCTAC-3' (Medlin et al., 1988), InoF: 5′-CGTCTCCTGGTTGGCCTGAAG-3'; InoR: 5′-CGCAAAAAGCTATTCCCGGCC-3'; NF: 5′-CGTTCTTAGTTGGTGGAGTGA-3′. CF1-CR2: 630 bp; CF2-CR1: 850 bp; Anl-CR2: 1026 bp; CF2-Bnl: 800 bp; Anl-InoR: 1390 bp; NF-CR3: 1300 bp; InoF-InoR: 680 bp; CF2-CR3: 1700 bp; CF1-CR1: 1400 bp.

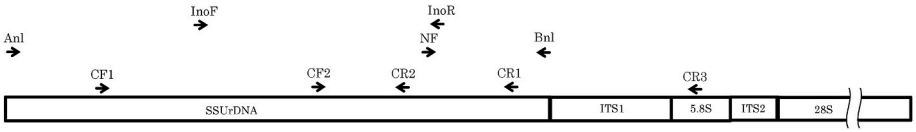

Fig. 1. Primer locations on SSUrDNA.

was constructed based on the edited sequences (1409 bp) using the maximum likelihood method (ML method). The General Time Reversible model (Tavaré, 1986) and the invariable site and Gamma distribution options were selected as the most suitable substitution model and optional parameters according to the Akaike information criterion (AIC). Bootstrap values were calculated by constructing 500 ML trees by the same dataset.

2.5. Microscopical examination

Giemsa-stained thin blood smears of 18 of 21 Japanese wild boars in Wakayama Prefecture were examined under a light microscope for intraerythrocytic parasites at x1,000 magnification. For the positive smear, % parasitemia was determined by counting the number of infected erythrocytes per 10,000 erythrocytes.

The single or multiple infection rate was determined by observing more than 100 infected erythrocytes on smears with 0.6% and 0.25% parasitemia. The mean size of intraerythrocytic parasites was determined by measuring more than 100 intraerythrocytic parasites in erythrocytes of single infection on smears with 0.6% and 0.25% parasitemia.

"Round or ring-shaped parasites", "oval parasites" and "piriform or pear-shaped parasites" were defined as parasites with a length-to-width ratio of <1.2, parasites with a length-to-width ratio of 1.2–1.8, and parasites with a length-to-width ratio of ≥ 1.8, respectively.

3. Results

3.1. Detection of Babesia protozoa by PCR and sequencing analysis

The expected sizes of the amplified products by FH-PCR and LH-PCR are approximately 630 bp and 850 bp, respectively.

In Wakayama Prefecture, 15 out of 21 samples obtained both a 630 bp-FH-PCR product and an 850 bp-LH-PCR product (Table 2). Two samples obtained only an 850 bp-LH-PCR product but not a 630 bp-FH-PCR product. Four samples obtained neither a 630 bp-FH-PCR product nor an 850 bp-LH-PCR product. For three samples, the nearly full length of SSUrDNA sequence from Anl to Bnl was determined by sequencing the PCR products amplified with Anl-CR2 and CF2-Bnl. The sequences, which were 1669 bp excluding Anl and Bnl, were completely identical to one another and are designated as InoBabSSUrDNASQ as described on Materials and Methods and registered in GenBank (Accession No. **LC196156**). The partial SSUrDNA sequences analysed for three other samples were also completely identical to the corresponding parts of InoBabSSUrDNASQ. All 21 samples were subjected to InoF-InoR-PCR (Table 2). Seventeen of the 21 samples were 680 bp-InoF-InoR-PCR-positive. These results suggested that 17 of 21 (81%) Japanese wild boars (*S. scrofa leucomystax*) in Wakayama Prefecture were infected with a *Babesia* species with the same SSUrDNA (Table 2). All seventeen 680 bp-InoF-InoR-PCR-positive samples were 850 bp-LH-PCR-positive, although only 15 of them were 630 bp-FH-PCR-positive.

In Kochi Prefecture, 18 out of 31 samples obtained an 850-bp-product by LH-PCR. All of the eighteen 850 bp-LH-PCR-positive samples obtained a 680-bp-product by nested PCR with CF1-CR1 and InoF-InoR. The nearly full length of SSUrDNA sequences for two samples and the partial SSUrDNA sequences for three other samples were determined

Table 2
Results of microscopic examination and PCR for wild boars captured in Wakayama prefecture in this study.

Wild Boar no.	Microscopic examination (%parasitemia)	PCR						Species of protoza detected
		CF1-CR2/FH-PCR		CF2-CR1/LH-PCR		InoF-InoR		
		PCR result	size (bp)	PCR result	size (bp)	PCR result	size (bp)	
WAK01	P (0.6)	P	630	P	850	P	680	*Babesia* sp.*
WAK02	P (0.01)	P	630	P	850	P	680	*Babesia* sp.
WAK03	P (0.04)	P	630	P	850	P	680	*Babesia* sp.
WAK04	N	N		N		N		
WAK05	N	P	700	P	850	P	680	*Babesia* sp./*Hepatozoon* sp.*
WAK06	N	P	630	P	850	P	680	*Babesia* sp.
WAK07	N	N		N		N		
WAK08	P (0.05)	P	630	P	850	P	680	*Babesia* sp.
WAK09	N	P	700	N		N		*Hepatozoon* sp.*
WAK10	P (0.03)	P	630	P	850	P	680	*Babesia* sp.
WAK11	P (0.02)	P	700/630	P	850	P	680	*Babesia* sp./*Hepatozoon* sp.
WAK12	P (0.06)	P	630	P	850	P	680	*Babesia* sp.
WAK13	P (0.04)	P	630	P	850	P	680	*Babesia* sp.
WAK14	P (0.25)	P	630	P	850	P	680	*Babesia* sp.
WAK15	N	P	700	N		N		*Hepatozoon* sp.
WAK16	nt	P	630	P	850	P	680	*Babesia* sp.
WAK17	nt	P	700	P	850	P	680	*Babesia* sp./*Hepatozoon* sp.
WAK18	nt	P	630	P	850	P	680	*Babesia* sp.
WAK19	P (nd)	P	700/630	P	850	P	680	*Babesia* sp./*Hepatozoon* sp.
WAK20	P (nd)	P	630	P	850	P	680	*Babesia* sp.*
WAK21	P (nd)	P	630	P	850	P	680	*Babesia* sp.*

P: positive; N: negative; nt: not tested; nd: not determined; *: full length sequenced.

and confirmed to be completely identical to InoBabSSUrDNASQ. These results suggested that 18 (850-bp-LH-PCR-positive) of 31 (58%) Japanese wild boars in Kochi Prefecture were infected with the same *Babesia* sp. found in wild boars in Wakayama Prefecture.

3.2. Detection of Hepatozoon protozoa by PCR and sequencing analysis

By FH-PCR analysis, 700 bp-PCR products were obtained for in total 6 samples from Wakayama Prefecture. By sequence analysis, the 700 bp-products of three samples were revealed to be completely identical to each other. For two other samples, the nearly full length of SSUrDNA from Anl to Bnl was determined. The SSUrDNA sequences were 1774 bp excluding Anl and Bnl and completely identical to each other (Accession No. **LC789560**). The top 6 sequences listed by the FASTA search for the sequence **(LC789560)** were those of *Hepatozoon felis,* followed by dozens of sequences registered as those of *Hepatozoon canis* and several other *Hepatozoon* spp. Thus, the sequence was identified as that of a *Hepatozoon* species most closely related to *Hepatozoon felis*. Subsequently, the sequence was compared with that of *Hepatozoon apri* (**LC314791**; 1002 bps), which was reported in 2017 as a new species found in Japanese wild boars in Gifu Prefecture on the mainland and in Tokushima Prefecture on Shikoku Island (Matsuo et al., 2016; Yamamoto et al., 2017). The sequence was shown to be completely identical to that of *Hepatozoon apri*, for the corresponding part. Therefore, the six 700 bp-FH-PCR-positive samples or boars were revealed to be parasitized with *Hepatozoon apri.* The presence of SSUrDNA of *Hepatozoon apri* appeared to have inhibited competitively FH-PCR amplification of SSUrDNA of *Babesia*. We did not analyze co-infection with the *Hepatozoon* sp. for the samples from Kochi Prefecture, due to insufficient blood sample volume.

3.3. FASTA Homology search and phylogenetic analysis for InoBabSSUrDNASQ

The top 100 sequences listed by the FASTA search included 58 sequences registered as *B. gibsoni,* 18 sequences registered as *Babesia canis*, and 1 sequence registered as *Babesia hongkongensis* and 23 other sequences registered as *Babesia* spp. Or *Babesia* clones in dogs, racoons, rats, cats, a wolf, lions, and a tick. The top SSUrDNA sequence (1561 bp) (**JX962780**) by the FASTA search corresponded to and was identical to 98th −1658th nucleotides of InoBabSSUrDNASQ except for two nucleotides (737th and 740th of InoBabSSUrDNASQ). This sequence has been registered as *B. gibsoni* found in a Chinese wild boar only in GenBank.

The constructed phylogenetic tree had 5 big groups, a group including *B. divergens* and *B. odocoilei*, a group of *Babesia* spp. found in feral racoons, a group of *B. gibsoni*, a group of *B. canis*, and a group including *B. bovis*, *B. bigemina* and *B. caballi* (Fig. 2A). The tree revealed that the *Babesia* sp. (InoBabSSUrDNASQ; **LC196156**) found in Japanese wild boars, along with the sequence (**JX962780**) found in a Chinese wild boar, appeared to form a sister clade nearest to but distinct from the clade of *B. gibsoni*, which includes a few isolates reported as *Babesia* sp. The monophyly of these two sister clades was supported by 78%, while the monophyly of each sister clade was supported by 100%. The *Babesia* sp. in Japanese wild boars was entirely different from the species in a

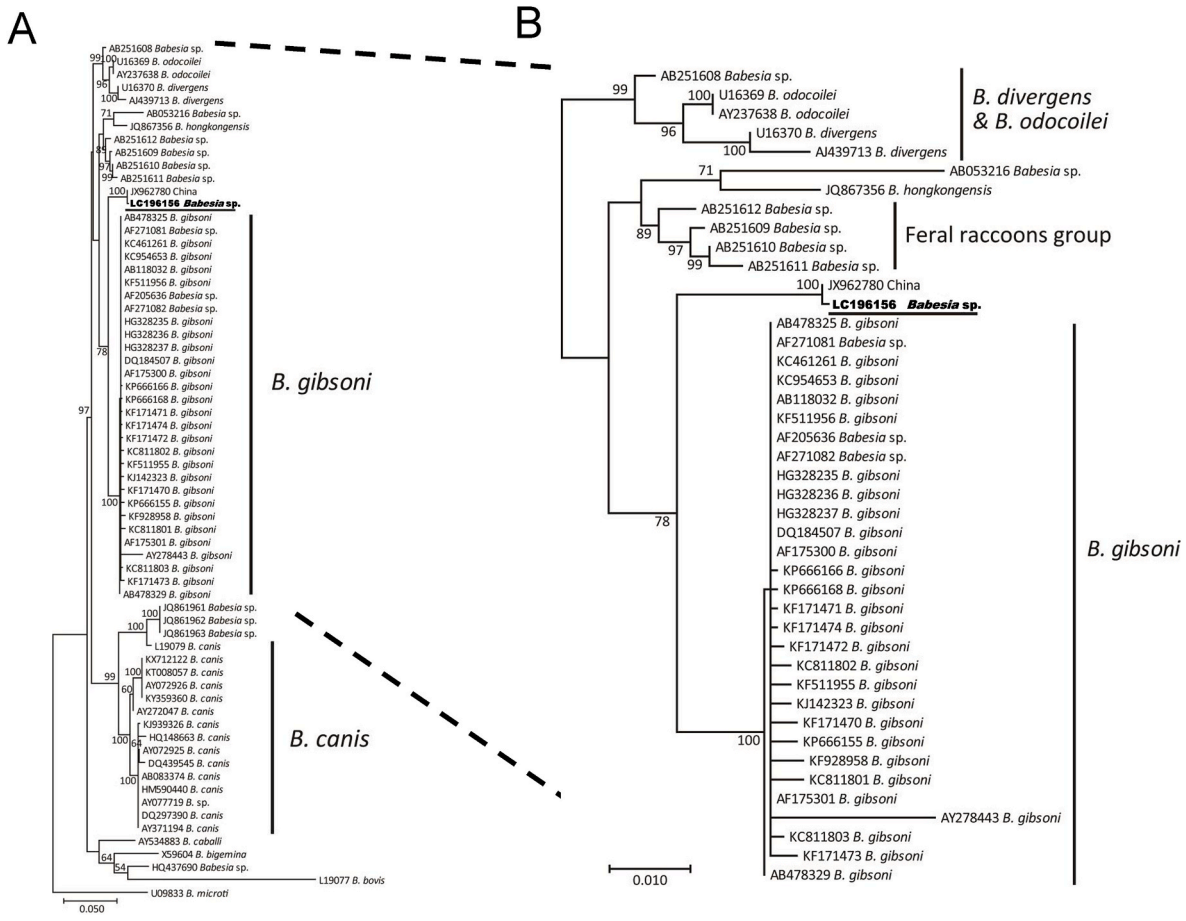

Fig. 2. Maximum-likelihood phylogenetic tree showing relationship between SSUrDNA sequences from *Babesia* sp. detected in this study and other selected *Babesia* spp.
The underbar indicates InoBabSSUrDNASQ (LC196156) of *Babesia* sp. isolated from Japanese wild boars. The SSUrDNA of *B. microti* (U09833) was used as outgroup. Only bootstrap values > 50% from 500 pseudo-replications are shown. The scale bar represents 0.010 substitutions per site. B is a higher magnification of A.

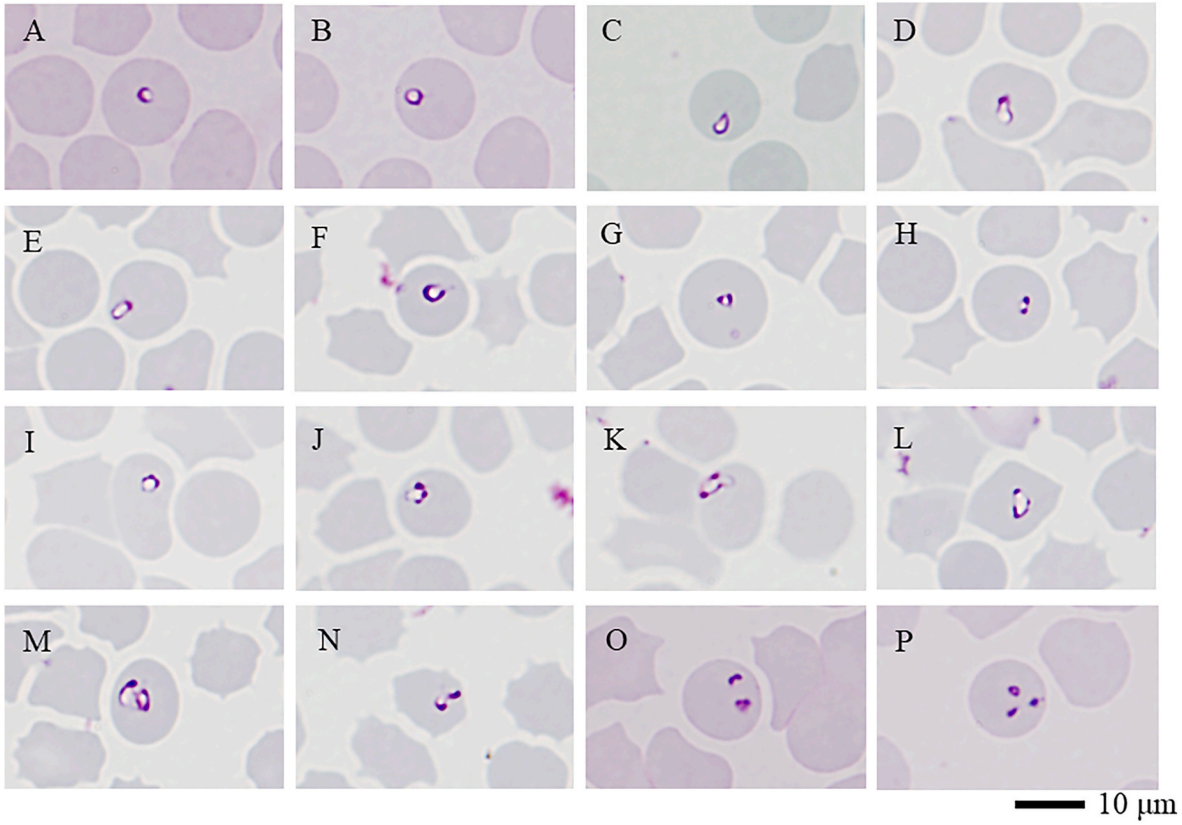

Fig. 3. Photomicrograph of Giemsa-stained thin blood smears showing typical forms of *Babesia*-like parasites from wild boars (*Sus scrofa leucomystax*) in Wakayama Prefecture.
A single ring-, oval or pear-shaped trophozoite (A–L), two pear-shaped, oval or ring-trophozoites (M–O) and three trophozoites (P) on an infected erythrocyte. The scale bar represents 10 μm.

European domestic pig, designated as *Babesia* sp. Suis (**HQ437690**) (Zobba et al., 2011), which was positioned in the clade including *B. bovis*, *B. bigemina* and *B. caballi*.

3.4. Microscopical examination

Intraerythrocytic parasites were found on 12 of 18 blood smears (Table 2). Parasitemia on 9 of these blood smears ranged from 0.01% to 0.6%. Parasitemia for 3 blood smears could not be determined due to the condition of the smears. The results of InoF-InoR- and LH- PCR and microscopic examination were consistent for 18 wild boars whose smears were observed except for two wild boars that were InoF-InoR-PCR-positive but intraerythrocytic parasite negative on smears (Table 2).

Giemsa-stained intraerythrocytic parasites of Japanese wild boars in Wakayama Prefecture are shown in Fig. 3. Ninety-one percent of infected erythrocytes contained a single ring-, oval- or pear-shaped parasite (Fig. 3A–L), while 9% of infected erythrocytes contained two ring-, oval- or pear-shaped parasites (Fig. 3M–O). However, bilobed parasites, or binary-divided parasites were rarely observed. A very few infected erythrocytes had three parasites (Fig. 3P). No erythrocytes with four parasites were found. Thus, typical so-called "Maltese cross"-shaped parasites were not found. Each parasite often possessed an elongated chromatin that sometimes contained 2 or 3 densely stained dots. A chromatin occasionally looked like forming a partial or complete outline of the parasite. "Round or ring-shaped parasites" with a length-to-width ratio of $<$1.2, "oval parasites" with a length-to-width ratio of 1.2–1.8, and "piriform or pear-shaped parasites" with a length-to-width ratio of $\geq$1.8 were 53%, 41% and 6%, respectively. "Round or ring-shaped parasites" measured 0.7–1.8 μm in diameter. "Oval parasites" and "piriform or pear-shaped parasites" measured 0.9–2.6 μm long by 0.6–1.6 μm wide. No *Hepatozoon*-like parasites were found on any of the smears.

4. Discussion

The SSUrDNA sequence of *Babesia* parasites found in Japanese wild boars (*Sus scrofa leucomystax*) in this study, designated as Ino-BabSSUrDNASQ, fell into the clade closestly related to but distinct from the clade containing *B. gibsoni*, along with the sequence (**JX962780**) that was registered as *B. gibsoni* isolate Pig/J78/. The sequence (**JX962780**) was described to originate from a wild boar in China in the part of features of GenBank information. It was, therefore, suggested that the same kind of *Babesia* sp. closestly related to but distinct from *B. gibsoni* parasitize not only Japanese but also Chinese wild boars.

Morikawa et al. (2021) recently reported 12 partial SSUrDNA sequences (1246 bp) (**LC593273**-84) from Japanese wild boars in Nagasaki and Kagoshima Prefecture on Kyushu Island and in Yamaguchi Prefecture on the mainland. All except one were completely identical to the corresponding part (268th – 1513th nucleotides) of Ino-BabSSUrDNASQ. The exception from Yamaguchi Prefecture was different by only one nucleotide (913th nucleotide). It can be said that Japanese wild boars on three of the four main islands, Honshu (the mainland), Kyushu, and Shikoku are highly parasitized with the same kind of *Babesia* sp. found in this study (Table 3).

Masatani et al. (2017) reported a partial SSUrDNA sequence (1495 bp) (**LC169083**-85) of *B. gibsoni*-like protozoa detected in 3 female *Amblyomma testudinarium* ticks attaching to a Japanese wild boar in Kagoshima Prefecture on Kyushu Island. The sequence was also identical to the corresponding part (68th – 1562nd nucleotides) of Ino-BabSSUrDNASQ except for 2 different nucleotides (70th and 96th

Table 3
Representative reports of porcine babesiosis caused by *B. trautmani, B. perroncitoi* and other *Babesia* species.

Year	Species	Location	Note	Ref.
1962	*B. trautmanni*	Northern Transvaal	Me(+), Md (−), Meas (−), Phots (−), PCR(−), Sq (−), n outbreak of porcine babesiosis (symptomatic) due to *B. trautmanni* in the farm.	Naude (1962)
1976	*B. trautmanni*	Ibadan, Nigeria	Me(+), Md (−), Meas (−), Phots (−), PCR(−), Sq (−), *B. trautmanni* detected in 9.6% of 135 pigs slaughtered in the survey of parasite-infection in the slaughter house.	Okon (1976)
1981	*B. perroncitoi*	Senegal	Me(+), Md (+), Meas (+), Phots (+), PCR(−), Sq (−), *B. perroncitoi* detected in pigs with symptoms of babesiosis.	Vercruysse and Parent (1981)
1982	*B. trautmanni* *B. perroncitoi*	Ibadan, Nigeria	Me(+), Md (−), Meas (−), Phots (−), PCR(−), Sq (−), Single infection of *B. trautmanni* or *B. perroncitoi* and coinfection of *B. trautmanni* and *B. perroncitoi* observed in pigs in the survey of parasite-infection in the slaughter house.	Dipeolu et al. (1982)
1993	*B. trautmanni*	Sardinia, Italy	Me(+), Md (+), Meas (+), Phots (−), PCR(−), Sq (−), An outbreak of porcine babesiosis (symptomatic) due to *B. trautmanni* in the farm.	Ligios and Scala (1993)
1997	*B. perroncitoi*	Inner Mongolia	Me(+), Md (+), Meas (+), Phots (−), PCR(−), Sq (−), *B. perroncitoi* detected in pigs with symptoms of babesiosis.	Guo et al. (1997)
1999	*B. trautmanni* *B. perroncitoi*	Ghana	Me(+), Md (−), Meas (−), Phots (−), PCR(−), Sq (−), Both single infection of *B. trautmanni* or *B. perroncitoi* observed in asymptomatic pigs except for some coughing pigs.	Permin et al. (1999)
2011	*B.* sp. *Suis*	Sardinia, Italy	Me(+), Md (+), Meas (+), Phots (+), PCR(+), Sq (+), *Babesia* sp. detected in a symptomatic pregnant pig *(Sus scrofa domesticus)*.	Zobba et al. (2011)
2013	*B. gibsoni*	China	*Babesia* sp. in (a) wild boar(s) in China; SSUrDNA (JX962780; 1561 bp) only registered in GenBank.	unpublished
2014	*B. bigemina*	Italy	Me(−), Md (−), Meas (−), Phots (−), PCR(+), Sq (+), *Babesia* sp. in wild boars; SSUrDNA (KF773716; 386 bp, KF773717; 347 bp) of 99% identity with *B. bigemina*.	Zanet et al. (2014)
2021	*B.* sp.	Japan	Me(−), Md (−), Meas (−), Phots (−), PCR(+), Sq (+) *Babesia* sp. detected in Japanese wild boars; SSUrDNA (LC593273~593284).	Morikawa et al. (2021)
2021	*B.* sp.	Corsica, France	Me(−), Md (−), Meas (−), Phots (−), PCR(+), Sq (−), *Babesia* sp. in wild boars in the infection survey of field animals.	Defaye et al., 2022
2021	*B.* sp. *Suis*	South Africa	Me(+), Md (+), Meas (+), Phots (+), PCR(+), Sq (+), *Babesia* sp. Suis in a symptomatic pig.	Avenant et al. (2021)
2022	*B.* sp.	Fujian Province, China	Me(−), Md (−), Meas (−), Phots (−), PCR(+), Sq (+), *Babesia* sp. in pigs in the *Babesia*-infection survey of dometic and field animals.	Zeng et al. (2022)
2023	*B.* sp.	Sri Lanka	Me(+), Md (−), Meas (+), Phots (−), PCR(+), Sq (−), *Babesia* sp. appearing *B. perroncitoi* by microscopic measurement in asymptomatic pigs in the farm.	Ranatunga et al. (2023)

Me: microscopic examination; Md: morphological description; Meas: measurement of size of parasites; Photos: microscopic photographs.
PCR: PCR for detection of *Babesia* sp.; Sq: sequence analysis of SSUrDNA of *Babesia* sp.

nucleotides). Thus, the *Babesia* sp. found in this study was suggested to be transmitted by *A. testudinarium* ticks, although it remains possible that these three *A. testudinarium* ticks were only feeding blood on a wild boar already parasitized with the *Babesia* sp.

B. trautmanni and *B. perroncitoi* have been so far reported as porcine *Babesia* spp. and the two species are primarily differentiated by their morphological characteristics (Soulsby, 1982; De Waal, 2004).

For *B. trautmanni,* the parasites are described as large oval, amoeboid and ring-shaped forms measuring 2.5–4 μm long by 1.5–2 μm wide. Parasites in pairs are frequently and four to six parasites are sometimes observed in an erythrocyte (Soulsby, 1982). On the other hand, for *B. perroncitoi*, the parasites are described as small, rounded and vacuolated forms measuring 0.7–1.9 μm in diameter or oval to piriform forms measuring 1.2–2.6 μm long by 0.7–1.9 μm wide (Soulsby, 1982). Genetic analyses have not been performed for either *B. trautmanni* or *B. perroncitoi*. A considerable number of studies have reported symptomatic or asymptomatic porcine babesiosis cases caused by *B. trautmanni* and/or *B. perroncitoi* with representative studies listed in Table 3. Morphological features were not in detail described or not described at all in most reports, even in reports that described either single infections or co-infections of both species in the same area (Dipeolu et al., 1982; Permin et al., 1999) (Table 3).

Zobba et al. (2011) reported *Babesia* parasites in a domestic pig (*Sus scrofa domesticus*) with signs indicative of babesiosis in North Sardinia, Italy in 2011. The sizes of intraerythrocytic parasites on blood smears of this isolate were variable, although only one type of SSUrDNA of *Babesia* parasites was obtained. Thus, the authors did not conclude morphologically whether this isolate was either *B. trautmanni* or *B. perroncitoi*, and tentatively named it *Babesia* sp. Suis. (Table 3). Recently, porcine babesiosis caused by *Babesia* sp. Suis was certificated by molecular analysis in South Africa (Avenant et al., 2021) (Table 3). It is noted that *Babesia* sp. Suis was found in a region where *B. trautmanni* had been reported in old days.

In 1997, Guo et al. (1997) reported that *Babesia* parasites in domestic pigs in Inner Mongolia was identified morphologically as *B. perroncitoi* without molecular analysis. More recently in 2023, Ranatunga et al. (2023) also reported *Babesia* parasites in domestic pigs in Sri Lanka suggested to be *B. perroncitoi* based on microscopic measurements, although the SSUrDNA sequence was not reported. For the *Babesia* sp. found in our study, morphological characteristics, including sizes of parasites, are not inconsistent with those of *B. perroncitoi*. However, it seems impossible to conclude or exclude that the *Babesia* sp. is *B. perroncitoi*, because molecular analysis of a type-strain of *B. perroncitoi* is lacking. However, it may be difficult to obtain typical *B. perroncitoi* that is fully scientifically approved. It is very intriguing to clarify if the SSUrDNA of these *Babesia* isolates reported as *B. perroncitoi* or *B. perroncitoi*-like is identical to the SSUrDNA (InoBabSSUrDNASQ) of *Babesia* sp. found in our study.

By genetic analyses, *B. bigemina* and a *Babesia* sp., closest to *Babesia* spp. found frequently in feral racoons, were shown to infect pigs or boars (Zanet et al., 2014; Zeng et al., 2022) (Table 3). It goes without saying that the molecular analysis is essential for studying porcine *Babesia.* However, not only molecular analysis but also morphological and biological examinations should be performed. In addition, addressing the historical naming of *Babesia* species, which were named before molecular analysis became common, is important; otherwise, these names may become obsolute and impractical.

Hepatozoon parasites recently detected in Japanese wild boars (*S. scrofa leucomystax*) in Gifu Prefecture on the mainland and in Tokushima Prefecture on Shikoku Island were named as *Hepatozoon apri*, a new species (Matsuo et al., 2016; Yamamoto et al., 2017). Because the *Hepatozoon* SSUrDNA sequence detected in wild boars (*S. scrofa leucomystax*) in Wakayama Prefecture in this study was identical to that of *Hepatozoon apri*, it was concluded that *Hepatozoon apri* was parasitized in wild boars (*S. scrofa leucomystax*) in Wakayama Prefecture. Thus, *Hepatozoon apri* appears to be widely distributed in wild boars (*S. scrofa leucomystax*) in Japan, as described by Yamamoto et al. (2017).

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Note

Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers: **LC196156** and **LC789560.**

CRediT authorship contribution statement

Shiho Ohmori: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Motoko Nagano-Fujii:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Kazuo Suzuki:** Resources, Formal analysis, Data curation. **Masataka Korenaga:** Resources, Formal analysis, Data curation. **Fumi Murakoshi:** Formal analysis. **Atsuko Saito-Ito:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Atsuko Saito-Ito reports financial support was provided by Takeda Science Foundation, Japan. Atsuko Saito-Ito reports financial support was provided by Shinryokukai General incorporated Assocation, Japan. Motoko Nagano-Fujii reports financial support was provided by Hyogo University of Health Sciences Grant for Research Promotion, Japan. Motoko Nagano-Fujii reports financial support was provided by Joint research program of Biosignal Research Center, Kobe University, Japan. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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