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INVERTEBRATE MICROBIOLOGY

The *Babesia divergens* Asia Lineage Is Maintained through Enzootic Cycles between *Ixodes persulcatus* and Sika Deer in Hokkaido, Japan

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ABSTRACT Parasites of the *Babesia divergens* Asia lineage, which are closely related to *B. divergens* in Europe and *Babesia* sp. strain MO1 in the United States, were recently reported in sika deer (*Cervus nippon*) in eastern Japan. To identify the tick vector(s) for this parasite, we conducted a field survey in Hokkaido, Japan, where the infection rate in sika deer is the highest in the country. A specific PCR system which detects and discriminates between lineages within *B. divergens* and between those lineages and *Babesia venatorum* showed that *Ixodes persulcatus* (11/822), but not sympatric *Ixodes ovatus* (0/595) or *Haemaphysalis* sp. (0/163) ticks, carried *B. divergens* Asia lineage. Genomic DNA was archived from salivary glands of partially engorged *I. persulcatus* females and three isolates of *B. divergens* Asia lineage were newly described. The 18S rRNA gene sequence of the isolates formed the Asia lineage cluster with those previously described in sika deer isolates. One salivary gland also contained parasites of *Babesia microti* U.S. lineage, which were subsequently isolated in a hamster *in vivo*. *B. venatorum* (strain Etb5) was also detected in one *I. persulcatus* tick. The 18S rRNA sequence of Etb5 was 99.7% identical to that of *B. venatorum* (AY046575) and was phylogenetically positioned in a taxon composed of *B. venatorum* isolates from Europe, China, and Russia. The geographical distribution of *I. persulcatus* is consistent with that of *B. divergens* in sika deer in Japan. These results suggest that *I. persulcatus* is a principal vector for *B. divergens* in Japan and Eurasia, where *I. persulcatus* is predominantly distributed.

IMPORTANCE The *Babesia divergens* Asia lineage of parasites closely related to *B. divergens* in Europe and *Babesia* sp. MO1 in the United States was recently reported in *Cervus nippon* in eastern Japan. In this study, specific PCR for the Asia lineage identified 11 positives in 822 host-seeking *Ixodes persulcatus* ticks, a principal vector for many tick-borne disease agents. Gene sequences of three isolates obtained from DNA in salivary glands of female ticks were identical to each other and to those in *C. nippon*. We also demonstrate the coinfection of *B. divergens* Asia lineage with *Babesia microti* U.S. lineage in a tick salivary gland and, furthermore, isolated the latter in a hamster. These results suggest that *I. persulcatus* is the principal vector for *B. divergens* as well as for *B. microti*, and both parasites may be occasionally cotransmitted by *I. persulcatus*. This report will be important for public health, since infection may occur through transfusion.

KEYWORDS *Babesia*, *Ixodes persulcatus*, tick-borne pathogens

Received 9 November 2017 **Accepted** 24 January 2018

Accepted manuscript posted online 26 January 2018

Citation Zamoto-Niikura A, Tsuji M, Qiang W, Morikawa S, Hanaki K-I, Holman PJ, Ishihara C. 2018. The *Babesia divergens* Asia lineage is maintained through enzootic cycles between *Ixodes persulcatus* and sika deer in Hokkaido, Japan. Appl Environ Microbiol 84:e02491-17. https://doi.org/10.1128/AEM.02491-17.

Editor Robert M. Kelly, North Carolina State University

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Human babesiosis is caused by intraerythrocytic protozoa belonging to the genus *Babesia*, which is maintained between ixodid ticks and various mammals in nature. Human babesiosis was first described in 1957 in a splenectomized Yugoslavian farmer (1). The cattle piroplasm *Babesia divergens* was subsequently identified as the causative agent, based on similar morphological features under light microscopy and successful transmission of human isolates to bovines (2). To date, approximately 40 human cases have been reported from Europe and attributed to *B. divergens* infection, although not all cases were diagnosed based on molecular methods. Almost all patients were asplenic and/or were immunocompromised at the time of infection, in which case the disease tends to be a life-threatening event, resulting in a high mortality rate of 42% (3). However, recent studies suggest that a wider range of infection has occurred in humans than previously recognized. Martinot et al. reported that an influenza-like infection was caused by *B. divergens* in immunocompetent patients in France (4). Furthermore, the existence of antibodies against *B. divergens* was evident in blood donors in Austria (2.1%) (5), in tick-exposed patients in Germany (3.6%) (6) and Belgium (33.2%) (7), and in forestry workers in France (0.1%) (8). In areas where *B. divergens* is endemic, *Ixodes ricinus*, which is distributed in many European countries and from North Africa to Scandinavia, is regarded as its vector. Vector competence is evidenced by experimental transstadial and transovarial transmission to susceptible animals (bovine and gerbil) (9–11), and by molecular epidemiology that indicates overlapping geographical distribution of this tick species and human cases (12–15).

Recently the view of human babesiosis has been changed to an emerging tick-borne disease, since patients infected with novel *Babesia* spp., as well as with piroplasms genetically related but not identical to well-known zoonotic *Babesia* spp., including *B. divergens*, are increasingly reported worldwide. In the United States, three cases of human babesiosis caused by *B. divergens*-like parasites were documented from Missouri (*Babesia* sp. strain MO1), Washington, and Kentucky (*Babesia* sp. strain KY). All three patients were previously splenectomized, and one babesiosis case was fatal (16–18). The piroplasm 18S rRNA gene sequences from all three patients were identical to each other and close to that of European *B. divergens* (U16370), with a sequence identity between them of 99.8% (1,721/1,724 bp) (16). Parasites with identical 18S rRNA gene sequences to the *B. divergens*-like species were subsequently isolated from eastern cottontail rabbits (*Sylvilagus floridanus*) on Nantucket Island, Massachusetts (19), and antibodies against *Babesia* sp. MO1 were evident in eastern cottontail rabbits in Tennessee (20) as well, suggesting that this animal is a reservoir for this *Babesia* sp.

Very recently, we reported that sika deer (*Cervus nippon*) in Japan carried a *Babesia* parasite genetically closely related to but different from European (EU) *B. divergens* and *Babesia* sp. MO1 (in the United States [U.S.] lineage) (21). In phylogenetic analysis, these parasites assembled into a monophyletic clade (*B. divergens/B. capreoli* group) and were divided into distinct lineages that reflected geographical origin of the parasites (EU, U.S., and Asia lineages). These results raised concern of possible infection in humans and the necessity of elucidating the vector ticks for the parasites in this country. However, neither of the (suspected) vectors for *B. divergens* in Europe and the United States, *I. ricinus* and *Ixodes dentatus* (22), respectively, is distributed in Japan.

To examine a large number of ticks, we established a PCR system which could detect and discriminate between EU/U.S. *B. divergens* and *B. divergens* Asia lineages, and between the *B. divergens* parasites and *Babesia venatorum* (formerly *Babesia* sp. strain EU1). *B. venatorum* is genetically similar to *B. divergens* and recently emerged in patients in Europe (23) and China (24). Since both zoonotic parasites have been frequently detected in sympatric ticks (12, 14, 15, 25–27), we conducted a field survey on ticks collected in Hokkaido, Japan, where deer infected with *B. divergens* are most abundant in this country, and screened DNA from the ticks using the discriminatory PCR we developed.

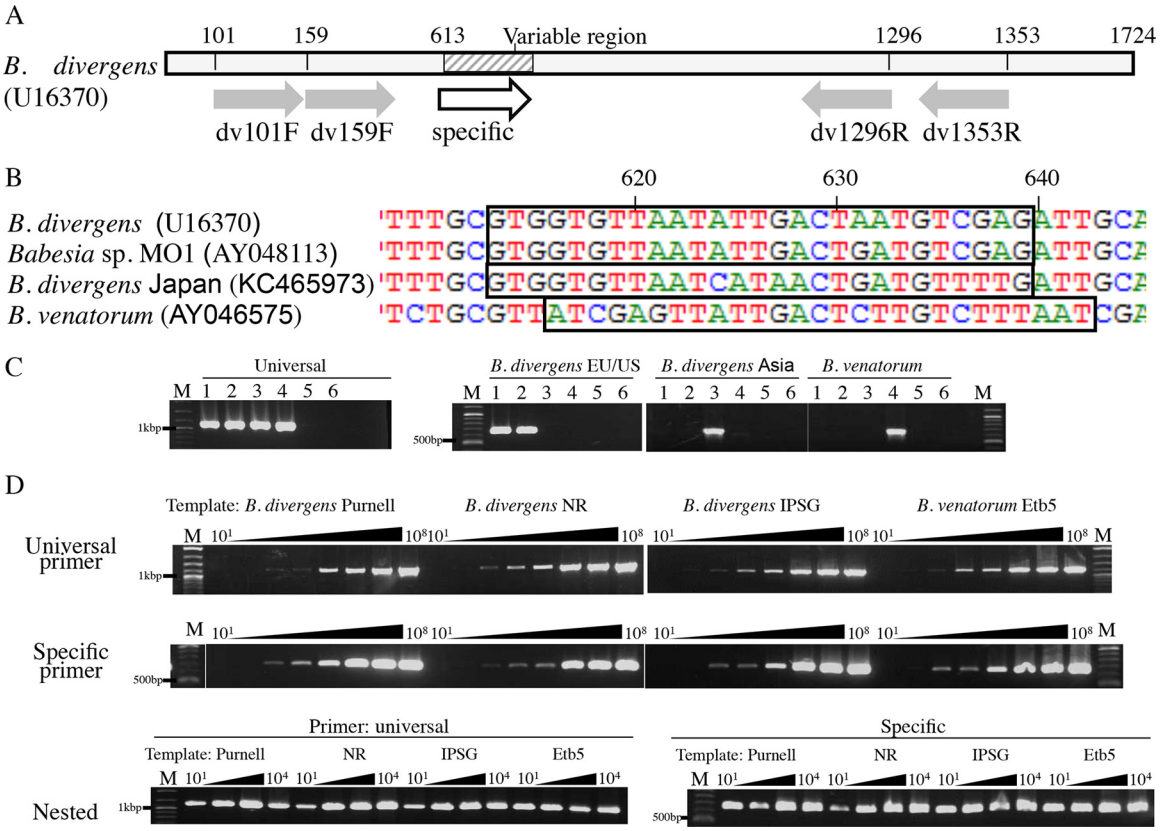

FIG 1 Type-specific PCR targeting the 18S rRNA genes of *B. divergens* and *B. venatorum*. (A) Schematic diagram of the PCR primer design. Numbers above the bar indicate nucleotide positions of designed primers in *B. divergens* (U16370). Gray and white arrows show positions of universal and specific primers, respectively. (B) Sequences of each lineage/species with boxes indicating the positions of the primers for dvEU (*B. divergens* and *Babesia* sp. MO1), dvJA (*B. divergens*, Japan) and dvJB (*B. venatorum*). (C) Specificity of the type-specific PCR. PCR amplification using universal primers dv159F and dv1296R or specific primers dvEU, dvJA, or dvJB/dv1296R. Plasmids carrying the 18S rRNA gene of *B. divergens* Purnell strain (EU lineage, lane 1), *Babesia* sp. NR strain (U.S. lineage, lane 2), *B. divergens* IpSG10 (Asia lineage, lane 3), *B. venatorum* Etb5 (lane 4), and negative controls (*B. microti*, lane 5, and *Theileria* sp., lane 6) were used as the template. M, marker. (D) Sensitivity of specific nested PCR. Conventional PCR using universal primers dv101F and dv1353R (upper panel) or specific primers dvEU, dvJA, or dvJB/dv1296R (middle panel) with the plasmid carrying the 18S rRNA gene of *B. divergens* strain Purnell (EU lineage), *Babesia* sp. strain NR (U.S. lineage), *B. divergens* IpSG10 (Asia lineage), and *B. venatorum* Etb5. Plasmids were diluted 10-fold from 1×10^1 to 1×10^8 copies and used (lanes 10^1 to 10^8). M, marker. Lower panel shows nested PCR amplification on the first 10^1 to 10^4 copy template PCR products from the universal primers shown above. Universal nested primers dv159F/dv1296R or specific nested primers dvEU, dvJA, or dvJB/dv1296R were used.

RESULTS

Development of specific PCR. By designing primers specific for the 18S rRNA genes of *B. divergens* Asia and EU/U.S. lineages and *B. venatorum* parasites, we developed a type-specific nested PCR. The specificity of this PCR system was examined by using plasmids carrying the 18S rRNA gene sequence of each parasite. The results are shown in Fig. 1C and demonstrate formation of a single positive signal that was specific for each parasite. Specificity was further tested by mixing two plasmid types together (1×10^8 copies of each) for every possible combination. In each case, only the target species was amplified (data not shown).

Sensitivity of the type-specific nested PCR was also examined by using 10-fold dilutions of plasmid (1×10^8 to 1×10^1 copies in a PCR mixture) as the template in the first-round PCR (Fig. 1D). Using either universal (top panel) or specific (middle panel) primers, products were observed at all template concentrations except at 1×10^1 and 1×10^2 copies of plasmid. In the nested PCR, in which first-round PCR products (universal primer) were used as the template, amplicons were visible at these lower concentrations (Fig. 1D, bottom panels).

The effectiveness of the dvJA primer was also evaluated in the presence of excess tick DNA by spiking tick DNA into PCR mixtures containing template *B. divergens* IpSG10

FIG 2 Tick survey areas in Hokkaido Island, Japan. Black and white circles show the areas where *I. persulcatus* ticks were PCR positive and negative, respectively, for *B. divergens* or *B. venatorum*. The areas where the ticks were collected during this study are underlined.

(Asia lineage) at a low copy number. Amplification of the target gene occurred in all samples whether or not excess tick DNA was present (not shown).

Detection of *B. divergens* and *B. venatorum* in *Ixodes persulcatus*. The ticks collected at the 7 areas—Kamishihoro, Akkeshi, Obihiro, Kushiro, Kiyosato, Esashi and Wakkanai—in Hokkaido (Fig. 2, Table 1) were all adults. The ticks were comprised of 3 species, *Ixodes persulcatus*, *I. ovatus*, and *Haemaphysalis* spp. Almost all collected ticks, except for *I. persulcatus* females, were processed for PCR examination. Broad-spectrum PCR targeting the 18S rRNA genes of *B. divergens* and *B. venatorum* (Fig. 1) (21) revealed that 11 of 822 examined *I. persulcatus* males were positive, while all DNA samples from *I. ovatus* ($n = 595$) and *Haemaphysalis* spp. ($n = 163$) were negative (Table 1, "Universal"). Specific PCR, which discriminates *B. divergens* (EU/U.S. and Asia lineages) and *B. venatorum*, revealed that the 11 positive *I. persulcatus* males carried *B. divergens* Asia lineage DNA (Table 1). Infection rates calculated from *I. persulcatus* males in four areas with positive results ranged from 0.5% (1/180, Kamishihoro) to 7.6% (1/13, Wakkanai). All positive samples examined by PCR using the dvJA primer were confirmed by sequencing the 18S rRNA, β-tubulin, and *CCT7* genes to be of the *B. divergens* Asia lineage (see Table 3).

To investigate additional ticks collected in other areas in Hokkaido (Fig. 2), archived DNA samples from *I. persulcatus* (previously tested for *B. microti* DNA; 28) were screened. By the broad-spectrum piroplasm PCR, 6 samples were found to be positive for *Babesia* DNA (Table 2). The specific nested PCR revealed that 5 samples and 1 sample were positive for *B. divergens* Asia lineage and *B. venatorum* (designated Etb5), respectively.

In all, *I. persulcatus* carrying *B. divergens* Asia lineage was detected as widely distributed across Hokkaido in 8 of the 14 sites examined (Fig. 2).

Genetic isolation of *B. divergens* Asia lineage from salivary glands of *I. persulcatus*. To investigate whether *I. persulcatus* was competent for transstadial transmission of *B. divergens*, we attempted to genetically isolate mature sporozoites, which are produced in the salivary glands when the ticks feed. A total of 98 salivary glands from *I. persulcatus* females collected in Kamishihoro, Akkeshi, and Obihiro were examined after the ticks fed on gerbils (Table 1, "Adult female"). The specific PCR on DNAs extracted from the salivary glands revealed that 1 tick from each area carried *B. divergens* Asia lineage, i.e., IpSG13-13-1 (Kamishihoro), IpSG10 (Akkeshi), and IpSG14-12-2 (Obihiro).

***B. divergens* 18S rRNA, β-tubulin, and *CCT7* gene sequences from *I. persulcatus*.** Partial 18S rRNA, β-tubulin, and *CCT7* gene sequences of the *B. divergens* detected in *I. persulcatus* males (whole body) and females (salivary glands) were successfully amplified and sequenced. Comparison of the partial 18S rRNA genes (1,567 bp) of the isolates

TABLE 1 Detection of *Babesia divergens* and *B. venatorum* in field-collected *Ixodes persulcatus* ticks

Area	*I. persulcatus*												*I. ovatus* adult			*Haemaphysalis* sp. adult		
	Adult male (whole body)						Adult female (salivary glands)											
			No. of positive PCR results for:						No. of positive PCR results for:									
	No. collected	No. examined	Universal	*B. divergens* Asia	*B. divergens* EU/U.S.	*B. venatorum*	No. collected	No. examined	Universal	*B. divergens* Asia	*B. divergens* EU/U.S.	*B. venatorum*	No. collected	No. examined	No. of positive PCR results, universal	No. collected	No. examined	No. of positive PCR results, universal
Kamishihoro[a]	184	180	1	1	0	0	166	31	1[b]	1[b]	0	0	78	78	0	1	1	0
Akkeshi[a]	335	334	7	7	0	0	321	34	1[b]	1[b]	0	0	309	309	0	142	142	0
Obihiro[a]	162	162	1	1	0	0	120	33	1[b,c]	1[b,c]	0	0	146	146	0	3	3	0
Kushiro	68	68	1	1	0	0	69	ND[d]	ND	ND	ND	ND	38	38	0	10	10	0
Kiyosato	49	49	0	0	0	0	47	ND	ND	ND	ND	ND	24	24	0	7	7	0
Esashi	18	16	0	0	0	0	14	ND	ND	ND	ND	ND	0	0	0	0	0	0
Wakkanai	13	13	1	1	0	0	16	ND	ND	ND	ND	ND	0	0	0	0	0	0
Total	829	822	11	11	0	0	753	98	3	3	0	0	595	595	0	163	163	0

[a]Samples from Zamoto-Niikura et al. (2016) (29) were included.
[b]Positive results were confirmed in salivary glands from partially engorged *I. persulcatus*.
[c]*B. microti* U.S. lineage parasites from tick salivary glands were isolated in a hamster.
[d]ND, not done.

TABLE 2 Detection of *B. divergens* and *B. venatorum* in DNA samples from ticks previously collected in additional areas in Hokkaido[a]

Collection area	Tick stage	*n*	Positive PCR result			
			Universal	*B. divergens* lineage		*B. venatorum*
				Asia	EU/U.S.	
Nemuro	Adult	139	1	1	0	0
	Nymph	196	0	0	0	0
Horonobe	Adult	42	2	2	0	0
Kiyosato	Adult	105	1	0	0	1
Shimokawa	Adult	15	0	0	0	0
Aibetsu	Adult	85	2	2	0	0
Furano	Adult	44	0	0	0	0
Hobetsu	Adult	36	0	0	0	0
Ebetsu	Adult	15	0	0	0	0
Total		677	6	5	0	1

[a]PCR was performed on the DNA samples previously prepared in Zamoto-Niikura et al. (2012) (28).

revealed that they were identical to each other, except for single nucleotide substitution at positions 256, 630, and 663 (position numbering based on GenBank reference sequence U16370). The 18S rRNA gene sequences were also identical to those of *B. divergens* in sika deer in Japan (GenBank accession numbers KC465973 to KC465977, AB857845, AB857846, and AB861504 to AB861507 [28]), except for the single-nucleotide polymorphism (SNP) noted above. In a BLAST search (July 2017), the 18S rRNA gene sequence of *B. divergens* isolate Nov-Ip316 in Russia (GenBank accession number GU057385, *I. persulcatus* origin) showed high sequence similarity (99.75%, 1,180/1,183 bp) to sequences from the *B. divergens* in *I. persulcatus* in Japan.

Partial β-tubulin sequences (1,347 bp, intron and exon) of all isolates in this study were identical to each other. Identical sequences were also found in sika deer in Japan (GenBank reference numbers KC465968 to KC465970 and AB861508 to AB861514).

CCT7 sequences (intron and exon) of *B. divergens* in *I. persulcatus* showed high identity, although SNPs (36 base substitutions in 1,647 bp) were observed. Among the 36 substitutions, 29 and 7 SNPs were seen in the exon and intron, respectively. All substitutions in the exon were in the third nucleotide position of the triplet genetic code, except for one substitution which occurred in the first position of the codon (position 669 in GenBank reference sequence AB367925). As a result, the *CCT7* amino acid sequences of the isolates were identical except for one amino acid residue at position 190 (serine or glycine).

18S rRNA and β-tubulin sequences of *B. venatorum* Etb5. By nested PCR, approximately 1,600 bp of 18S rRNA and 1,200 bp of β-tubulin gene sequences of *B. venatorum* Etb5 were successfully amplified and directly sequenced. A BLAST search of the partial 18S rRNA gene sequence (1,588 bp) revealed a similarity of 99.7% (1,584/1,588 bp) to those of *B. venatorum* (formerly *Babesia* sp. EU1) from a human (GenBank accession number AY046575), deer (GQ888709), and *I. ricinus* ticks (HM113372 and AY553915) in Europe and to *B. venatorum* from a human (KF724377) in China and *I. persulcatus* (LC005775) in Mongolia. A BLAST search of the Etb5 β-tubulin sequence (1,237 bp) identified sequences of *B. odocoilei* (GenBank accession number KC465972; 91%, 1,006/1,105 bp) and *B. divergens* (KC465967; 90%, 980/1,086 bp) as having the highest similarity.

Phylogenetic analysis based on 18S rRNA gene sequences. A neighbor-joining phylogenetic tree based on the 18S rRNA gene sequences (1,123 bp) obtained herein and those of related parasites available in GenBank was constructed (Fig. 3). The phylogenetic tree contained two large clades. One included *B. venatorum* Etb5, detected in this study, and *B. venatorum* (formerly *Babesia* sp. EU1) from Eurasian countries (*B. venatorum* group), and another clade (*B. divergens/B. capreoli* group) included *B. divergens*, *B. capreoli*, and *Babesia* sp. MO1. The newly identified *B. divergens*

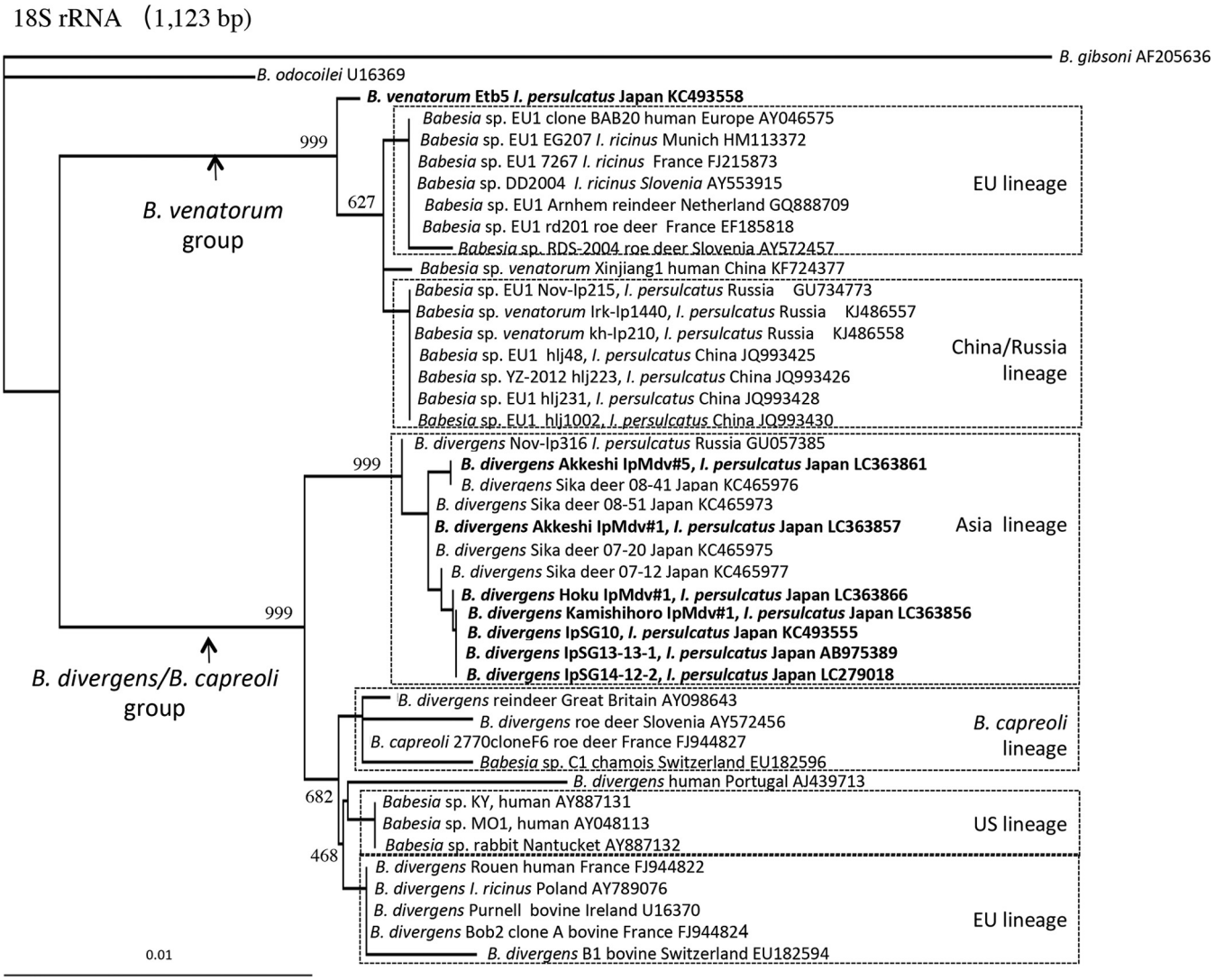

FIG 3 Neighbor-joining phylogenetic tree based on 18S rRNA gene sequences. Names in bold indicate parasites isolated in this study. The number on each branch shows the occurrence in 1,000 bootstrap replicates.

parasites from *I. persulcatus* adults in Japan fell into the *B. divergens* Asia lineage cluster and were closely related to isolates from sika deer in Japan (Fig. 3).

The *B. venatorum* clade consisted of 2 or more lineages correlating with geographical origin, namely, EU and China/Russia lineages (Fig. 3). The EU lineage included *B. venatorum* (formerly *Babesia* sp. EU1) from the first index patient (GenBank accession number AY046575), *I. ricinus* ticks, and deer in Europe. The China/Russia lineage included *B. venatorum* from *I. persulcatus* in Russia and China. The newly identified *B. venatorum* Etb5 formed a distinct lineage from the EU and China/Russia lineages, with a high bootstrap value.

Phylogenetic analysis based on β-tubulin gene sequences. To further investigate the phylogenetic placement of the *Babesia* parasites found in *I. persulcatus* in this study, a neighbor-joining phylogenetic tree was constructed based on the β-tubulin gene sequences (Fig. 4). The phylogenetic trees of the intron and exon sequences both showed similar topologies, with high bootstrap values compared to those in the tree based on the 18S rRNA gene (Fig. 4). A distinct branch comprising *B. divergens* and *Babesia* sp. MO1 (*B. divergens/B. capreoli* group) β-tubulin separated into 3 lineages that reflect the geographic distribution of the parasites (United States, Europe, and Japan).

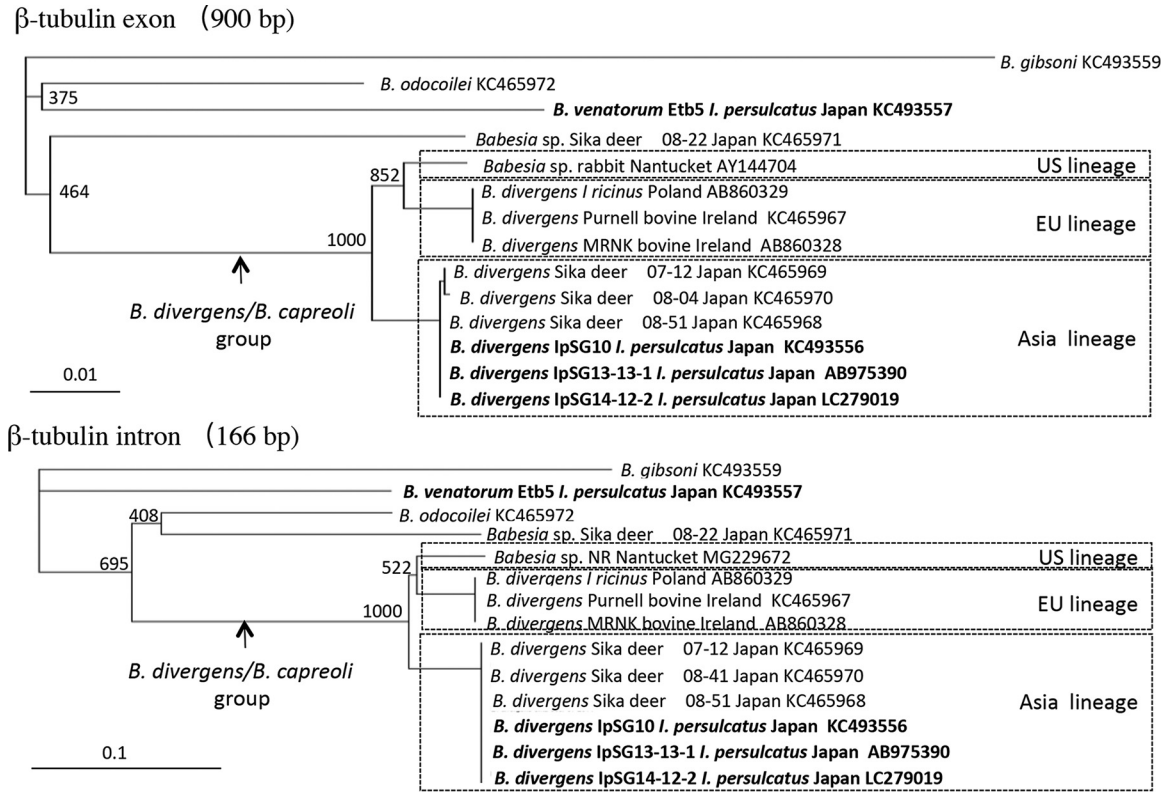

FIG 4 Neighbor-joining phylogenetic tree based on β-tubulin gene sequences. Names in bold indicate parasites isolated in this study. The number on each branch shows the occurrence in 1,000 bootstrap replicates.

Comparing the lengths of the distance bar and bootstrap values in the β-tubulin tree to those for the 18S rRNA gene, these lineages appear distinct.

Isolation of *B. microti* U.S. lineage IpSG14-12-2. Salivary glands containing *B. divergens* IpSG14-12-2 were positive for *B. microti* U.S. lineage when examined by PCR (Fig. 5), and therefore a portion of the homogenate was inoculated into a naive hamster. Ten days after inoculation, parasites were observed in hamster erythrocytes in a stained blood film under microscopy (Fig. 6). The parasite was subsequently isolated (*B. microti* strain IpSG14-12-2) and confirmed to be genetically of *B. microti* U.S. lineage by sequencing the *CCT7* gene (LC333115).

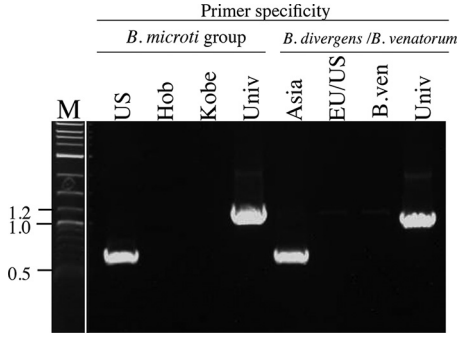

FIG 5 IpSG14-12-2, isolated from salivary glands of an *I. persulcatus* tick, contained both *B. microti* and *B. divergens* DNA, detected by type-specific PCR targeting 18S rRNA genes of *B. microti*, *B. divergens*, and *B. venatorum*. Specific primers for U.S., Hobetsu, and Kobe lineages within the *B. microti* group and universal primers for all lineages were used (lanes US, Hob, Kobe and Univ, respectively). Specific primers for Asia and EU/U.S. lineages of *B. divergens* and *B. venatorum* within the *B. divergens*/*B. venatorum* group and universal primers were used (lanes: Asia, EU/US, B.ven and Univ, respectively). M, marker.

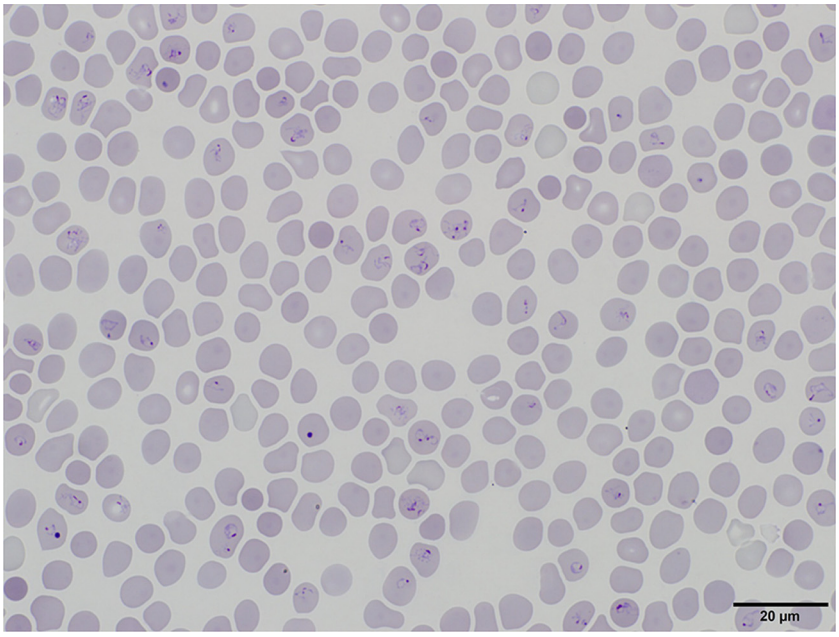

FIG 6 Intraerythrocytic *B. microti* U.S. lineage parasites (IpSG14-12-2) isolated in a hamster from infected *I. persulcatus* salivary glands in Japan. Giemsa-stained blood film. Bar, 20 μm.

Genetic stability of the specific primer region of the 18S rRNA gene. Since specific primers were designed from a variable region of the 18S rRNA gene, we examined genetic stability by comparing this region in sequences available in GenBank. In the region where dvEU and dvJA primers were derived, 1 and 2 SNPs, respectively, were found (Fig. 7A). Guanine replaced an adenine at position 19 of dvEU and at position 15 in dvJA. Thymidine substituted for adenine at position 18 in dvJA. The latter SNP was also found in some of the *B. divergens* Asia lineage sequences determined in this study from *I. persulcatus* (Fig. 7B).

DISCUSSION

We describe herein *I. persulcatus* in Japan carrying parasites of the *B. divergens* Asia lineage, which was recently identified from sika deer in this country (21). By using a specific PCR system established in this study (Fig. 1), *I. persulcatus* ticks carrying the parasite were identified as widely distributed over the north, central, and eastern parts of Hokkaido, while samples from *I. ovatus*, a sympatric and abundant tick, were all negative for this parasite (Fig. 2, Tables 1 and 2). The 18S rRNA and β-tubulin gene sequences of the *B. divergens* Asia lineage isolated from salivary glands of *I. persulcatus* were identical to those found in sika deer blood (Fig. 3 and 4), suggesting that the parasite in Japan, and perhaps also throughout the temperate zones of the eastern Eurasian continent, is maintained in an enzootic cycle involving *I. persulcatus* ticks and sika deer.

We modified a previously established PCR system (Fig. 1) to specifically detect and easily classify the 18S rRNA gene sequences of *B. divergens* Asia and EU/U.S. lineages and *B. venatorum*. In Hokkaido these *Babesia* species, as well as *B. microti* U.S. and Hobetsu lineages, are carried in ixodid ticks (28, 29). Therefore, a specific PCR system was crucial in this study for detecting *B. divergens* Asia lineage in ticks without the need for sequencing. Furthermore, this system will be greatly advantageous for large-scale epidemiological surveys for *B. divergens*, especially in investigating sika deer, because *Theileria* infection is also abundant in this host (30, 31). In most cases *B. divergens* infection in sika deer is found as a coinfection with *Theileria*, with the *Theileria* parasitemia generally much higher. The massive *Theileria* infection may consequently mask the *B. divergens* infection so that PCR using broad primers designed for piroplasm DNA often fails to detect the more limited *B. divergens* sequences (unpublished data).

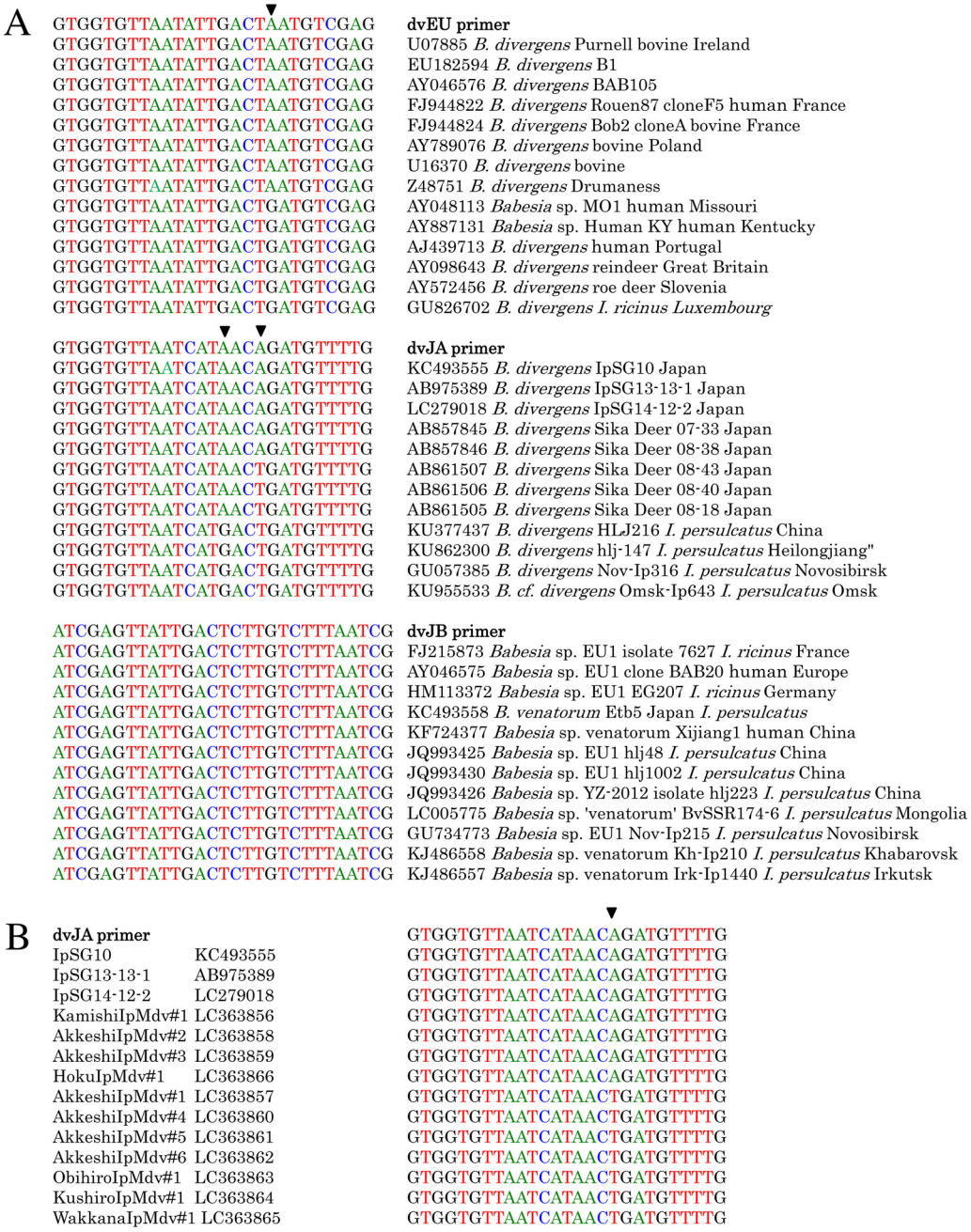

FIG 7 SNPs in the 18S rRNA gene region where specific primers were designed. (A) Corresponding regions of *B. divergens* and *B. venatorum* available in GenBank. (B) Corresponding region of the *B. divergens* Asia lineage detected in *I. persulcatus*, Japan.

I. persulcatus carrying *B. divergens* Asia lineage was proven to be spread over north, central, and eastern parts of Hokkaido (8/14 areas; Fig. 2 and Tables 1 and 2). This tick species is also distributed broadly over the eastern half of Japan, where sika deer carrying the lineage are frequently found (21), suggesting that *I. persulcatus* is the main vector for this parasite in this country. Rar et al. (25) reported that *I. persulcatus* collected in the Novosibirsk region, Russia, also carried *B. divergens* (Nov-Ip316). In our analysis, the 18S rRNA gene sequence of the Russian isolate Nov-Ip316 (GenBank accession number GU057385) was nearly identical to that of *B. divergens* from *I. persulcatus* in Japan (except for 3 out of 1,183 bases), and it consequently fell into the Asia lineage clade in the phylogenetic tree (Fig. 3). Thus, it is very likely that the Asia

lineage of *B. divergens* is transmitted primarily by *I. persulcatus* throughout the temperate zones of the eastern Eurasian continent where *I. persulcatus* is commonly distributed.

Evidence showing an association of *B. divergens* infection with ticks that bite people raises a concern about the emergence of human babesiosis in the region. Notably, Qi et al. reported *B. divergens* infection in anemic patients in China (Shandong province) examined by PCR and sequencing (32). Although we were unable to include the Chinese *B. divergens* isolate in our genetic analysis due to its short reported sequence, nonetheless this information suggests that *B. divergens* in Asia may be infectious to humans and cause mild illness.

The phylogenetic tree based on the 18S rRNA gene sequences revealed that *B. divergens* is a complex composed of four lineages: the Asia, EU, and U.S. lineages of *B. divergens*, and one minor assemblage (Fig. 3 and 4), which corresponds to a previous study (21). The minor assemblage (*B. capreoli* lineage) includes parasites of different names, such as *B. capreoli*, *Babesia* sp., and *B. divergens*. The phylogenetic tree based on the β-tubulin (exon and intron) gene sequences also supports the separation of *B. divergens* into three geographic lineages, U.S., EU, and Asia.

The various lineages are related to different vector tick species. The EU lineage of *B. divergens*, a main causative agent of human babesiosis in Europe, is transmitted primarily by *I. ricinus* ticks. In North America, *I. dentatus* has been reported to carry the U.S. lineage of *B. divergens* (22). For the Asia lineage of *B. divergens*, *I. persulcatus* is found to be the principal vector (Tables 1 and 2). All of these tick species, including *I. persulcatus*, are genetically very closely related (*I. ricinus* species complex) and are found across the temperate zones of the Northern Hemisphere with distinct geographic distributions (33). We speculate that an ancestor of the *I. ricinus* complex was infected with *B. divergens* and expanded its habitat. As a result of recent coevolution between the parasites and vector ticks, we observe that each of the three lineages of *B. divergens* is primarily vectored by regional ticks belonging to the *I. ricinus* species complex. A similar coevolutionary perspective has been described to explain the close relationship between the three sublineages in the *B. microti* phylogroup (34, 35). The Europe-Central Asia, East Asia, and North American *B. microti* sublineages are primarily transmitted by *I. ricinus*, *I. persulcatus* (28), and *I. scapularis* (*I. dammini*) (36), respectively. To elucidate their precise coevolutionary history, transmission studies and analysis of a greater number of samples from the various geographical origins and various tick species are needed.

The speculation of the coevolutionary history described above is also supported by the fact that *B. divergens* Asia lineage is specifically transmitted by *I. persulcatus*. In this survey, the majority of the ticks collected in Hokkaido were *I. persulcatus* and *I. ovatus*, which accounted for about 90% of all ticks collected (Table 1). This collection was advantageous for the purpose of the survey, since these species are the common ticks that feed on sika deer (37). Furthermore, the geographical distributions of both ticks largely overlap in Hokkaido (37–40). Therefore, it is considered likely that these two tick species have an equal opportunity to ingest deer blood infected with *B. divergens*. However, PCR examination revealed that only *I. persulcatus* carried *B. divergens* Asia lineage. None of the *I. ovatus* ticks tested positive, even in the Akkeshi and Obihiro areas where the number of *I. ovatus* ticks examined was comparable to that of *I. persulcatus* (Table 1). These results strongly suggest a species-specific interaction between the tick and parasite in nature. *I. ovatus* (subgenus *Partipalpiger*), an ancient type of ixodid tick, is genetically different from the *I. ricinus* complex (41, 42). We speculate that the ancestral *I. ricinus* complex has been infected recently with *B. divergens*, long after *I. ricinus* evolved and separated from the *I. ovatus* clade.

In this study, we demonstrated that a tick could be coinfected with *B. divergens* and *B. microti*. When *B. divergens*-positive salivary gland homogenate (IpSG14-12-2) from an *I. persulcatus* female that also tested positive for *B. microti* was inoculated into a naive hamster, protozoa emerged in the erythrocytes (Fig. 6). Based on the sequence of the *CCT7* gene, the parasite was genetically identical to the *B. microti* U.S. lineage, which is

maintained between rodents and *I. persulcatus* in Japan (29). Although we did not exclude the possibility that the *I. persulcatus* female ingested both *B. divergens* and *B. microti* at same time as a nymph, the reservoirs for the two piroplasms—sika deer and rodents, respectively—are quite different. Rather, we speculate that a persistently *B. divergens*-infected *I. persulcatus* tick fed on a rodent infected with *B. microti* U.S. lineage as a nymph, since *B. divergens* is reported to survive through all three stages of a tick generation. Donnelly and Peirce (9) demonstrated that all stages of the F1 generation resulting from an infected *I. ricinus* female tick could transmit *B. divergens* to cattle. Furthermore, in some cases the infection persisted until the F2 larval stage. Bonnet et al. (10) also showed that *B. divergens* could persist in *I. ricinus* beyond more than one molt by detecting *B. divergens* DNA in the nymphal stage after feeding on nonparasitized blood as infected larvae. On the other hand, *B. microti* is unable to persist in *I. ricinus* ticks beyond one instar (43).

Another possible mechanism for the mixed infection is that there may be an unknown reservoir(s) which is susceptible to both *B. divergens* and *B. microti*. In the United States, parasites identical to *B. divergens* sp. MO1 (U.S. lineage) were isolated from an unexpected host, the cottontail rabbit (44). In Hokkaido, Japan, lagomorphs, including the pika (*Ochotona hyperborea*) and mountain hare (*Lepus timidus*), are also distributed (45), but they have not been investigated for piroplasm infections. It may be worthwhile to survey such wild animals to elucidate the life cycle of piroplasms, despite the rare opportunity to do so.

B. venatorum, formerly *Babesia* sp. EU1, was first described in 2003 as the agent infecting asplenic patients in Italy and Austria (23). An additional human case was reported in Germany (46). Through epidemiological surveys in Europe (47–49) and successful transmission experiments (50, 51), *I. ricinus* was recognized as a competent vector for *B. venatorum*. In this study, specific PCR and sequencing identified the presence of *B. venatorum* in *I. persulcatus* in Japan. Although only one *B. venatorum*-infected *I. persulcatus* tick was detected in this study, very closely related sequences were also reported from *I. persulcatus* in the Novosirsk region of Russia (GenBank accession number GU734773; 25) and in China (JQ993425, JQ993428, and JQ993430) (Fig. 3), suggesting geographically wide distribution of the parasite over eastern Eurasia where *I. persulcatus* exists.

The genetic regions where the specific 18S rRNA gene primers were designed are variable, and a few SNPs were noted when the corresponding regions of the 18S rRNA genes of various *B. divergens* isolates were aligned with primers dvJA and dvEU. Nevertheless, amplicons were generated from the *Babesia* sp. NR813 strain, whose sequence is identical to those of *Babesia* sp. MO1 and *Babesia* sp. KY, using primer dvEU in the PCR system (Fig. 1C and D). Similarly, 18S rRNA gene sequences of *B. divergens* Asia lineage were newly amplified in this study by nested specific PCR using the dvJA primer, even though some of these possessed a single nucleotide substitution (Fig. 7A and B). These results suggested that single substitution in those positions had little effect, possibly because the SNPs were internal and not at the critical 3′ end. Future studies might include modification of the primers or conditions (e.g., different annealing temperature) to safeguard against possible effects attributable to the primer sequence.

MATERIALS AND METHODS

Field collections. Unfed host-seeking ticks were collected by flagging vegetation alongside trails in forests on Hokkaido Island, where sika deer previously were found to be most prevalently infected with *B. divergens* (33%) in Japan (21). The survey areas in this study (Kamishihoro, Akkeshi, Obihiro, Kushiro, Kiyosato, Esashi, and Wakkanai) (Table 1) are shown in Fig. 2. Species identification was performed by morphological examination under microscopy of the collected ticks, as described by Takada (38) and Ehara (39). In addition, genetic identification (mitochondrial 16S rRNA and cytochrome oxidase gene sequencing) was performed on ticks which were morphologically suspect or positive for *Babesia* spp. We also examined DNA extracted from ticks (either individual or pooled samples from 2 to 5 ticks) that were collected from other areas in Hokkaido in a previous study (28) (Fig. 2, Table 2).

Extraction of DNA from ticks. Ticks were individually crushed with a pestle homogenizer (Scientific Specialties, Inc.) and suspended in 300 μl of TNE buffer (10 mM Tris-HCl, 150 mM NaCl, and 100 mM

EDTA; pH 8.0) containing 0.1% sodium dodecyl sulfate (SDS). The suspensions were digested with 100 μg/ml proteinase K at 55°C overnight. DNAs were purified by phenol extraction followed by ethanol precipitation. To facilitate visualization of the DNA pellets, Glyco blue (Ambion) was used as the carrier. Pellets were resuspended in 50 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA; pH 7.5). The final double-stranded DNA (dsDNA) concentration of the samples was approximately 2×10^3 ng/μl (Qubit; Thermo Fisher Scientific).

Type-specific PCR. To detect and discriminate between *B. divergens* Asia and EU/U.S. lineages (21) and between *B. divergens* and *B. venatorum*, a PCR for specific amplification was developed based on 18S rRNA gene sequences (Fig. 1) and modification of previously described primers (primers dvEU and dvJB are 8 bases longer, respectively, than the primers BDV and BOD described by Duh et al. [52]). The forward primers dvEU (5′-GTGGTGTTAATATTGACTAATGTCGAG-3′; specific for the EU and U.S. lineages within *B. divergens*), dvJA (5′-GTGGTGTTAATCATAACAGATGTTTTG-3′; specific for the Asia lineage in *B. divergens*), and dvJB (5′-ATCGAGTTATTGACTCTTGTCTTTAATCG-3′; specific for *B. venatorum*) were designed and used with reverse primer dv1296R (5′-CGGACGAACCTTTTTACGGACACTAG-3′) (21) (Fig. 1).

Questing ticks carried parasites at levels too low for detection in the first-round PCR, so for the epidemiological study nested specific PCR was performed on the first-round products using primers dv101F (5′-ACAACAGTTATAGTTTCTTTGGTATTCG-3′) and dv1353R (5′-GCCTTAAACTTCCTTGCGGCTTAGAGC-3′) (21), which broadly anneal to the 18S rRNA gene sequence of *B. divergens* and *B. venatorum* DNA (Fig. 1).

Parasites in the activated salivary glands could be detected by conventional PCR.

18S rRNA, β-tubulin, and chaperonin containing TCP1 subunit eta (*CCT7*) gene amplification and sequencing. Nested PCR using the primers dv101F/dv1353R and then dv159F/dv1296R was performed to universally amplify the 18S rRNA gene of *B. divergens* and closely related zoonotic parasites (*B. venatorum*) according to Zamoto-Niikura et al. (21) (Fig. 1). Sequences of β-tubulin and chaperonin containing TCP1 subunit eta (*CCT7*) genes of *B. divergens* were amplified according to Zamoto-Niikura et al. (21).

Each PCR mixture contained 200 μM each of deoxynucleoside triphosphate (dNTP), 0.4 μM of each primer, 1 μl of DNA, and 0.5 U of Ex Taq DNA polymerase (TaKaRa Bio) in 20 μl total volume. Positive controls were purified amplicons of the target sequence, and negative controls contained no DNA. Thermal cycling was carried out in a Mastercycler ep (Eppendorf) with 30 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s, and final extension at 72°C for 5 min. Nucleotide sequences of the 18S rRNA, β-tubulin and *CCT7* gene amplicons were determined directly on the PCR products with the primers used for amplification and additional internal primers for *CCT7*, i.e., BdivCCTSQ1F (5′-TTTACAGGTCCKAGGGGCATGGACAAGC-3′), BdivCCTSQ2F (5′-GCTGAGACGTCACTAAATTCRAAGCTACT-3′), BdivCCTSQ3F (5′-AAGGCCACYGGAGCRTCCATACAGACCAC-3′), and BdivCCTSQ1R (5′-ACAAGCMGYGCGGAAGTACTTAATGATGACTTGTG-3′).

Specificity and sensitivity of the type-specific PCR. Partial 18S rRNA gene sequences from *B. divergens* strain Purnell (53), *Babesia* sp. (*B. divergens*) strain NR813 (44), *B. divergens* IpSG10, *B. microti* U.S. lineage isolated from *I. persulcatus* in Japan (strain IpSG13-1-2) (29), *B. venatorum* strain Etb5, and *Theileria* sp. (GenBank accession number AB012199) from *I. persulcatus* in Japan were amplified by PCR (29) and cloned individually into pCR2.1 (Thermo Fisher Scientific). After sequence confirmation, the concentration of each plasmid was diluted to 1×10^8 copies/μl. The copy number of the plasmid was calculated based on the concentration and length of plasmid using an online copy number calculator (http://cels.uri.edu/gsc/cndna.html).

The specificity of the type-specific primers was examined on all of the plasmids generated above containing 18S rRNA gene inserts for the various piroplasms, using 10^8 copies of the plasmid as the template in a PCR mixture (Fig. 1C). The PCR procedure was as described above. Specificity was further evaluated by PCR using a mixture of 10^8 copies of *B. divergens* strain Purnell plasmid with 10^8 copies of *B. microti*, *B. venatorum* strain Etb5, or *Theileria* sp. plasmid as the template with dvEU and dv1296R primers. PCR was also similarly conducted with the specific primers for *B. divergens* IpSG or *B. venatorum*, using mixtures of two plasmids as the template. Finally, specificity was evaluated by mixing plasmid-carrying *B. divergens* IpSG10 (Asia lineage) at 1×10^1, 1×10^2, and 1×10^3 copies with 6×10^3 ng of tick DNA (3 times as concentrated as normal) for the template in nested PCR and using the dvJA primer in the second PCR.

Serial 10-fold dilutions were prepared in water from the 10^8 copy stocks, and 1 μl of each dilution was used in a PCR mixture to examine the sensitivity of the type-specific PCR for *B. divergens* strain Purnell, *B. divergens* NR813, *B. divergens* IpSG, and *B. venatorum* strain Etb5 (Fig. 1D). The PCR procedure was as described above.

Isolation of *B. divergens* genomic DNA from salivary glands of *I. persulcatus* females. *I. persulcatus* female ticks collected in areas of Akkeshi, Kamishihoro, and Obihiro where *Babesia* is endemic were used for genetic isolation of *B. divergens*. Ticks were fed on gerbils (a noncompetent host) for 4 days to activate quiescent salivary gland sporozoites to multiply. Thereafter, the partially engorged ticks were removed manually. Salivary glands were dissected from ticks individually under a stereomicroscope and homogenized in cold phosphate-buffered saline (PBS) with a tissue grinder (glass wall tissue grinder; Radnoti) (54). DNA extraction from the homogenates and subsequent PCRs were performed according to the methods described above.

Detection and isolation of *B. microti* U.S. lineage from tick salivary glands. Since *I. persulcatus* is demonstrated to be a vector for *B. microti* U.S. lineage in Japan (29), DNA extracted from salivary glands was examined for the presence of this parasite by specific PCR based on the β-tubulin gene sequence

TABLE 3 Accession numbers determined in this study

Species	Strain	Origin	Accession no. for:		
			18S rRNA	β-Tubulin	*CCT7*
B. divergens	IpSG10	*I. persulcatus* female	KC493555	KC493556	AB975388
	IpSG13-13-1	*I. persulcatus* female	AB975389	AB975390	AB975391
	IpSG14-12-2	*I. persulcatus* female	LC279018	LC279019	LC279020
	KamishihoroIpMdv#1	*I. persulcatus* male	LC363856	LC363867	LC363878
	AkkeshiIpMdv#1	*I. persulcatus* male	LC363857	LC363868	LC363879
	AkkeshiIpMdv#2	*I. persulcatus* male	LC363858	LC363869	LC363880
	AkkeshiIpMdv#3	*I. persulcatus* male	LC363859	LC363870	LC363881
	AkkeshiIpMdv#4	*I. persulcatus* male	LC363860	LC363871	LC363882
	AkkeshiIpMdv#5	*I. persulcatus* male	LC363861	LC363872	LC363883
	AkkeshiIpMdv#6	*I. persulcatus* male	LC363862	LC363873	LC363884
	ObihiroIpMdv#1	*I. persulcatus* male	LC363863	LC363874	LC363885
	KushiroIpMdv#1	*I. persulcatus* male	LC363864	LC363875	LC363886
	WakkanaIpMdv#1	*I. persulcatus* male	LC363865	LC363876	LC363887
	HokuIpMdv#1	*I. persulcatus* male	LC363866	LC363877	LC363888
	NR Halpha1	Eastern cottontail rabbit	ND[a]	MG229672	MG229673
B. venatorum	Etb5	*I. persulcatus* male	KC493558	KC493557	ND
B. gibsoni		Canine	ND	KC493559	ND
B. microti	IpSG14-12-2	*I. persulcatus* female	ND	ND	LC333115

[a]ND, no data.

as previously described (55). Salivary gland homogenates testing positive for *B. microti* were inoculated into hamsters as previously described (29).

Phylogenetic analysis. Partial 18S rRNA (1,123 bp) and β-tubulin (exon, 900 bp; intron, 166 bp) gene sequences of *B. divergens* from salivary glands of *I. persulcatus* females (IpSG10, IpSG13-13-1, and IpSG14-12-2) and *B. venatorum* (Etb5) from an *I. persulcatus* male were aligned with closely related sequences available from GenBank. 18S rRNA gene sequences of *B. divergens* from *I. persulcatus* males were also included (the sequences identical to the ones used were not included). Phylogenetic trees of the β-tubulin gene were constructed separately, based on the exon and intron sequences of the gene. Accession numbers are shown in the phylogenetic trees (Fig. 3 and 4). *Babesia gibsoni* was used as the outgroup. The multiple sequence alignments and construction of the phylogenetic trees using the neighbor-joining method were done by ClustalW (56). Consensus phylogenetic trees were built from 1,000 bootstrap repetitions.

Genetic stability of the 18S rRNA variable region gene sequences used in primer design. Specific species primers were designed from the 18S rRNA gene sequence spanning a variable region (Fig. 1A) for *B. venatorum* and the various *B. divergens* strains. Corresponding sequences of related isolates from geographically distant countries (or regions) of origin were downloaded from GenBank, aligned and compared (Fig. 7).

Laboratory animals. A specific-pathogen-free (SPF) gerbil 30 weeks of age (MON/JmsGbsSlc, retired) and Syrian hamster (slc:syrian) 3 weeks of age were purchased from Japan SLC, Inc.

Reference strains. For sequence comparison, genomic DNA from *B. divergens* strain MRNK (21), *B. divergens* from *I. ricinus* collected in Poland (21), and *Babesia* sp. strain NR (44) was used.

Accession number(s). Sequences determined in this study were deposited in the International Nucleotide Sequence Database (INSD) (GenBank or DDBJ) under the accession numbers listed in Table 3.

ACKNOWLEDGMENTS

We thank Haruyuki Hirata (Rakuno-Gakuen University) for his assistance. The research was supported by AMED under grant number JP17fk0108217.

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