FULL PAPER *Parasitology*

Epidemiological Survey of *Theileria orientalis* Infection in Grazing Cattle in the Eastern Part of Hokkaido, Japan

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ABSTRACT. *Theileria orientalis* is one of the benign species of *Theileria* that is widely distributed in Japan and is sometimes responsible for serious economic losses in the livestock industry. In the present study, we surveyed the current status of *T. orientalis* infection in grazing cattle in the eastern areas of Hokkaido (Taiki, Otofuke, Shintoku, and Shin-Hidaka districts) using molecular methods, as well as traditional methods, of diagnosis. The genes encoding the major piroplasm surface protein (MPSP) and p23 of *T. orientalis* were identified using highly detectable polymerase chain reaction (PCR). Results of the MPSP-PCR assay indicated that grazing cattle in these districts, after about 1.5 months pasturage, showed high rates of infection, ranging from 10.0–64.8%. Although the main MPSP and p23 genotypes detected were the Ikeda- or Chitose-types, an MPSP gene closely relating to that found in Okinawa prefecture, and a p23 gene closely relating to the Australian (Warwick) Buffeli-type gene, were found in the cattle in Shintoku and Shin-Hidaka districts. The present survey indicated that there were at least five types of *T. orientalis* classified by their MPSP genes in Hokkaido, Japan, and that *T. orientalis* infection rates are still high in this region.

KEY WORDS: cattle, Hokkaido, MPSP, p23, *Theileria orientalis*.

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Bovine theileriosis in Japan is caused by *Theileria orientalis* which is a tick-transmitted, intraerythrocytic protozoan belonging to the phylum Apicomplexa [23]. In Hokkaido, the northern island of Japan, most cattle are pastured from spring to autumn to reduce rearing costs, but this is associated with a high risk of contracting theileriosis. *T. orientalis* is a member of the relatively benign Theileria group (*Theileria sergenti/buffeli/orientalis*). It shows a lower pathogenicity in cattle than *T. parva* and *T. annulata*, but causes symptoms including fever, anemia, and anorexia [7]. However, the livestock industry in Japan still suffers enormous economic losses due to this disease [9, 22], because no commercially effective medicines or vaccines are currently available for its control in Japan. Application of flumethrin pour-on to the grazing cattle is the main method used to control the parasite, by reducing the number of vector ticks during the pasturage season [20]. Shimizu *et al.* [20] reported that the mortality and morbidity in grazing cattle in Japan were less than 0.1% and about 2.5%, respectively. Although the tick densities in Hokkaido are lower than in other areas [26], many farmers still suffer from the consequences of bovine theileriosis and related losses in livestock production.

The purpose of the present study was to investigate the current status of bovine theileriosis in Hokkaido and to discover the significance of this disease in grazing cattle. The genes encoding the major piroplasm surface protein (MPSP) and p23 of *T. orientalis* were subjected to epidemiological molecular analysis. The MPSP and p23 genes are expressed in the intraerythrocytic stage of the parasite, called piroplasm, and are conserved to some degree among field isolates of *T. orientalis* [6, 8, 17]. *T. orientalis* populations have recently been found to consist of seven types, based on a series of all the registered MPSP gene sequences [10, 27]. Another gene, which encodes a 23-kDa piroplasm membrane protein, was also reported to show genetic diversity [18, 28]. We therefore collected a total of 501 blood samples from 230 grazing cattle in the eastern areas of Hokkaido, Japan (Taiki, Otofuke, Shintoku, and Shin-Hidaka districts) in 2007 and 2008. We surveyed the current status of *T. orientalis* infection in the cattle using molecular methods, including newly designed PCR primers and DNA sequencing analyses, together with classical methods of diagnosis.

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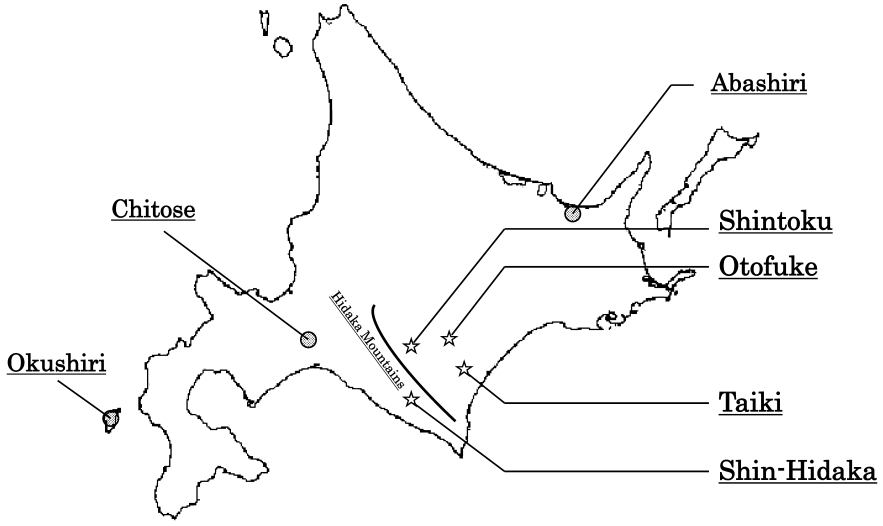

Fig. 1. Geographical map of Hokkaido, Japan. Stars indicate the locations of the four districts (Otofuke, Shintoku, Taiki, and Shin-Hidaka) where the blood samples were collected. Filled circles show the locations (Abashiri, Chitose, and Okushiri districts) where previous epidemiological surveys of *T. orientalis* have been carried out [10, 11, 14].

MATERIALS AND METHODS

Collection of blood samples: The cattle examined in the present study were grazed on 4 public farms in the eastern areas of Hokkaido (Taiki, Otofuke, Shintoku, and Shin-Hidaka districts) (Fig. 1), from May to July in 2007 and 2008. In the Taiki district, Holstein cattle aged 12–20 months were firstly allowed to graze from 4th June, 2007, and 89 or 91 blood samples were collected 4 times (on 29th May, 25th June, 9th July, and 23rd July, 2007) from the cattle. These samples were collected in order to investigate the changes in *T. orientalis* infection throughout the grazing period. Thirty-six and 83 blood samples were collected from cattle in the Shintoku (firstly grazing cattle aged approximately 9 months) and Shin-Hidaka districts (cattle of various ages with various grazing histories) on 17th July and 25th July, 2007, respectively. The other 20 samples were collected from cattle in the Otofuke district (cattle of various ages, including 4 firstly grazing cattle) on 14th July, 2008. The animals sampled included Japanese Black, Angus, Hereford, Japanese Short-horn, and Holstein cattle (Table 1). The sampling was performed at approximately 1.5 months after the start of pasturage. Thus, a total of 501 blood samples were collected from 230 grazing cattle in these districts.

Diagnoses of parasitemia and anemia: Approximately two m*l* of blood were collected from the tail veins of cattle and added to ethylenediaminetetraacetic acid, to a final concentration of 0.15–0.22%. Thin blood smears were made and then stained with Giemsa solution, for classical microscopic diagnosis. Parasitemia was observed in *T. orientalis*-infected red blood cells (RBC) by microscopy. The blood smear was classified based on the followed method, referred to as the Ishihara's method in the Japanese literature: –; no detection, +; 1 parasite in 10 fields (<0.05%), ++; >1 parasite in 10 fields (0.05–0.5%), +++; more than 1 parasite in 1 field (0.5–5%), ++++; more than 10 parasites in 1 field (> 5%). Additionally, the RBC count, hematocrit (HCT) value, and hemoglobin (Hb) concentration of respective blood samples were determined using a Celltac α (Nihon Koden, Tokyo, Japan), to detect anemia. Anemia was diagnosed if the RBC, HCT, or Hb values were $<5 \times 10^6$ cells/μl, 24%, or 8 g/d*l*, respectively [5].

DNA extraction and plasma preparation: Genomic DNA was extracted from 100 μl of blood sample using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany), and 200 μl of the DNA solutions (approximately 6 $\mu g/\mu l$) were obtained and stored at –30°C. Blood plasma fractions were obtained by centrifugation at 700 × g for 15 min at 4°C, and then stored at –30°C, for subsequent analysis by enzyme-linked immunosorbent assay (ELISA).

PCR: A pair of universal primers (857 base pairs (bp)) [8, 24], and three kinds of type-specific primer pairs for the Ikeda strain (826 bp), Chitose strain (831 bp), and *T. buffeli* Warwick stock (825 bp) [10, 13, 27] were used for the PCR identification of *T. orientalis*. All the primer pairs were targeted to the MPSP (p32/33/34) gene of *T. orientalis*, and were able to amplify the indicated sizes of DNA fragments by PCR.

In the present study, we designed two new kinds of primer pairs universal for *T. orientalis*, by aligning all of the registered sequences of MPSP and p23 genes listed in Figs. 2 and 3. Based on the designation, the first "MPSP" primers, MPSP-F (5'-CTTTGCCTAGGATACTTCCT-3') and MPSP-R (5'-ACGGCAAGTGGTGAGAACT-3'), amplified a 776-bp DNA fragment from the MPSP gene of *T. ori-*

entalis, while the second "p23" primers, p23-F (5'-GTACACACCTTGAAATCTGGC-3') and p23-R (5'-CAAGAGAGGCAACAACAACGA-3'), amplified a 601-bp DNA fragment from the p23 gene. These primer pairs targeted completely conserved regions in these database sequences, and the PCR analyses were expected to be adopted for the surveillance of *T. orientalis* infections.

These diagnostic PCR analyses were performed using 2 μl of stored DNA template extracted from the blood sample, mixed with 18 μl of reaction buffer, consisting of 0.2 μl of Ex Taq DNA polymerase (Takara, Tokyo, Japan), 0.2 μl of 10 μM each primer, 10 μl of 2 × Ampdirect plus (PCR buffer: Shimadzu Biotech., Kyoto, Japan), and 7.4 μl of double distilled water (DDW). These reactions were performed using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.) for 35 cycles. After an initial denaturation step for 10 min at 94°C, all the cycles consisted of denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C, followed by an additional 4 min at 72°C. The amplified PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, U.S.A.), and then visualized under an ultraviolet light.

ELISA: ELISA was performed as described previously [21] with some modifications to check the presence of *T. orientalis*-specific immunoglobulin G (IgG) in the collected blood plasma fractions. Crude parasite antigens including a mixture of Ikeda- and Chitose-type piroplasms kindly provided by Kaketsuken (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) were used. In brief, each well of a micro-titer plate (Nalge Nunc, Roskilde, Denmark) was coated with 1 μg/m*l* of the antigen in 100 μl of 0.05 M carbonate-bicarbonate buffer (pH 9.6), and then incubated overnight at 4°C. Samples were washed five times with 0.05% Tween-20 in phosphate-buffered saline (PBS-T) between all steps. The whole well was then blocked with 200 μl of 4% skimmed milk in PBS-T for 1 hr at 37°C. Plasma samples diluted to 1:400 with 1% skimmed milk in PBS-T were added as the first antibodies in 100 μl, and incubated at 37°C for 1 hr. Horseradish peroxidase-conjugated rabbit serum anti-bovine IgG (Bethyl Laboratories Inc., Montgomery, TX, U.S.A.) was diluted to 1:10,000 with 1% skimmed milk in PBS-T, and 100 μl of the second antibody was added to each well. After incubation at 37°C for 1 hr, 100 μl of SureBlue Reserve TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, U.S.A.) were added, and the mixture was incubated at room temperature for 20 min. After adding an equal volume of 1N HCl to each well to stop the reaction, the optical density (OD) was measured at a wavelength of 450–630 nm, using an MTP-120 ELISA reader (Corona Electric, Ibaraki, Japan). The sample was considered to be positive for *T. orientalis* infection when the OD value was > 0.5.

DNA sequencing and phylogenetic analysis: DNA sequencing analysis of PCR fragments was carried out to examine the diversities of the MPSP and p23 genes derived from the blood samples. At least six blood samples were randomly selected from the diagnostic PCR-positive samples from each of the Taiki, Shintoku, and Shin-Hidaka farms. The DNA fragments were amplified by a further PCR step for subsequent DNA sequencing. PCR was performed using 2 μl of the selected DNA template mixed with 18 μl of reaction buffer, consisting of 0.2 μl of Expand HiFi Plus Enzyme Blend (Roche Applied Science, Basel, Switzerland), 0.2 μl of 10 μM of each primer, 4 μl of 5 × Expand HiFi Plus Reaction Buffer with 7.5 mM $MgCl_2$, 0.4 μl of 10 mM PCR Grade Nucleotide Mix (Roche Applied Science), and 13 μl of DDW. All of the PCR cycles consisted of an initial denaturation step for 2 min at 94°C , followed by 35 cycles for 1 min at 94°C, annealing for 30 sec at 58°C, and extension for 1 min at 72°C, followed by an additional 7 min at 72°C. The amplified PCR products were cloned into a pCR2.1 vector, according to the manufacturer's instructions for the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). DNA sequencing of the inserts was performed by a DNA sequencing service (Sigma-Aldrich, Tokyo, Japan). The CLUSTAL X program (University College Dublin, Dublin, Ireland) was used to align the obtained sequences, and a neighbor joining method using an NJplot program [16] constructed 2 phylogenetic trees based on the MPSP and p23 genes. The accuracy of the branches of the phylogenetic trees was confirmed by a bootstrap method [1]. The representative sequences obtained in the present study were registered in the GenBank database (National Center for Biotechnology Information, National Institutes of Health), as indicated in Figs. 2 and 3.

RESULTS

A study of grazing cattle in Taiki district: In Taiki district (Fig. 1), grazing was started on 4th June in 2007. On 29th May, before the cattle were put out to graze, only 2 out of 89 cattle (2.2%) were positive for *T. orientalis* infection, based on microscopic observation, sero-diagnostic ELISA, and PCR detections of the MPSP and p23 genes, as shown in Tables 1 and 2. However, the number of *T. orientalis*-positive cattle had increased dramatically, to 48.4% and 49.5%, based on PCR detections of the MPSP and p23 genes, respectively, by 25th June, 3 weeks after being put out to pasture. By 23rd July, more than half of the cattle (64.8% by MPSP-PCR and ELISA) were infected with *T. orientalis*. Importantly, the percentage of cattle with anemia increased from 0.0% (29th May) to 22.0% (23rd July) in a group of MPSP PCR-positive cattle (Table 1).

The newly designed MPSP and p23 primers used for PCR analyses could detect the parasite in a greater number of blood samples than the classical universal primers could, as shown in Table 2. PCR-based typing for the Ikeda, Chitose, and Buffeli stocks showed that at least the individuals of *T. orientalis* carrying the Ikeda-type (type 2 in Fig. 2) and Chitose-type MPSP genes (type 1) were distributed in the farm in Taiki district, and approximately half of the infected cattle exhibited a mixed infection with the Ikeda- and Chitose-

Table 1. Classical diagnoses and disease occurrence of *T. orientalis*-positive cattle grazing in the east part of Hokkaido

Districts	Species	Date (total cattle)	Number of positive cattle (%)		
			Microscopic test[a)]	ELISA[a)]	Anemia[b)]
Taiki	Holstein	5/29 (89)	2 (2.2)	2 (2.2)	0 (0.0)
		6/25 (91)	13 (14.3)	14 (15.4)	3 (6.8)
		7/9 (91)	40 (44.0)	37 (40.7)	5 (8.6)
		7/23 (91)	47 (51.6)	59 (64.8)	13 (22.0)
Otofuke	Japanese Black/Angus	7/14 (20)	5 (25.0)	1 (5.0)	0 (0.0)
Shintoku	Japanese Black/Angus	7/17 (36)	8 (22.2)	4 (11.1)	0 (0.0)
Shin-Hidaka	Hereford/Japanese Short-horn/Holstein	7/25 (83)	57 (68.7)	39 (47.0)	16 (32.6)

a) Number (%) of cattle showing positive reactions (of total cattle).
b) Number (%) of cattle showing clinical anemia in a group of MPSP PCR-positive cattle as shown in Table 1.

Table 2. PCR diagnoses of *T. orientalis*-positive cattle grazing in the east part of Hokkaido

Districts	Date (total cattle)	Number of positive cattle (%)					
		Universal[a)]	MPSP[a)]	p23[a)]	Ikeda[b)]	Chitose[c)]	I&C[d)]
Taiki	5/29 (89)	0 (0.0)	2 (2.2)	2 (2.2)	2 (100.0)	0 (0.0)	0 (0.0)
	6/25 (91)	13 (14.3)	44 (48.4)	45 (49.5)	35 (79.5)	7 (15.9)	7 (15.9)
	7/9 (91)	38 (41.8)	58 (63.7)	58 (63.7)	57 (98.3)	23 (36.7)	23 (36.7)
	7/23 (91)	44 (48.4)	59 (64.8)	58 (63.7)	57 (96.6)	32 (54.2)	32 (54.2)
Otofuke	7/14(20)	1 (5.0)	2 (10.0)	2 (10.0)	2 (10.0)	0 (0.0)	0 (0.0)
Shintoku	7/17 (36)	6 (16.7)	10 (27.8)	8 (22.2)	7 (70.0)	7 (70.0)	6 (60.0)
Shin-Hidaka	7/25 (83)	21(25.3)	49 (57.8)	51 (59.0)	41 (83.7)	31 (63.3)	26 (53.1)

a) Number (%) of cattle showing positive reactions (of total cattle).
b) Number (%) of cattle showing a PCR-positive reaction for Ikeda-type in a group of MPSP PCR-positive cattle.
c) Number (%) of cattle showing a PCR-positive reaction for Chitose-type in a group of MPSP PCR-positive cattle.
d) Number (%) of cattle showing both reactions for Ikeda-and Chitose-types in a group of MPSP PCR-positive cattle.

type parasites (54.2% in a group of MPSP PCR-positive cattle on 23rd July) (Table 2). The detection rate of the Ikeda-type gene (96.6% on 23rd July) was consistently higher than that of the Chitose-type gene (54.2% on 23rd July), while another gene of the *T. buffeli*-type (type 3) was not detected by the present PCR analysis.

Based on the results from Taiki on 25th June and 9th July, we divided the samples without microscopically detected parasites into 2 groups (Table 3): Group A had a negative reaction in the MPSP-PCR assay (–/–), while Group B had a positive reaction in the PCR assay (–/+). Parasitemia and anemia were assessed approximately 2 weeks after the initial division. In Group A, 25.6% of the cattle had become positive in both the microscopic and MPSP-PCR tests, but showed no anemia. On the other hand, 88.6% of the cattle in Group B showed positive reactions in both tests, and importantly, 22.6% and 58.1% of the positive cattle exhibited anemia and high levels of parasitemia (> +++), respectively (Table 3). These findings suggest that the MPSP-PCR method is able to detect infection 2 weeks earlier than traditional methods.

Geographical variation in infection of grazing cattle among Otofuke, Shintoku, and Shin-Hidaka districts: To examine whether the high rate of *T. orientalis* infection observed in Taiki district was also seen in the eastern areas of Hokkaido, blood samples were collected from grazing cattle on three different farms in Otofuke, Shintoku, and Shin-Hidaka districts (Fig. 1), approximately 1.5 months after they were put out to pasture. The results of MPSP-PCR analysis indicated high infection rates of 10.0%, 27.8%, and 57.8% on these farms, respectively, as shown in Table 2. The MPSP-PCR-positive cattle showed a high rate of anemia (32.6%) in the farm in Shin-Hidaka district, while anemia was not observed in the cattle on the Otofuke and Shintoku farms (Table 1). As in Taiki district, the newly developed MPSP- and p23-PCR analyses were more useful than the classical universal PCR analysis for detecting the parasite, and MPSP-PCR-positive cattle were infected with at least the Ikeda type or the Chitose type, or a mixture of these 2 types (Table 2).

Table 3. Infection status of the groups 2 weeks after initial division based on MPSP-PCR results

Initial division	Infection status after 2 weeks		
Parasitemia[a]/PCR[b]	Parasitemia[a]/PCR[b]	Numbers of cattle (%)	Anemia (%)[c]
Group A (n=39)[d] –/–	–/–	29 (74.4)	1 (3.4)
	+/+	5 (12.8)	0 (0.0)
	++/+	4 (10.3)	0 (0.0)
	+++/+	1 (2.6)	0 (0.0)
	++++/+	0 (0.0)	0 (0.0)
	subtotal	10 (25.6)	0 (0.0)
Total		39 (100.0)	1 (3.4)
Group B (n=35)[d] –/+	–/+	4 (11.4)	0 (0.0)
	+/+	3 (8.6)	0 (0.0)
	++/+	10 (28.6)	0 (0.0)
	+++/+	9 (25.7)	3 (33.3)
	++++/+	9 (25.7)	4 (44.4)
	subtotal	31(88.6)	7 (22.6)
Total		35 (100.0)	7 (20.0)

a) Parasitemia was determined by microscopic examination using the Ishihara method.
b) Judgment was based on MPSP-PCR, but p23-PCR showed similar results to those of MPSP-PCR.
c) Number (%) of cattle showing clinical anemia in the same group.
d) Samples were sorted from the data on microscopic test-negative cattle on the Taiki farm on 6/25 and 7/9 (Table 1).

Phylogenetic analyses of isolated MPSP and p23 genes: For DNA sequencing of the MPSP and p23 genes from field isolates, 24, 6, and 10 samples were randomly selected from the PCR-positive blood samples from the Taiki, Shintoku, and Shin-Hidaka farms, respectively. Forty PCR fragments derived from the MPSP and p23 genes were isolated, and the complete sequences were determined. Two phylogenetic trees were generated. Thirty-five and 34 samples of the MPSP and p23 genes, respectively, showed complete or high sequence identities with the registered sequences of Ikeda-type *T. orientalis* (D11046 and D84447, respectively), as shown in Figs. 2 and 3. In Taiki district, only Ikeda types were detected for both the MPSP and p23 genes. On the other hand, two MPSP and three p23 Chitose-type genes (D12689 and D844462, respectively) were obtained from three and one of the grazing cattle on the Shintoku and Shin-Hidaka farms, respectively (Figs. 2 and 3). Type-4 (Brisbane type) and type-5 MPSP genes (Cheju type) which were closely related to the types previously isolated in Okinawa prefecture, were found in one animal each from the Shin-Hidaka and Shintoku farms (Fig. 2). One Shin-Hidaka animal showed the rare type-4 MPSP gene, and the third Buffeli-type p23 gene (AB021223) which is readily distinguishable from the Ikeda- and Chitose-type p23 genes was also isolated (Fig. 3).

DISCUSSION

Based on the results from 25th June in Taiki district, 48.4% of grazing cattle were MPSP-PCR-positive (Table 2), indicating that about a half of the cattle became infected with *T. orientalis* during a brief 3-week span after being put out to pasture. After 1.5 months pasturage, we also observed high infection rate in all the other districts. Because suitable medicines and vaccines are currently unavailable, this infectious disease is becoming an increasingly serious problem for the livestock industry in Japan. The increase in *T. orientalis* infection is related to the seasonal activities of the vector ticks in the pastures [2]. Although Ixodid ticks are commonly found in the eastern part of Hokkaido [4], the presence of *Haemaphysalis longicornis*, a tick known to be a major vector of *T. orientalis*, has not been investigated [3]. Further studies are needed to identify the vector(s) capable of transmitting the infectious organism in these cold, eastern areas of Hokkaido.

In our study, 88.6% of the cattle that were initially microscopic test-negative but MPSP-PCR-positive became microscopic test-positive 2 weeks later (Table 3). This result demonstrates that a high incidence of *T. orientalis* infection can exist even when the piroplasms of *T. orientalis* cannot be detected by microscopic observation. It also indicates that the newly designed MPSP and p23 PCR methods provide useful means for detecting hidden infections before the appearance of symptoms in the cattle. The clinical application of these methods could help livestock producers to avoid economic damage caused by bovine theileriosis. In contrast, the detection rates of these PCR methods (10% and 57.8% in MPSP-PCR) for the blood samples obtained from the Otofuke and Shin-hidaka farms were lower than those of the microscopic test (25% and 68.7%, respectively) (Tables 1 and 2). Since there is a possibility of infection with unidentified new *T. orientalis* members, further investigations are necessary.

Two major types of MPSP genes categorized as the Chi-

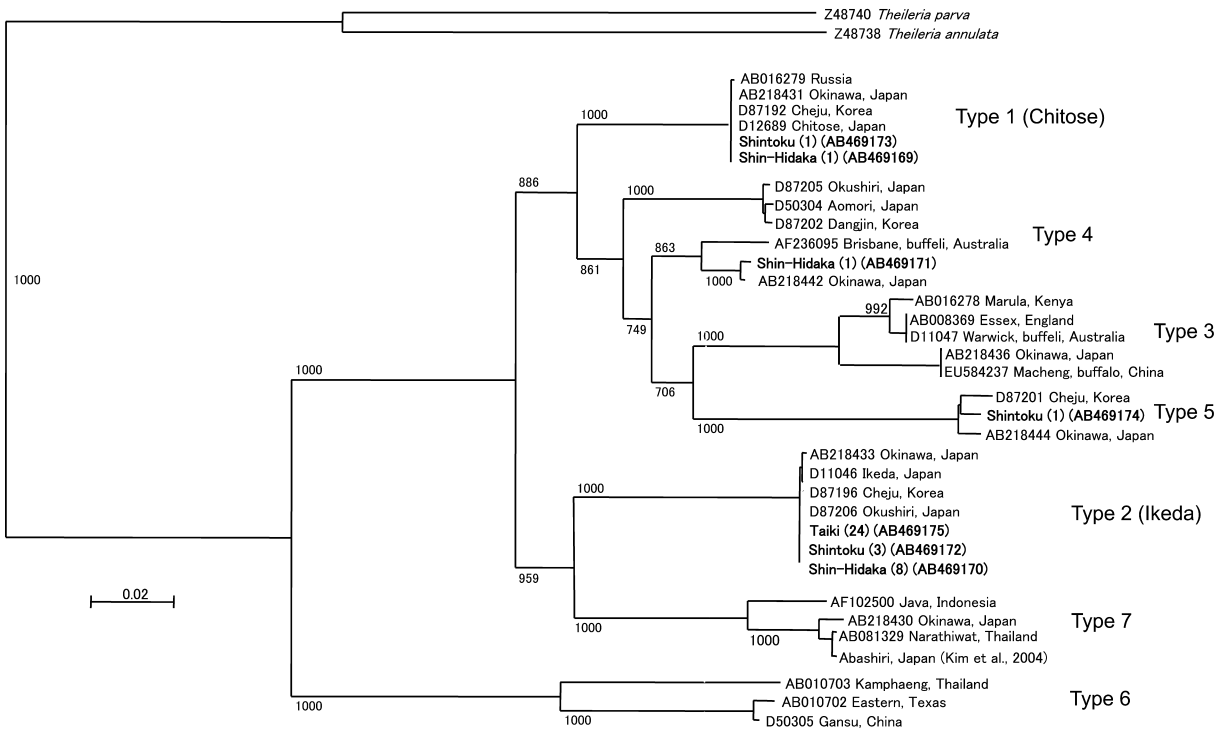

Fig. 2. Phylogenetic tree of the MPSP genes based on blood samples collected from Taiki, Shintoku, and Shin-Hidaka districts, together with previously registered sequences from the GenBank database. The sequences of the MPSP genes determined in the present study are expressed in bold-faced type, together with the number of collected samples. The representative sequences of isolated MPSP genes refer to the GenBank accession numbers, as indicated at the end of each branch. The numbers shown at the branch nodes indicate the bootstrap values. Different to the previous discrimination [10], a population of type-4 MPSP genes might be divided into two further groups due to the complexity of the branches.

tose and Ikeda types have been recognized from field isolates in Japan [13], and it has been also reported that these populations are distinguishable by MPSP allele-specific PCR methods [13, 15, 24]. The Chitose-type genotype is widely distributed [6, 12, 13], while the Ikeda-type parasite is commonly found in Korea and Japan but to a lesser extent in China [6], and not at all in Italy [19]. Most of field isolates consist of a mixture of 2–3 MPSP-type parasites, and single MPSP-type infections rarely occur [23]. In the present study, unique types of MPSP and p23 genes were found on the farms in Shintoku and Shin-Hidaka districts, in addition to the Chitose and Ikeda types. Type-3 (Buffeli-type), type-6 (Gansu-type), and type-7 (Abashiri-type) MPSP genes were not detected in the present study. The cow (an Angus) from the farm in Shintoku district that harbored *T. orientalis* with the type-5 MPSP gene was originally born on the farm, but its ancestors had been introduced from Miyazaki and Kagoshima prefectures, located in Kyushu Island, in the southern part of Japan. Type-4 and -5 MPSP genes have previously been found in Okinawa prefecture [27], and were also detected in the Kyushu area in our latest study (manuscript in preparation). In Shin-Hidaka district, the cow (a Hereford) with the type-4 MPSP and Buffeli-type p23 genes was also born on the study farm. The Buffeli-type p23 gene was derived from *T. buffeli* and has only previously been reported in the isolates from Australia [17]. The farm in Shin-Hidaka district had previously introduced cattle from Australia. This is the first report of the Buffeli-type p23 gene being isolated in Japan. Taken together with the results of a previous report concerning the isolation of a type-7 MPSP gene from Abashiri district (Fig. 1) [10], the present survey indicates that there are at least five types of *T. orientalis* (Types 1, 2, 4, 5, and 7) that can be categorized by their MPSP genes.

Terada *et al.* [25] reported that the indigenous Japanese Black breed of cattle was more resistant to *T. orientalis* infection than the exotic Holstein. Japanese Black cattle in Otofuke and Shintoku districts showed no sign of anemia, while the exotic Holsteins and Herefords in Taiki and Shin-Hidaka districts showed a high rate of anemia. Although differences in topography of the pastures, numbers and species of ticks, and age of the examined cattle among these districts make it difficult to evaluate the significance of the breed in the sensitivity to infection, it is possible that Japanese Black cattle are more resistant to *T. orientalis* than Holsteins and Herefords.

The present survey indicated that a population of *T. orientalis* carrying at least five types of MPSP genes and three types of p23 genes exists in Hokkaido. While the diversities of these immunogenic genes suggest that the control of *T.*

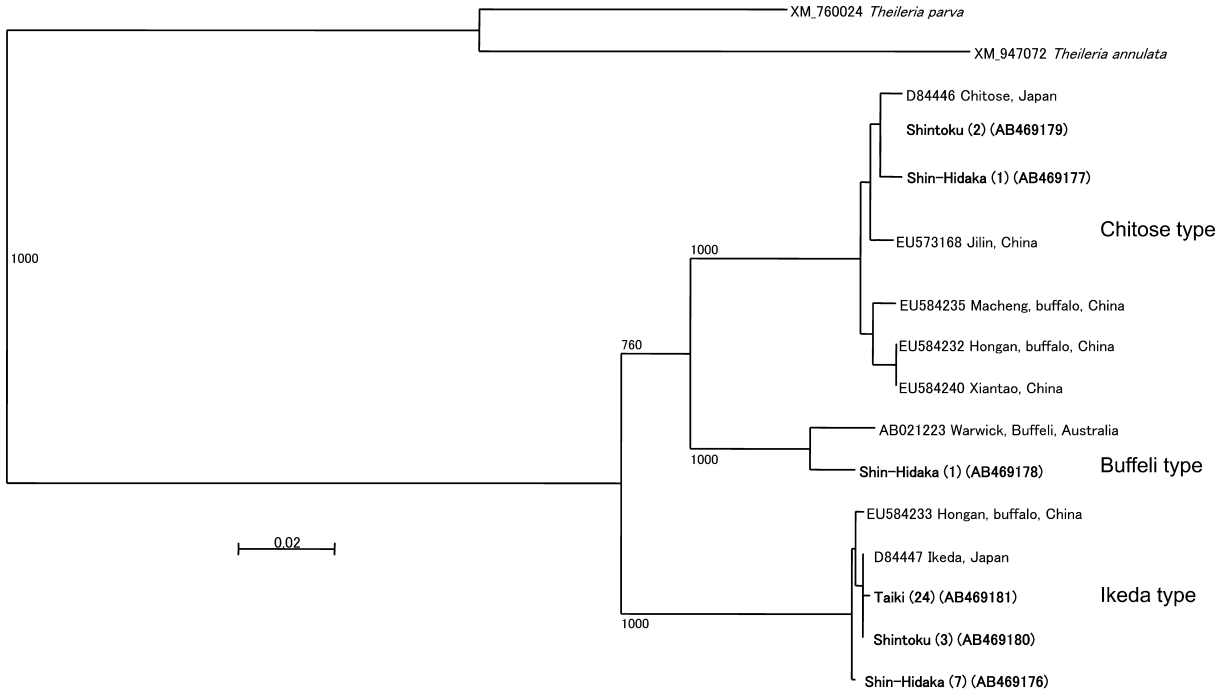

Fig. 3. Phylogenetic tree of the p23 genes of *T. orientalis* based on the blood samples from Taiki, Shintoku, and Shin-Hidaka districts, together with previously registered sequences from the GenBank database. The sequences of the p23 genes determined in the present study are expressed in bold-faced type, together with the number of collected samples. The representative sequences of isolated p23 genes refer to the GenBank accession numbers, as indicated at the end of each branch. The numbers shown at the branch nodes indicate the bootstrap values.

orientalis infection could be difficult, molecular epidemiological surveys are essential to provide the information needed for the successful developments of vaccines and diagnostic measures. The present study also demonstrated that *T. orientalis* infection is still a potentially serious problem in Hokkaido, Japan.

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