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Dual Presence of *Anaplasma phagocytophilum* and Its Closely Related *Anaplasma* sp. in Ixodid Ticks in Hokkaido, Japan, and Their Specific Molecular Detection

Adrian Patalinghug YBAÑEZ[1,2], Kotaro MATSUMOTO[1], Toshio KISHIMOTO[3], Naoaki YOKOYAMA[4] and Hisashi INOKUMA[1,2]*

[1]*Department of Veterinary Clinical Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan*
[2]*United Graduate School of Veterinary Sciences, Gifu University, Gifu 501–1193, Japan*
[3]*Okayama Prefectural Institute for Environmental Science and Public Health, Okayama 701–0298, Japan*
[4]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan*

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ABSTRACT. *Anaplasma phagocytophilum* causes human granulocytic anaplasmosis (HGA) and tick-borne fever in ruminants. A closely related and potentially novel *Anaplasma* sp. in Japan was recently characterized. The aims of the study were to provide molecular evidence for the presence of these 2 species in Japan, and to develop a reliable PCR method based on the nucleotide differences within the citrate synthase (*gltA*) gene. DNA samples from 182 ixodid ticks (134 *Ixodes persulcatus*, 35 *Haemaphysalis douglasii* and 13 *I. ovatus*) collected from 2 sites in Hokkaido, Japan, were screened for *A. phagocytophilum* and its closely related *Anaplasma* sp. (herein designated as *Anaplasma* sp. Japan) using 16S rRNA PCR, revealing a combined prevalence rate of 27.5% (50 samples). The positive samples were then used to evaluate a newly developed *gltA*-based nested PCR method. Selected positive samples were further characterized using the *groEL* gene for confirmation and phylogenetic analyses. Two groups of sequence results were obtained: those that had closer identities with (1) *A. phagocytophilum* (99.5–99.6% for 16S rRNA, 97.5% for *gltA* and 98.4% for *groEL*), and those that had closer identities with (2) *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan (99.3% for 16S rRNA, 96.4–98.7% for *gltA* and 97.5–97.9% for *groEL*). The present study confirmed the distinct presence of *A. phagocytophilum* and its closely related *Anaplasma* sp. in Japan, and developed a new PCR detection method based on *gltA* that can distinguish the 2 organisms.
KEY WORDS: 16S rRNA, *Anaplasma phagocytophilum, Anaplasma* sp., *gltA, groEL*.

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Anaplasma phagocytophilum is the designated name replacing 3 species of granulocytic bacteria: *Ehrlichia phagocytophila, E. equi* and the agent of human granulocytic ehrlichiosis [36]. It is also the agent of pasture fever or tick-borne fever of ruminants [32]. It infects humans and horses in the US and Europe and is also detected in some parts of the Middle East and Asia [26, 41]. Among the possible wildlife reservoirs of *A. phagocytophilum* are the sika deer (*Cervus nippon yesoensis*) [16], white-tailed deer (*Odocoileus virginianus*) [22], white-footed mouse (*Peromyscus leucopus*) [22], and redwood chipmunk (*Tamias ochrogenys*) [9].

The potential tick vectors of *A. phagocytophilum* vary in each region and include *Ixodes ricinus* in Iran [3], Turkey [1], France [8] and *several European countries* [4], *I. persulcatus* in China [5], *Haemaphysalis longicornis* and *I. persulcatus* in Korea [18], *I. persulcatus* and *I. ricinus* in Russia [19, 21, 31], *I. scapularis*, *Dermacentor variabilis* and *I. pacificus* in the U.S.A. [11, 12, 35], *Hyalomma marginatum*, *Rhipicephalus turanicus* and *Boophilus kohlsi* in Israel [17], and *D. marginatus, I. ricinus, R. bursa, H. punctata* and *H. concinna* in Spain [2, 23].

In Japan, the potential tick vectors include *I. persulcatus, I. ovatus* and *H. megaspinosa* [25, 39]. Molecular detection and characterization (based on P44/MSP2 gene) of *A. phagocytophilum* strains from ixodid ticks in Japan revealed closer identities with the strains found in other countries [25, 37]. However, lower sequence identities were observed on 16S rRNA characterization [38].

The distinct phylogenetic position of an *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan (herein designated as *Anaplasma* sp. Japan) was established based on multigene analyses using 16S rRNA, *groEL* and *gltA* genes [38]. Many studies using 16S rRNA-based PCR in detecting this species may have previously considered it as *A. phagocytophilum* despite sequence and phylogenetic analyses suggesting a phylogenetic divergence. Moreover, the PCR assay used in these previous studies appears to detect both organisms because *Anaplasma* sp. Japan was detected while using *A. phagocytophilum* DNA as a positive control [15, 16, 39]. However, there are no current studies that clarify the distinct presence of both organisms in Japan.

In the present study, DNA samples from ixodid ticks were analyzed based on the 16S rRNA, *gltA* and *groEL* genes of

*CORRESPONDENCE TO: INOKUMA, H., Department of Clinical Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555 Japan.
e-mail: inokuma@obihiro.ac.jp

Table 1. Oligonucleotide sequences of primers used in this study

Primer Name	Oligonucleotide (5'–3')	Reference
16S rRNA PCR		
EC9	TACCTTGTTACGACTT	Kawahara *et al.*, 2006 [16]
EC12A	TGATCCTGGCTCAGAACGAACG	Kawahara *et al.*, 2006 [16]
SSAP2 f	GCTGAATGTGGGGATAATTTA	Kawahara *et al.*, 2006 [16]
SSAP2 r	ATGGCTGCTTCCTTTCGGTTA	Kawahara *et al.*, 2006 [16]
AP1f	CATGCAAGTCGAACGGGTTA	Sashika *et al.*, 2006 [29]
AP1r	CATCAACACGGAGATAAATTATC	Sashika *et al.*, 2006 [29]
gltA species-specific PCR		
AP1SPglF1	ATGB*TAGAAAAR*GCTGTTTTR*GM*GTGT	This study
AP1168SPglR1	TCATACCATTGM*GATR*CCCATCC	This study
APgl151F2	GCTTGCAGATCAGAGATAACTTTCATTGAT	This study
APgl756R2	AGTGGCCACTCCYGCGCACAAACA	This study
APJ10F2	AAK*GCTGTTTTAGCGTGTGGTGATCTT	This study
APJ932R2	ATTTTCGCCCTCGGGTCGTGA	This study
gltA PCR for a longer Sequence		
APSPGL282F1	AACCGCGTTGTGTACGACATTA	Ybañez *et al.*, 2012 [38]
CS7F2	ATGR*TAGAAAAW*GCTGTTTT	Ybañez *et al.*, 2012 [38]
APSPGL1682R1	AAAACCAATATAGCAGCAGCACTCT	Ybañez *et al.*, 2012 [38]
APSPGL1644R2	ATAGCAATGGACATCGTACTAACTGG	Ybañez *et al.*, 2012 [38]
AP1084SPglR2	TCTTAGCR*CTATACCTGAGTAAAAGTC	This study
groESL PCR for a longer Sequence		
EEgro1F	GAGTTCGACGGTAAGAAGTTCA	Chae *et al.*, 2000 [6]
EEgro2R	CAGCGTCGTTCTTACTAGGAAC	Chae *et al.*, 2000 [6]
APGRSP41F3	GAATCTAGCTATGTTGCATGATAATGT	Ybañez *et al.*, 2012 [38]
APSPGR1697R1	CAGCATAAACACGCACTACGAA	Ybañez *et al.*, 2012 [38]
APSPGR234F2	CGTAGTAGGACTTTCCGGTTTTTG	Ybañez *et al.*, 2012 [38]
APSPGR1680R2	TGCACAGCATAAACACGCACTA	Ybañez *et al.*, 2012 [38]

*Degenerate primers: Y=C or T, R= A or G, W=A or T, K=G or T, M=A or C.

A. phagocytophilum and the closely related *Anaplasma* sp. Japan. The dual presence and molecular distinction of both species in Japan was confirmed. Moreover, a highly sensitive and specific PCR based on *gltA* was developed to distinguish these 2 species.

MATERIALS AND METHODS

Samples: A total of 182 ixodid ticks (134 *I. persulcatus*, 35 *H. douglasii* and 13 *I. ovatus*) were collected by flagging in several locations in Eastern Hokkaido, Japan, in 2010 and 2011. The DNA sample was extracted from each tick using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, U.S.A.), eluted with 200 μl of TE buffer, and stored at −30°C until further use. DNA from the following species were also used as controls: 3 strains of *A. phagocytophilum* from humans (Webster strain), horses (supplied by Dr. P. Brouqui, France) and cattle (supplied by Dr. G. Jouncour, France), *Anaplasma* sp. Japan from 10 sika deer [15], *E. canis* (supplied by Dr. S. Harrus, Israel), *E. muris* (strain NA1) [33], *Ehrlichia* sp. from *I. ovatus* (strain HF565; supplied by Dr. H. Fujita, Japan), *A. bovis* [29], *A. centrale* [14], *A. platys* [13], *A. marginale* [27], *Theileria orientalis*, *Babesia bovis* and *B. ovata* [30]. Moreover, plasmid DNAs from 2 of the positive samples were used as templates for the serial dilution PCR assay (herein designated as plasmid-IP11 and plasmid-HD10).

16S rRNA screening PCR and purification methods: Primers EC9 and EC12A were used for primary amplification [16]. Subsequently, the PCR products were used as templates for the species-specific second amplification. Primer sets SSAP2f/SSAP2r [16] and AP-f1/AP-r1 [29] were used to amplify a 641- and 770-bp DNA fragment of *A. phagocytophilum* and *Anaplasma* sp. Japan, respectively. These oligonucleotide primers are listed in Table 1. The positive controls used were DNA extracted from *A. phagocytophilum* (Webster strain) and *Anaplasma* sp. Japan from sika deer. Double distilled water (DDW) was used as negative control. Amplification products were visualized using 1.5% agarose gel after migration, and were purified using either a QIAquick PCR purification kit (QIAGEN) or a QIAquick Gel Extraction Kit (QIAGEN).

Species-specific gltA PCR: A species-specific PCR method was developed based on the *gltA* gene (designated as *gltA* PCR in this study; Fig. 1). The sequences of the oligonucleotide primers used in the present study are indicated in Table 1. Primers for the *gltA* PCR were designed based on several sequences of *A. phagocytophilum* and *Anaplasma* sp. Japan from the GenBank database. The method was initially tested using field samples that were positive with *A. phagocytophilum* (from France) and *Anaplasma* sp. Japan [38]. DNA from samples that tested positive in the earlier 16S rRNA PCR

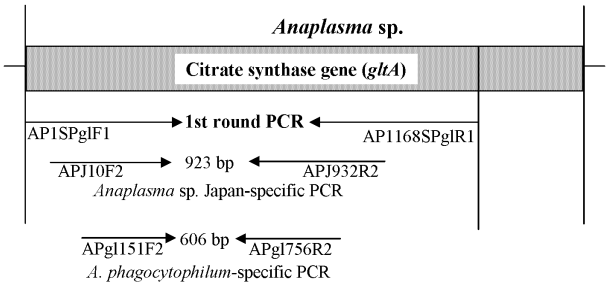

Fig. 1. Strategy of the newly developed PCR based on *gltA*.

were also tested using the *gltA* PCR. In the initial amplification, a final volume of 10 μl was used. It was composed of 4.5 μl DDW, 1 μl of dNTPs (2 mM each), 1 μl 10× PCR buffer, 0.4 μl of $MgCl_2$ (50 mM), 0.5 μl of primer AP1SPglF1 (80 pmol/reaction), 0.5 μl of primer AP1168SPglR1 (40 pmol/reaction), 0.1 μl *Taq* DNA polymerase (5 U/μl) (Invitrogen, Carlsbad, CA, U.S.A.), and 2 μl of DNA template. The cycling conditions used were the following: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec and 72°C for 1.5 min, and final extension at 72°C for 5 min. In the 2nd amplification, a final volume of 25 μl was used. It was composed of 15.35 μl DDW, 2.5 μl of dNTPs (2 mM each), 2.5 μl 10× PCR buffer, 1.0 μl of $MgCl_2$ (50 mM), 1.25 μl of each primer (10–20 pmol/reaction), 0.15 μl *Taq* DNA polymerase (5 U/μl) (Invitrogen), and 1 μl of the 1st PCR amplicon. The *gltA* PCR used the following inner primer sets: APgl151F2/APgl756R2 for *A. phagocytophilum* (606 bp) and APJ10F2/APJ932R2 for the *Anaplasma* sp. Japan (923 bp). Similar cycling conditions as the 1st PCR were used, except for the annealing step, which used a step-down protocol starting from 4 cycles of 74°C, followed by a 2°C incremental decrease for every 2 cycles each, to a final annealing of 68°C for the remaining cycles.

Specificity and sensitivity of gltA PCR: The *gltA* PCR was tested against the controls mentioned in the Samples section above to further evaluate its specificity. To evaluate its sensitivity, standard (using the inner primers) and nested PCR assays using a 10-fold serial dilution of templates were performed. Briefly, amplicons from the longer *gltA* fragments of *A. phagocytophilum* and its closely related *Anaplasma* sp. were ligated into a pCR 2.1 plasmid and subsequently cloned into One Shot Top10 chemically competent *Escherichia coli* using a TOPO TA Cloning kit (Invitrogen). Transformed *E. coli* were grown overnight in a shaker at 37°C in Luria Bertani (LB) broth supplemented with 1 μl/m*l* of 50 mg/m*l* ampicillin (Wako Pure Chemical Industries, Osaka, Japan). Plasmids were extracted and purified from pelleted *E. coli* using a QIAprep Spin Miniprep Kit (Qiagen), and eluted with 50 μl of purified DDW. The initial DNA concentrations for plasmid-IP11 (192.5 *n*g/μl) and plasmid-HD10 (230.7 *n*g/μl) were measured using the Thermo Scientific NanoDrop 2000. Using the initial concentration values, the number of plasmid DNA copies/μl was computed using an online program hosted by the University of Rhode Island Genomics and Sequencing Center (http://www.uri.edu/research/gsc/resources/cndna.html). Concentrations were then diluted using DDW to a starting concentration of 1 *n*g/μl (approximately 2×10^8 copies/μl), and 10-fold serially diluted until 10^{-8} for use as templates for the PCR assay.

PCR for longer gltA and groEL sequences for phylogenetic analyses: For a longer *gltA* sequence, the inner primer AP1084SPglR2 was designed to produce a 1,084 bp fragment. Amplification cycling conditions were similar to those for the *gltA* PCR, except for the annealing step of the 2nd amplification in which 54°C was used. For *groEL* amplification of *A. phagocytophilum*, APGRSP41F3/EEGRO2R and EEGRO1F/EEGRO2R were used as outer and inner primer pairs, respectively. The cycling conditions used were similar to those of previously published methods [6, 38]. On the other hand, *groEL* amplification of *Anaplasma* sp. Japan was performed using a published method [38].

Sequencing and analyses: Direct sequencing method was performed using the same PCR inner primers. In cases where the obtained sequence result was of low quality, the

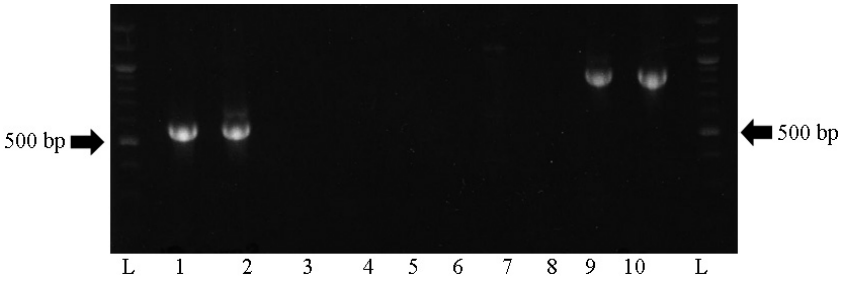

Fig. 2. Specificity of the newly developed *gltA*-based PCR assay. Lanes 1–5 used the specific 2nd round PCR for *A. phagocytophilum*, while lanes 6–10 used the specific 2nd round PCR for the closely related *Anaplasma* sp. Japan. The same 1st amplicons were used for lanes 1 and 6 (*A. phagocytophilum* from *I. persulcatus* no. 56); lanes 2 and 7 (*A. phagocytophilum* from *I. persulcatus* no. 73); lanes 3 and 8 which represent negative control (double distilled water); lanes 4 and 9 (*Anaplasma* sp. Japan from *H. douglasii*); and lanes 5 and 10 (*Anaplasma* sp. Japan from infected sika deer). L represents the DNA ladder.

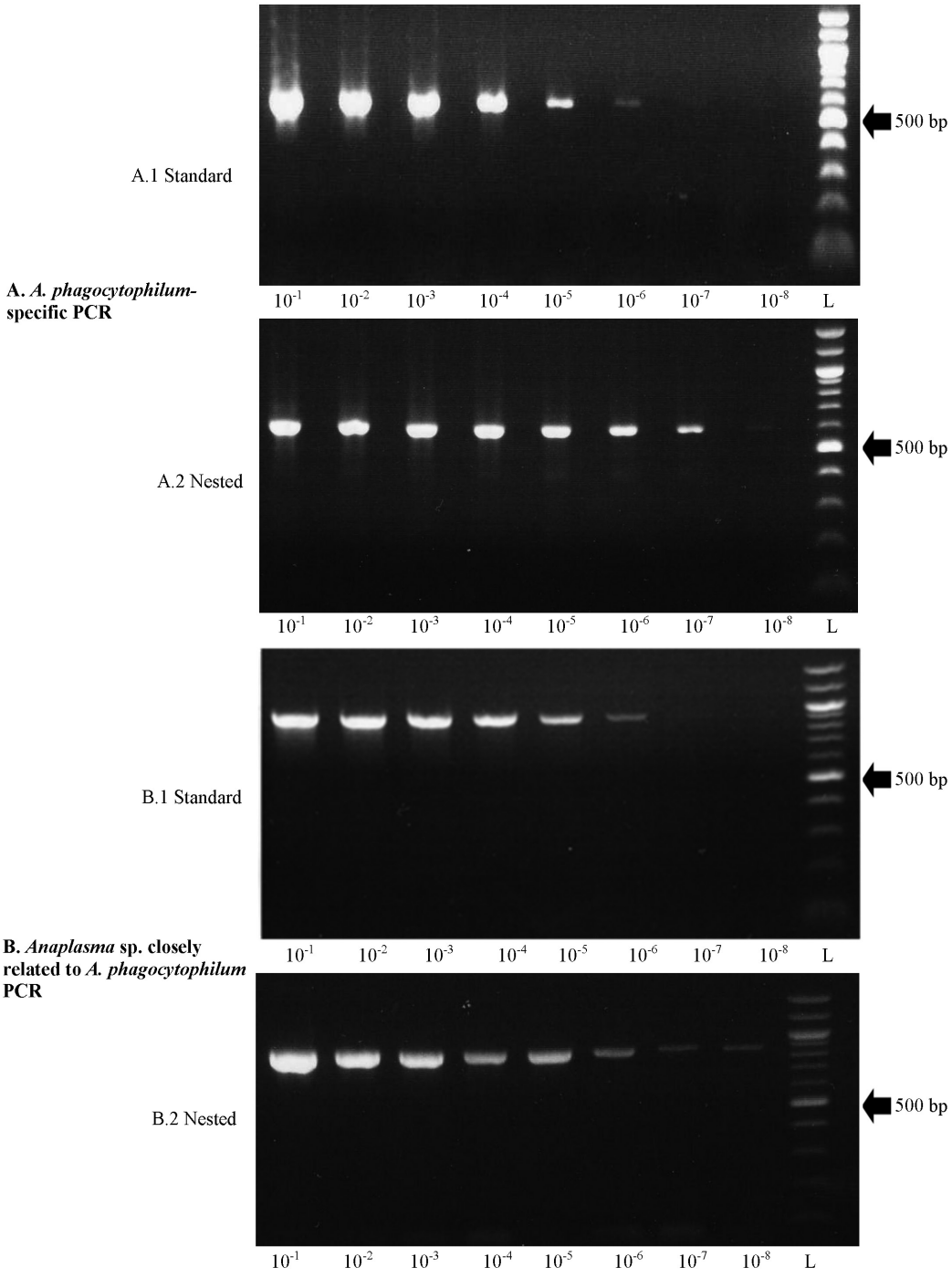

Fig. 3. Sensitivity detection limits of the newly developed *gltA*-based PCR assay by 10-fold serial dilution of plasmid DNA of (A) *A. phagocytophilum* and the closely related (B) *Anaplasma* sp. Japan. Template dilutions are indicated under each lane. L represents DNA ladder.

amplicon was cloned into TOPO TA vector (Invitrogen), and sequenced using the primers provided with the kit. Nucleotide sequence results were initially checked using the BLAST program hosted by the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison with other known sequences. Percent identities were computed using EMBOSS pairwise alignment tool (using the local method) hosted by the European Bioinformatics Institute (http://www. ebi.ac.uk/Tools/emboss/align/index.html). Gaps were not considered in the final

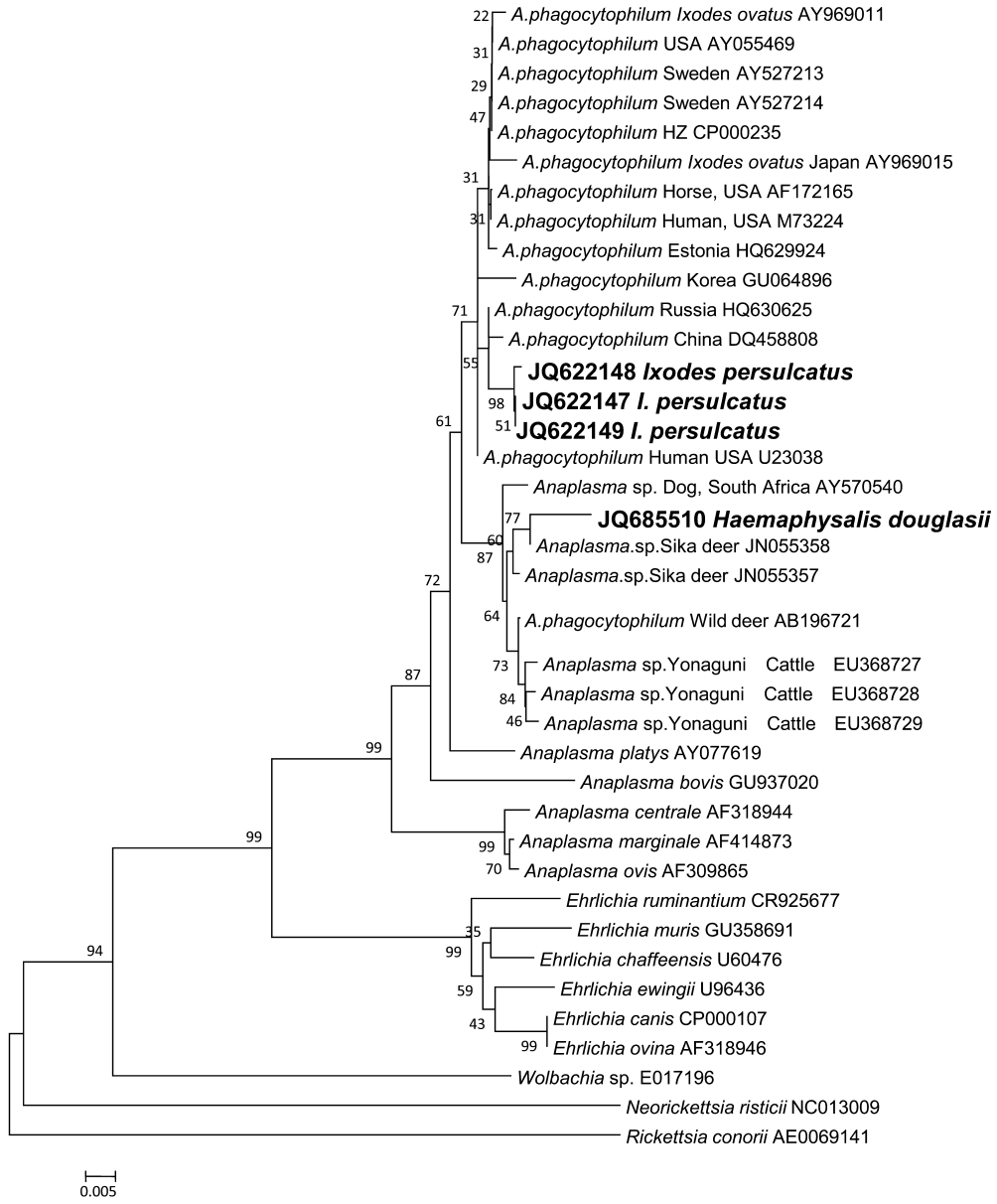

Fig. 4. Phylogenetic relationship of *A. phagocytophilum* and the closely related *Anaplasma* sp. Japan with other Anaplasmataceae organisms based on 16S rRNA. The tree was analyzed using nucleotide sequences by the neighbor-joining method and was supported by 1,000 bootstrap replications. *Rickettsia conorii* was used as an outgroup.

computation of percent identities. The multiple alignment analyses were performed using MUSCLE program [7] with the default parameters (also hosted on the European Bioinformatics Institute website). Prior to phylogenetic analyses, the aligned sequences were tested for suitability with the neighbor-joining (NJ) and maximum likelihood (ML) methods as suggested by Hall [10]. NJ and ML analyses were performed using the MEGA software version 5.05 [34]. The tree stability was estimated by bootstrap analysis for 1,000 replications. Separate analyses using deduced amino acid (AA) sequence characters for *groEL* and *gltA* genes were performed for comparison.

Nucleotide accession numbers: The accession numbers of the nucleotide sequences used for comparison are indicated beside the organism's name (Figs. 4–6). All nucleotide sequences obtained in the present study were registered in GenBank with the following accession numbers: *Anaplasma* sp. from *I. persulcatus* #11 [JQ622143 (*gltA*)], *Anaplasma* sp. from *H. douglasii* #10 [JQ685511 (gltA), JQ685510 (16S rRNA), JQ685509 (groEL)], *A. phagocytophilum*

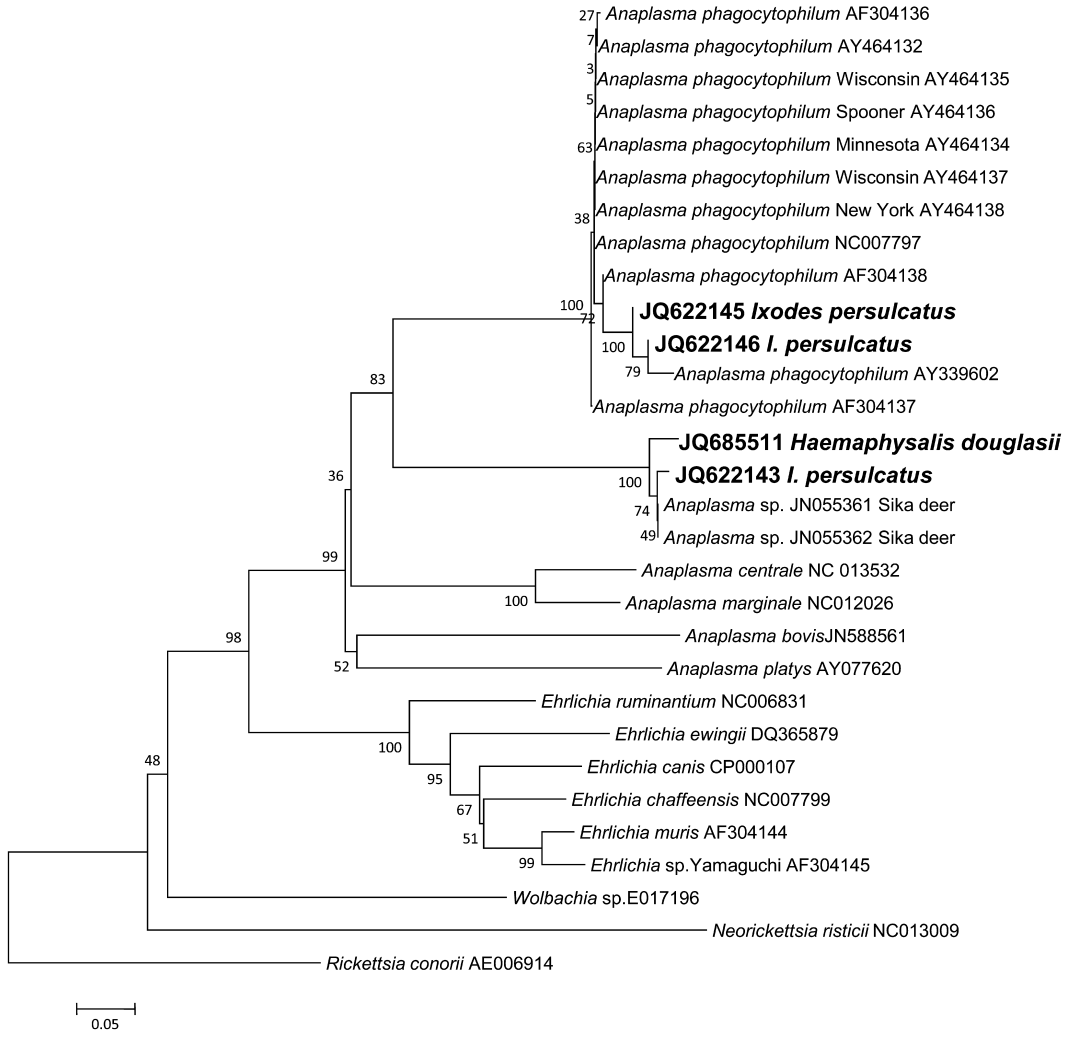

Fig. 5. Phylogenetic relationship of *A. phagocytophilum* and its closely related *Anaplasma* sp. in Japan with other Anaplasmataceae organisms based on *gltA*. The tree was analyzed using nucleotide sequences by the neighbor-joining method and was supported by 1,000 bootstrap replications. *Rickettsia conorii* was used as an outgroup.

from *I. persulcatus* #11 [JQ622145 and JQ622146 (gltA), JQ622147 (16S rRNA), JQ622144 (*groEL*)], and *A. phagocytophilum* from *I. persulcatus* #73 and #56 [JQ622148 and JQ622149 (16S rRNA), respectively].

RESULTS

In the 2nd amplification of the *gltA* PCR, only the targeted *A. phagocytophilum* using APgl151F2/APgl756R2 and *Anaplasma* sp. Japan using APJ10F2/APJ932R2 showed positive bands (Fig. 2). Moreover, control DNAs from other species were found to be negative (figure not shown). The serial dilution PCR assay using plasmid DNA template from *A. phagocytophilum* and *Anaplasma* sp. Japan revealed the same detection limits of approximately 200 copies/PCR for the standard protocol and 2 copies/PCR for the nested protocol (Fig. 3).

The 16S rRNA PCR revealed a prevalence rate of 27.5% (50 samples). The 16S rRNA sequence results of 4 selected samples revealed 2 groups: (1) 3 amplicons (550 bp) from *I. persulcatus* DNA that had closer identities (99.5–6%) with *A. phagocytophilum*, and (2) 1 amplicon (767 bp) from *H. douglasii* that had closer identity with *Anaplasma* sp. Japan. The amplicons from *I. persulcatus* were 100% identical to each other, except for 1 which differed only by 1 bp (99.8%).

Using the *gltA* PCR on the 16S rRNA-positive samples, 32 and 31 samples were found to be positive for *A. phagocytophilum* and *Anaplasma* sp. Japan, respectively. Thirteen samples were found to be dually infected (Table 2). A complete *gltA* sequence (1,236 bp; JQ622143) obtained in 1 clone was 99.4–99.5 and 98.8% identical to *Anaplasma* sp. Japan (JN055361 and JN055362) based on nucleotide and deduced amino acid (AA) sequences, respectively. It was also 68.7 and 66.4% identical to *A. phagocytophilum*

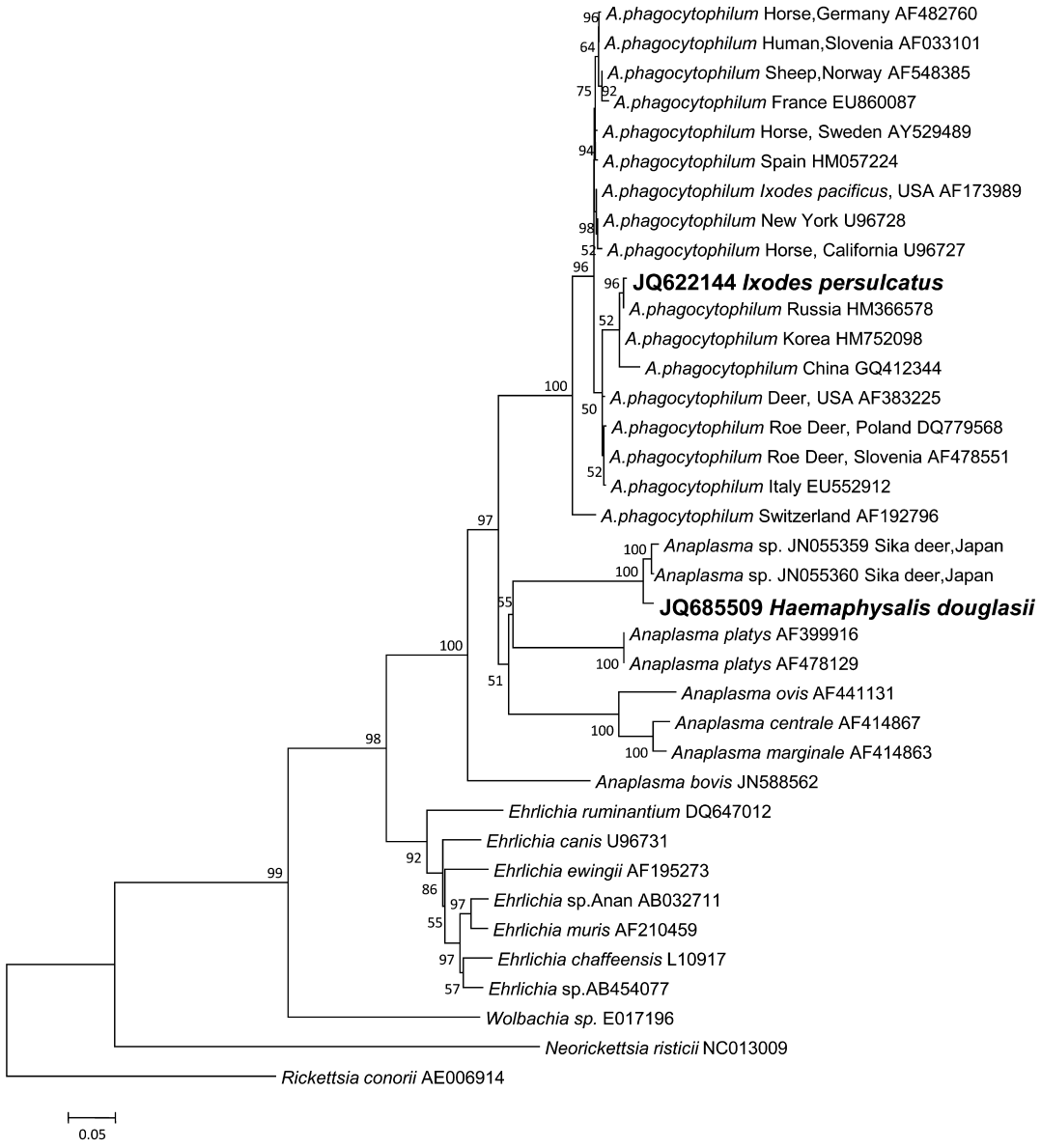

Fig. 6. Phylogenetic relationship of *A. phagocytophilum* and its closely related *Anaplasma* sp. in Japan with other Anaplasmataceae organisms based on *groEL*. The tree was analyzed using nucleotide sequences by the neighbor-joining method and was supported by 1,000 bootstrap replications. *Rickettsia conorii* was used as an outgroup.

(AY464138) based on nucleotide and deduced AA sequences, respectively.

A shorter sequence (918 bp; JQ685511) obtained from *H. douglasii* was 96.4–96.6 and 96.7% identical to *Anaplasma* sp. Japan based on based on nucleotide and deduced AA sequences, respectively. Two other clones from *I. persulcatus* (1,092 bp each; JQ622145, JQ622146) were 99.5% identical to each other, and were 97.5 and 68.3% identical to *A. phagocytophilum* (AF304138) and *Anaplasma* sp. Japan (JN055361, JN055362) based on nucleotide sequences, respectively. Based on deduced AA sequences, the 2 clones revealed 96.7 and 65.4–65.7% identities with *A. phagocytophilum* and *Anaplasma* sp. Japan, respectively. All 3 of the clones were amplified from the same DNA source from *I. persulcatus*.

Using the *groEL* PCR, a 1,600-bp sequence was obtained from *I. persulcatus* (JQ622144), and was 98.4 and 99.4% identical to *A. phagocytophilum* (AF172163) based on nucleotide and deduced AA sequences, respectively. A complete 1,653 bp-*groEL* sequence was also obtained from *H. douglasii* (JQ685509), and was 97.5–97.9 and 98.4–99.1% identical to *Anaplasma* sp. Japan based on nucleotide and deduced AA sequences, respectively.

Phylogenetic analyses from the 16S rRNA, *gltA* and

Table 2. PCR detection results (using *gltA*)

Ticks			PCR result (Positive %)		
Collection site	Species	Tested	*A. phagocytophilum*	*Anaplasma* sp.-Japan	Dual
Sikaoi	*Ixodes persulcatus*	97	23 (12.6%)	11 (6.0%)	11 (6.0%)
Nakaoseobetsu	*I. persulcatus*	37	6 (3.3%)	8 (4.4%)	1 (0.5%)
	Haemaphysalis douglasii	35	3 (1.6%)	6 (3.3%)	1 (0.5%)
	I. ovatus	13	0 (0%)	6 (3.3%)	0 (0%)
Total		182	32 (17.6 %)	31 (17.0%)	13 (7.0%)

groEL genes revealed 2 clusters, one grouped with *A. phagocytophilum*, and another grouped with *Anaplasma* sp. Japan (Figs. 4–6). The branches were supported by moderate to high bootstrap values regardless of whether NJ or ML was used. Similar results were also observed when deduced AA characters were used in the *gltA* and *groEL* phylogenetic trees (figures not shown).

DISCUSSION

The newly developed species-specific PCR method based on *gltA* is highly specific and sensitive in detecting *A. phagocytophilum* and *Anaplasma* sp. Japan. The new method was able to distinguish both species in the 2nd round PCR, and excluded other *Ehrlichia*, *Anaplasma, Theileria* and *Babesia* spp. The use of *gltA* in designing a PCR method appears to be more advantageous because it is highly conserved (found in almost all bacteria) [28]. Moreover, it exhibits higher variation between species than the 16S rRNA [13, 38]. Several studies on *Anaplasma* have been conducted using this gene [13, 20, 38, 40].

The combined prevalence rate of *A. phagocytophilum* and its closely related *Anaplasma* sp. in the present study (27.5% in all ticks, or 26.9% if only *I. persulcatus* is considered) is higher than the previously reported rate of 7.1% in *I. persulcatus* ticks [24]. Prevalence rates of *A. phagocytophilum* in cattle of 1.0–3.4% have also been reported [15, 24]. However, the previous reports used different PCR methods, which may also differ in sensitivities or specificities, leading to varying results. Also, they may not have distinguished *A. phagocytophilum* from the closely related *Anaplasma* sp. Japan, and hence reported both species as one and the same. In the present study, 12 *I. persulcatus* and 1 *H. douglasii* were found to be positive for the 2 organisms. This is the first report of dual detection of *A. phagocytophilum* and the closely related *Anaplasma* sp. Japan in *H. douglasii* in Japan or elsewhere. This suggests that coinfection of both organisms in a tick is possible, and that the closely related *Anaplasma* sp. may share similar potential vectors with *A. phagocytophilum*.

The present study confirmed the dual presence of *A. phagocytophilum* and an *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan. In a related study, samples found to be *Anaplasma*-positive using a 16S rRNA-based PCR revealed negative results using a PCR based on p44/msp2 [24]. Thus, the new reliable PCR method developed in this study can be used to clarify results as well as to conduct further studies to assess the epidemiological status of the 2 organisms in Japan. Humans and animals exposed to pastureland are at constant risk of contact with ixodid ticks, and thus risk of infection with these 2 organisms. While the pathogenesis of *A. phagocytophilum* in humans and animals has been well studied and documented, studies on the closely related *Anaplasma* sp. Japan have been limited. Further studies are recommended because its occurrence in Japan poses a possible public health threat due to its relatedness with the human pathogen, *A. phagocytophilum*.

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