NOTE *Parasitology*

Divergence of *p33/34* Gene of *Theileria* Found in *Cervus nippon* in Japan

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ABSTRACT. The 18S rRNA gene and the piroplasm major immunodominant protein gene (*p33/34*) of *Theileria* from various subspecies of sika deer in 8 different locations of Japan were analyzed. The similarity between 633 bp partial sequences of the 18S rRNA gene among various subspecies of sika deer was found to be between 99.7% and 100%. While the percent identities of the 412 bp partial *p33/34* gene sequence and deduced amino acid sequences between *Theileria* of sika deer from Yamaguchi Prefecture and those found in deer from other Prefectures, were comparatively low, 68.7% to 70.1% and 64.1% to 70.0% respectively. These findings suggest that there are at least two genetically distinct strains of *Theileria* of sika deer in Japan.

KEY WORDS: *Cervus nippon*, divergence, *Theileria* sp.

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Theileria parasites have been known to be present species from Japanese sika deer (*Cervus nippon*) for several decades, although no detailed description of the parasite was made by earlier researchers [3, 17, 20]. In our recent study, the 18S rRNA genes of *Theileria* species detected in Japanese sika deer, *Cervus nippon centralis* in Yamaguchi and *Cervus nippon yesoensis* in Hokkaido were analyzed [8]. Phylogenetic analysis of the gene sequences have revealed that Theileria species obtained from sika deer comprise a clade that is clearly distinct from the clade of *Theileria* found in cattle [8]. A number of subspecies of sika deer occur in Japan, including *C. n. centralis* in Honshu island - the mainland of Japan; *C. n. yesoensis* in Hokkaido; *C. n. nippon* in Kyushu; *C. n. mageshimae* in Mageshima island; *C. n. yakushimae* in Yakushima island; *C. n. keramae* in Keramashima island and *C. n. pulchellus* in Tsushima island [16]. *Theileria* infection of these subspecies of sika deer is not well understood. In this study, the 18S rRNA gene and the piroplasm major immunodominant protein gene (*p33/34*) from *Theileria* were analyzed to assess potential geographic divergence possibly into different subspecies related to different sika deer found in various locations in Japan.

Genomic DNA was extracted from spleen samples of 53 *C. n. centralis* animals. Three of the *C. n. centralis* were from Yamaguchi Prefecture and had tested positive for *Theileria* species in our previous study (Y22, Y47 and Y52) [8]. Other *C. n. centralis* were hunted at Saitama (n=1), Hiroshima (n=8) and Tottori (n=10) prefectures in 2002 and 2003. The samples from three C. n. yesoensis were collected in Hokkaido in 2003. The samples of *C. n. nippon* were collected from Fukuoka (n=10) and Miyazaki (n=10) Prefectures. Five samples from *C. n. pulchellus* in Tsushima Island were collected in 2003. Three samples from *C. n. yakushimae* were collected in Yakushima Island in 2004. Immediately upon sacrificing the animal, a small piece of spleen was collected, kept in a cold box kept at 4°C and transported to Yamaguchi University. Tick species of sika deer from Yamaguchi Prefecture were previously examined, including *Haemaphysalis longicornis, Haemaphysalis yeni, Haemaphysalis flava, Haemaphysalis megasoinosa, Haemaphysalis kitaokai* and *Amblyomma testudinarium* [7]. However, no information was available for tick infestation of sika deer in other location.

Total DNA was extracted from each deer sample using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). *Theileria* infection was screened by PCR with the primer set Ba305F (5'-GTG-AAA-CTG-CGA-ATG-GCT-CA-3') and Ba932R (5'-CCA-TGC-TGA-AGT-ATT-CAA- GAC-3') that are specific to 18S rRNA genes of both *Babesia* and *Theileria* species [9]. The PCR condition used was the same as described in our previous reports [9]. When positive amplification was observed with appropriate size in the 18S rRNA-based PCR, these samples were also analyzed for the presence of *p33/34* genes by PCR with the primer set Th331F (5'-AAG-CCA- CTK-WTG-TTC-AAG-AA-3') and Th782R (5'-TCG-ACA-AGT-GGY-TTG-TAR-TC-3'). This set of primers was designed based on the alignment data of *p33/34* genes of other *Theileria* species registered in GenBank. The PCR condition of the *p33/34* gene amplification was the same as that of 18SrRNA-based PCR.

Amplified PCR products were extracted with a QIAPCR purification kit (QIAGEN) for direct sequence analysis. DNA sequencing was performed using a Perkin-Elmer ABI Prism 377 automated DNA sequencer at the DNA Core Facility of the Center for Gene Research, Yamaguchi University, as described previously [9]. The determined sequences of the agent and the registered sequences of other related species were analyzed for phylogenetic relationships with other registered sequences in GenBank. Multiple alignment analysis, pairwise percent identities of the sequences, distance matrix calculations, and construction of

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phylogenetic trees were performed with the Clustal W program [23] version 1.8 in the DNA data bank of Japan (DDBJ; Mishima, Japan [http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-e.html]). The distance matrices for the aligned sequences with all gaps ignored were calculated using the Kimura two-parameter method [13], and the neighbor-joining method was used for constructing a phylogenetic tree [19]. The robustness of the tree obtained was estimated by bootstrap analysis using 100 repetitions with the same program. Tree figures were generated using Tree View version 1.61 [18].

The GenBank accession numbers of *Theileria* 18S rRNA gene sequences used to analyze the data are as follows: *T. cervi*, U97054-6; *Theileria* sp. detected from Mhorr gazelle (BK115); AF158710, *T. verifera*; AF097993; *T. annulata*, M64243; *T. lestoquardi*, AF081135; *T. parva*, L02366; *T. taurotragi*, L19082; *Theileria* sp. Type A; U97047, *Theileria* sp. Type B; U97048, *Theileria* α sp. Type C; U97051, *Theileria* sp. Type D; U97052, *Theileria* sp. Type E; U97053, *Theileria* sp. Type H; U97050, *Theileria* α sp. Ipoh-Malaysia; AB000273, *Theileria* sp. China; AF036336, *Theileria* sp. Thung Song; AB000270, *T. buffeli* Warwick; AB000272, *T. buffeli* Marula; Z15106, *T. sergenti* Fukushima, AB016074; *T. sergenti* Ikeda AB000271; *Theileria* sp. from *C. n. centralis* in Yamaguchi Prefectures. AF529271 to AF529273. The GenBank accession numbers of the *p33/34* gene sequences of other species used to analyze the data are as follows: *T. buffeli* Warwick, D11047; *T. buffeli* Marula, AB016278; *T. orientalis*, AF097993; *T. sergenti* Fukushima, AB016280; *T. sergenti* Ikeda D11046; *T. annulata*, U22887 and U22888, and *T. parva*, U22889. The GenBank accession numbers of the deduced amino acid sequence of *p33/34* used to analyze the data are as follows: *T. buffeli* Warwick, BAA01796; *T. buffeli* Marula, BAA23206; *T. orientalis*, BAA31949; *T. sergenti* Fukushima, BAA31951; *T. sergenti* Ikeda BAA01795; *T. annulata*, AAB60238 and AAB60239, and *T. parva*, AAC46910.

A PCR product of approximately 670 bp was obtained from one out of three samples of *C. n. yesoensis* in Hokkaido, and one out of five samples from *C. n. pulchellus* in Tsushima Island using the 18S rRNA gene based-PCR. Similar-sized single bands were also detected from one *C. n. centralis* sample from Saitama, two samples from Hiroshima and one sample from Tottori Prefectures. One *C. n. nippon* sample in Fukuoka and two samples from Miyazaki Prefectures also showed positive bands. No positive bands were detected from *C. n. yakushimae* in Yakushima Island. Analysis of the 633 bp partial sequences, excluding the primer region of PCR products, indicated that all the sequences obtained from the various geographically diverse species mentioned above were 100% identical. However, these partial sequences showed 1 or 2 nucleotide differences (percent identities 99.7% to 99.8%) from those of *Theileria* detected from C. n. centralis in Yamaguchi Prefecture. When compared to the sequences of *Theileria* sp. obtained from cattle, the nucleotide sequences of these parasites detected from sika deer showed comparatively lower percent identities, between 96% and 98%. The 18S rRNA gene sequences of *Theileria* detected from sika deer in Japan were included in a phylogenetic tree with other known *Theileria* species (Fig. 1). Parasites obtained from sika deer comprise a clade that is clearly distinct from those. On the other hand, the isolates from *C. n. centralis* from Yamaguchi Prefecture branch out separately in the tree with a bootstrap value of 100. The phylogenetic position of *Theileria* from sika deer infers closer evolutionary relationships to the bovine parasites than to *T. cervi*, which forms its own distinct clade.

The 18S rRNA gene is a standard marker for the phylogenetic analysis of piroplasma, including *Babesia* and *Theileria*. Numerous researchers have confirmed the phylogenetic relationships of *Theileria* by analyzing this gene [1, 2, 4–6, 14, 24]. *P33/34* gene is also known to be a useful marker for classifying bovine *Theileria* spp. [10–12]. In this study, *p33/34* gene of Theileria detected from sika deer was also analyzed. *P33/34* gene based-PCR was performed using the DNA samples that tested positive with the 18S rRNA PCR. Each sample showed a single positive band of approximately 450 bp. All the sequences were determined by using a direct sequence method in the present study; however, multiple infections with different species or strains of *Theileria* could be occurred. Thus cloning and sequence methods should be used to evaluate the multiple infections. The percent identities of the *p33/34* gene and of the deduced amino acid sequences were calculated by comparing the 412 bp partial sequences excluding the primer region of PCR products of various *Theileria* spp, as summarized in Table 1. The percent identities of the *p33/34* gene and deduced amino acid sequences among *Theileria* of sika deer from Hokkaido, Saitama, Tottori, Hiroshima, Fukuoka, Miyazaki and Tsushima were between 97.8% to 100%, and 96.3% to 100%, respectively. However, this gene and the amino acid sequences of *Theileria* obtained in Yamaguchi Prefecture showed lower percent similarity of 68.7% to 70.1%, and 64.1% to 70.0%, respectively, compared with those from other Prefectures.

The partial *p33/34* gene of *Theileria* from sika deer in Yamaguchi showed deletion of 3 nucleotides compared with others. The nucleotide sequences of *Theileria* detected from sika deer also showed comparatively less similarity between 59.1% to 73.5% identity with registered sequences of *Theileria* spp in cattle including *T. sergenti, T. orientalis, T. bufferi, T. annulata* and *T. parva*. Phylogenetic trees constructed based on the *p33/34* gene and deduced amino acid sequences of *Theileria* species are shown in Fig. 2. *Theileria* acquired from sika deer in Japan were divided into two groups in the tree: *Theileria* from sika deer in Yamaguchi Prefecture, and those in Hokkaido, Saitama, Tottori, Hiroshima, Fukuoka, Miyazaki and Tsushima Prefectures. Each group makes an independent clade compared to the *Theileria* sp. in cattle.

These findings suggest there are at least two different strains of *Theileria* that parasitize sika deer in Japan. The *p33/34* gene encodes the piroplasm major immunodominant

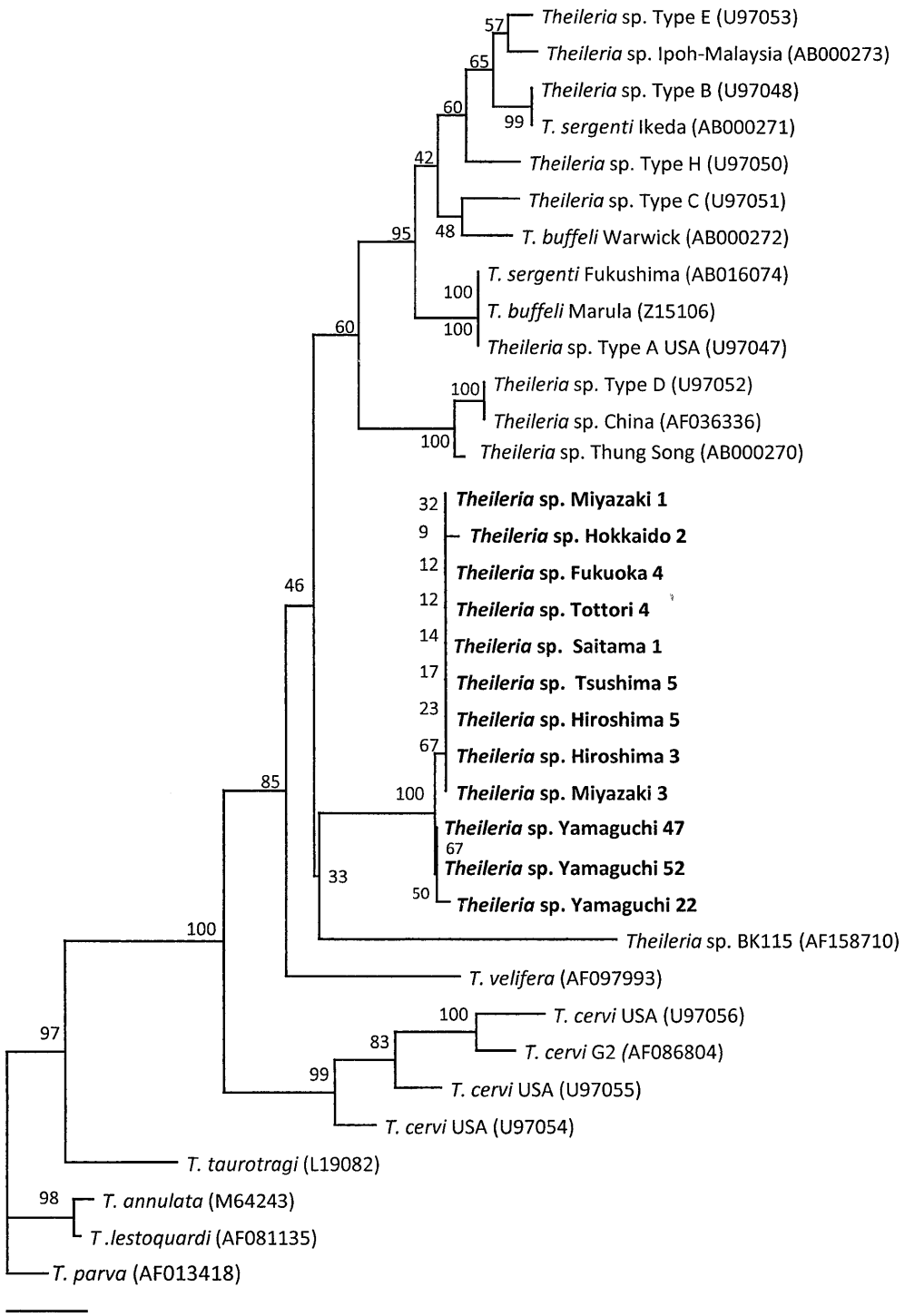

Fig. 1. Phylogenetic relationship of various *Theileria* spp. based on the nucleotide sequences of the 18S rRNA gene. The neighbor-joining method was used to construct the phylogenetic tree with the Clustal W program. The scale bar represents 1% divergence. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown.

Table 1. Degree of similarity between *p33/34* gene sequences and deduced amino acid sequences

	% Similarity*																
Theileria sp.	Yamaguchi-22	Yamaguchi-47	Yamaguchi-52	Hokkaido-2	Saitama-1	Hiroshima-3	Hiroshima-5	Tottori-4	Fukuoka-4	Miyazaki-1	Miyazaki-3	Tsushima-5	*T.sergenti* Fukushima	*T.buffeli* *T.orientalis* Warwick		*T.annulata*	*T.parva*
Yamaguchi-22	–	99.5	99.5	69.4	69.4	69.1	69.1	69.4	69.6	69.1	69.1	69.1	73.2	69.5	69.3	63.0	64.7
Yamaguchi-47	99.0	–	99.3	69.2	69.1	69.2	69.2	69.2	69.4	68.7	69.2	69.2	70.3	69.0	68.6	61.9	59.2
Yamaguchi-52	99.3	98.0	–	69.9	69.6	69.6	69.6	69.9	70.1	69.4	69.1	69.6	73.5	69.8	69.5	63.0	64.7
Hokkaido-2	68.4	64.1	69.1	–	99.0	99.8	99.8	100.0	99.8	98.3	97.8	99.8	68.2	66.8	67.0	63.8	65.3
Saitama-1	70.0	65.1	70.0	97.5	–	99.3	99.3	99.0	99.3	99.0	99.0	99.3	67.7	66.3	66.5	63.1	65.1
Hiroshima-3	69.2	64.1	70.0	99.2	98.3	–	100.0	99.8	99.5	98.8	98.0	100.0	68.2	67.0	67.2	63.8	65.5
Hiroshima-5	68.4	64.1	69.1	99.3	98.3	100.0	–	99.8	99.5	98.8	98.0	100.0	68.2	67.0	67.2	63.8	65.5
Tottori-4	68.4	66.7	69.1	100.0	97.5	99.2	99.3	–	99.8	98.3	97.8	99.8	69.2	68.1	68.1	64.7	66.4
Fukuoka-4	69.1	65.4	69.9	99.3	98.3	98.3	98.5	99.3	–	98.8	98.3	99.5	68.2	66.5	66.8	63.6	65.8
Miyazaki-1	69.1	65.4	69.9	96.4	96.7	96.3	96.3	96.4	97.1	–	98.8	98.8	67.5	65.8	66.0	62.8	64.8
Miyazaki-3	70.0	65.1	70.0	94.2	96.7	95.0	95.0	94.2	95.0	98.0	–	98.0	67.7	65.5	66.2	62.6	64.8
Tsushima-5	68.4	64.1	69.1	99.3	98.3	100.0	100.0	99.3	98.5	97.1	95.0	–	68.2	67.0	67.2	63.8	65.5
T.sergenti Fukushima (AB016280, BAA31951)	69.9	62.7	69.9	62.6	60.8	61.7	61.7	64.6	61.8	61.8	61.7	61.7	–	83.7	83.7	60.4	61.0
T.orientalis (AB008369, BAA23206)	65.9	61.8	65.9	62.6	61.7	62.5	62.5	64.6	61.8	61.0	59.2	62.5	81.3	–	98.8	61.1	59.1
T.buffeli Warwick (D11047, BAA01796)	65.4	60.0	65.4	62.8	60.8	61.7	61.7	63.9	62.0	61.3	60.0	61.7	81.3	97.1	–	60.5	59.3
T.annulata (DU22888, AAB60238)	58.4	55.0	58.4	57.6	58.0	58.0	58.0	57.6	57.6	56.8	57.1	58.0	50.8	49.2	47.5	–	77.3
T.parva (U22889, AAC46910)	58.7	50.0	58.7	59.5	59.2	59.2	59.2	60.2	59.5	59.5	59.2	59.2	50.8	48.3	49.0	72.1	–

*: The values found in the upper right cells are the levels of similarity between *p33/34* gene partial (412bp) nucleotide sequences and the values found in the lower left cells are the levels of similarity between *P33/34* deduced amino acid sequences.

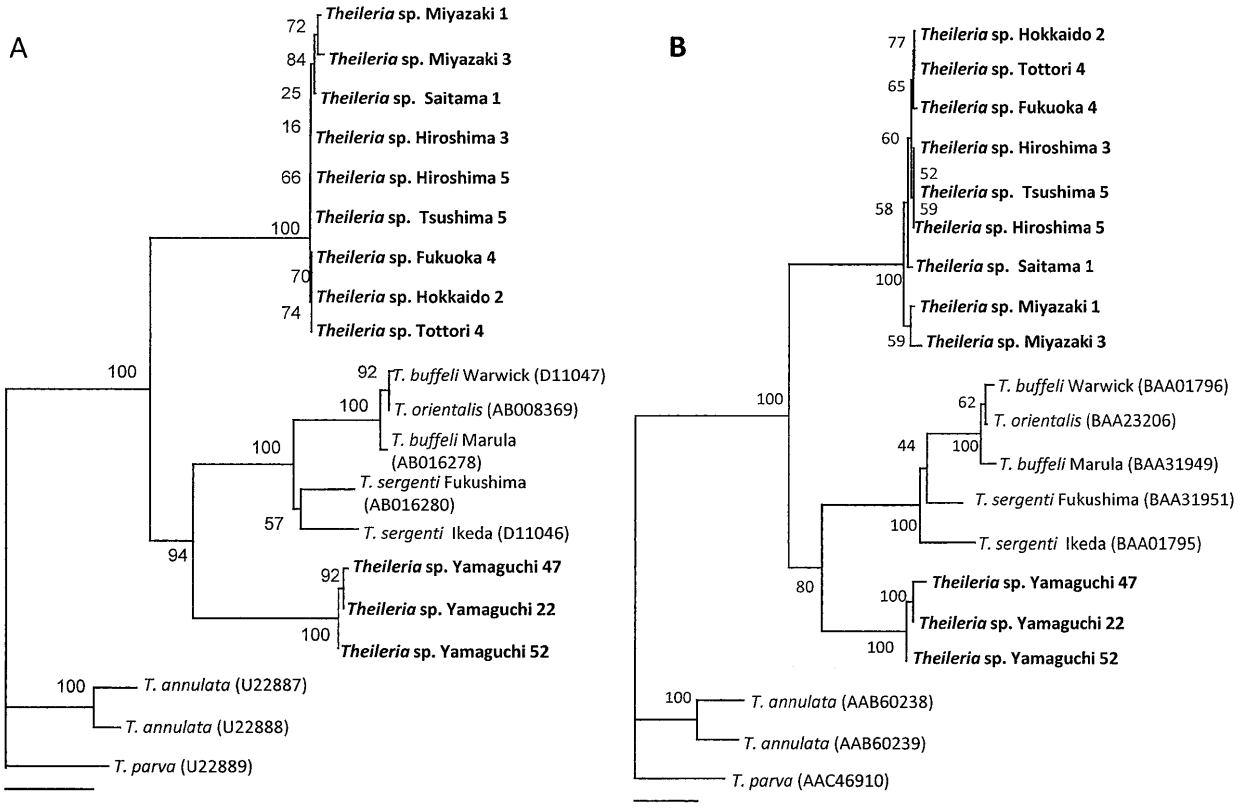

Fig. 2. Phylogenetic relationship of various *Theileria* spp. based on the nucleotide sequences of the piroplasma major immunodominant protein gene (*p33/34*) (A) and deduced amino acid sequences (B). The neighbor-joining method was used to construct the phylogenetic tree with the Clustal W program. The scale bar represents 10% divergence. The numbers at nodes are the proportions of 100 bootstrap resampling that support the topology shown.

protein, and as a result it could be changed under heavy selective pressure by the immunity system of the host. Based on recent analysis of mitochondrial DNA, sika deer in Japan may be divided into two groups - eastern and western [15, 21, 22]. Sika deer in Yamaguchi Prefecture belong to the western group of sika deer, which also include those found in Hiroshima, Tottori, Fukuoka, Miyazaki and Tsushima. The exact origin of the *Theileria* detected from sika deer in Yamaguchi found to be different from others is unknown. Because sika deer also live in neighboring countries of Japan, including Korea, China and Russia, genes of *Theileria* species in these regions should be phylogenetically analyzed to better clarify the divergence of *p33/34* gene among *Theileria* detected from subspecies of sika deer. Other possibility of the divergence of *Theileria* is difference of vector tick of each strain. As *Theileria* spp. perform gametogony in tick body, genetical variation of *Theileria* could be easily occurred in tick stage. Although tick species infested on sika deer were not well examined in the present study, vector ticks of sika deer in each location should be clarified. Analysis of *Theileria* spp. in vector ticks is also interesting. Morphological feature and pathogenesis of the two different strains of *Theileria* detected from sika deer in Japan should also be clarified in the future studies.

Nucleotide sequence accession number. The 18S rRNA gene sequences obtained from sika deer have been deposited in the GenBank database under the accession numbers; EU126895 for *Theileria* sp. from *C. n. yesoensis* in Hokkaido; EU126896 for *Theileria* sp. from *C. n. centralis* in Saitama; EU126897 and EU126898 for *Theileria* sp. from *C. n. centralis* in Hiroshima; EU126899 for *Theileria* sp. from *C. n. centralis* in Tottori; EU126900 for *Theileria* sp. from *C. n. nippon* in Fukuoka; EU126901 and EU126902 for *Theileria* sp. from *C. n. nippon* in Miyazaki; EU126903 for *Theileria* sp. from *C. n. pulchellus* in Tsushima island. The nucleotide sequences of the *p33/34* gene obtained from sika deer have been deposited in the GenBank database under the accession numbers; EU126904 for *Theileria* sp. from *C. n. yesoensis* in Hokkaido; EU126905 for *Theileria* sp. from *C. n. centralis* in Saitama; EU126906 and EU126907 for *Theileria* sp. from *C. n. centralis* in Hiroshima; EU126908 for *Theileria* sp. from *C. n. centralis* in Tottori; EU126909 to EU126911 for *Theileria* sp. from *C. n. centralis* detected from sika deer in Yamaguchi; EU126912 for *Theileria* sp. from *C. n. nippon* in Fukuoka; EU126913 and EU126914 for *Theileria* sp. from *C. n. nippon* in Miyazaki; EU126915 for *Theileria* sp. from *C. n. pulchellus* in Tsushima island.

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