

## Novel Genetic Variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale*, and a Novel *Ehrlichia* sp. in Wild Deer and Ticks on Two Major Islands in Japan

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**Wild deer are one of the important natural reservoir hosts of several species of *Ehrlichia* and *Anaplasma* that cause human ehrlichiosis or anaplasmosis in the United States and Europe. The primary aim of the present study was to determine whether and what species of *Ehrlichia* and *Anaplasma* naturally infect deer in Japan. Blood samples obtained from wild deer on two major Japanese islands, Hokkaido and Honshu, were tested for the presence of *Ehrlichia* and *Anaplasma* by PCR assays and sequencing of the 16S rRNA genes, major outer membrane protein *p44* genes, and *groESL*. DNA representing four species and two genera of *Ehrlichia* and *Anaplasma* was identified in 33 of 126 wild deer (26%). DNA sequence analysis revealed novel strains of *Anaplasma phagocytophilum*, a novel *Ehrlichia* sp., *Anaplasma centrale*, and *Anaplasma bovis* in the blood samples from deer. None of these have been found previously in deer. The new *Ehrlichia* sp., *A. bovis*, and *A. centrale* were also detected in *Hemaphysalis longicornis* ticks from Honshu Island. These results suggest that enzootic cycles of *Ehrlichia* and *Anaplasma* species distinct from those found in the United States or Europe have been established in wild deer and ticks in Japan.**

Human ehrlichioses (also called anaplasmoses), which are emerging potentially fatal infectious diseases, are caused by obligatory intracellular gram-negative bacteria in the family *Anaplasmataceae* (17, 42). Members of this family also cause economically devastating diseases such as heartwater and bovine anaplasmosis in livestock and diseases such as canine ehrlichiosis and Potomac horse fever in companion, military, and police working animals (12, 17, 28, 49). In the family *Anaplasmataceae*, five *Ehrlichia* species and five *Anaplasma* species are officially recognized (17). All of them are maintained in nature through an enzootic cycle between bloodsucking ticks and vertebrate hosts, primarily wild mammals. Vertebrate host infection is essential for them, since these bacteria are rarely transmitted vertically in ticks (7, 34). Seven *Ehrlichia* and *Anaplasma* species are recognized in the continent of North America (*Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Anaplasma phagocytophilum*, *Anaplasma platys*, *Anaplasma marginale*, and *Anaplasma centrale*) (3, 4, 30, 31, 37, 51). Thus far, only *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Anaplasma phagocytophilum* have been known to cause potentially fatal human diseases in the United States (9, 17, 42). In Europe, six *Ehrlichia* and *Anaplasma* species (*A. phagocytophilum*, *E. chaffeensis*, *E. canis*, *A. platys*, *A. centrale*, and *A. marginale*) have been reported (1, 8, 16, 18, 39, 45, 46, 53). However, human infection in Europe was molecularly confirmed only for *A. phagocytophilum* (8), but several serologically confirmed human monocytic and granulocytic ehrlichioses have been reported (8, 15, 57). In Africa,

six species (*Ehrlichia ruminantium*, *E. canis*, *A. marginale*, *Anaplasma bovis*, *A. platys*, and *A. centrale*) have been reported (2, 13, 29, 41, 50). Human ehrlichiosis caused by *Ehrlichia* sp. was serologically detected in Mali (58). In Asia, seven *Ehrlichia* and *Anaplasma* species (*E. canis*, *E. chaffeensis*, *Ehrlichia muris*, *A. marginale*, *A. centrale*, *A. platys*, and *A. phagocytophilum*) were reported (10, 11, 22, 24, 25, 27, 38, 59, 61). Human ehrlichiosis caused by trematode-borne *Neorickettsia sennetsu* in the family *Anaplasmataceae* has been reported in Japan and Malaysia (49), and serologic evidence of human infection with *A. phagocytophilum* and *E. chaffeensis* was reported from Korea (20, 43).

Wild mammals are the primary reservoirs of *Ehrlichia* and *Anaplasma* species infection. In the United States, wild deer are the primary reservoirs of *E. chaffeensis* and *E. ewingii* (3, 31, 60, 63, 64), and small rodents and deer are reservoirs of *A. phagocytophilum* (6, 31, 33, 56). In addition, an *Anaplasma* sp. called the white-tailed deer (WTD) agent (3, 31) infects wild deer. In Europe, *A. phagocytophilum* DNA has been detected in deer and varieties of other wild animal species (14, 40, 45, 47). In Asia, wild mammalian reservoirs have been identified for *Ehrlichia muris* and a newly characterized 'Candidatus Neoehrlichia mikurensis' in small wild rodents (23–25). Although several studies have reported the presence of *A. phagocytophilum*, *E. chaffeensis*, the HF strain closely related to *E. chaffeensis*, and *Anaplasma bovis* in ticks in Asia (10, 11, 27, 44, 52, 62), the natural mammalian reservoir of these species has not been identified in Asia. In the present study, we sought to determine whether and what types of *Ehrlichia* and *Anaplasma* species infect deer in Japan using multiple PCR, followed by sequencing of the PCR products. Here, we present the first evidence of *Ehrlichia* and *Anaplasma* species among naturally

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TABLE 1. Primers

Specificity	Primer name	Sequence	PCR product size (bp)
16S rRNA; all members	EC9	5'-TACCTTGTACGACTT	1,462
	EC12A	5'-TGATCCTGGCTCAGAACGAACG	
16S rRNA <i>E. muris</i>	EM87f	5'-GGTTCGCTATTAGTGGCAGA	517
	EM584r	5'-CAGTATTTAAAAGCCGCTCCA	
16S rRNA <i>Ehrlichia</i> HF strain	HF51f	5'-AAGTCGAACGGACAATTACC	923
	HF954r	5'-GTTAGGGGGATACGACCTTC	
16S rRNA <i>N. mikurensis</i>	IS58-62f	5'-GGAATAGCTGTTAGAAATGACA	488
	IS58-594r	5'-CTATCCTCTCTCGATCTCTAGTTT	
16S rRNA <i>A. phagocytophilum</i>	SSAP2f	5'-GCTGAATGTGGGGATAATTTAT	641
	SSAP2r	5'-ATGGCTGCTTCCTTTCGGTTA	
16S rRNA <i>A. centrale</i>	AC1f	5'-CTGCTTTTAATACTGCAGGACTA	426
	AC1r	5'-ATGCAGCACCTGTGTGAGGT	
16S rRNA <i>A. bovis</i>	AB1f	5'-CTCGTAGCTTGCTATGAGAAC	551
	AB1r	5'-TCTCCCGACTCCAGTCTG	
<i>p44</i>	p3709	5'-GCTAAGGAGTTAGCTTATGA	~400
	p4257	5'-AGAAGATCATAACAAGCATTG	
<i>p44</i>	p3761	5'-CTGCTCTKCCCAARACCTC	~300
	p4183	5'-CAATAGTYTTAGCTAGTAACC	

infected wild deer in Asia. These findings identify four species of *Ehrlichia* and *Anaplasma*, including a novel *Ehrlichia* species and novel *A. phagocytophilum* strains. Analysis of specimens from *Hemaphysalis longicornis* ticks associated with deer revealed the presence of three of these four species, implying the established deer-tick enzootic cycle.

#### MATERIALS AND METHODS

**Deer and tick specimens.** Sera were collected from 126 wild deer for examination: samples from 79 deer (*Cervus nippon yesoensis*) from Hokkaido Island, northern Japan, collected during 1975, 1989, and 1991; and samples from 47 deer (*Cervus nippon nippon*) from Shimane Prefecture on Honshu Island, southwestern Japan, collected during 2001 and 2002. Both locations were within the Piedmont physiographic region and were approximately 1,500 km apart. Sixty-eight *Hemaphysalis longicornis* ticks were collected by flagging in Shimane Prefecture, Japan, in 1999. Twenty-five *H. longicornis* ticks were removed from deer (*Cervus nippon nippon*) in Nara Prefecture, Honshu Island, Japan, in 2004.

**PCR.** DNA from the deer sera and homogenates of ticks was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA). Extracted DNA was used as the template for nested PCR amplification of 16S rRNA genes. The primer pair EC9 and EC12A (Table 1) used for the first PCR amplifies all known *Anaplasma* and *Ehrlichia* species. DNA from *E. muris* strain AS145 and *A. phagocytophilum* strain HZ were used as positive controls, and doubly distilled H<sub>2</sub>O was used as the negative control. The primers for the second-round PCR were specific for *E. muris*, *Ehrlichia* strain HF, '*Candidatus* Neoehrlichia mikurensis,' *A. phagocytophilum*, *A. centrale*, and *A. bovis* (Table 1). The *A. phagocytophilum* *p44* gene was amplified by nested or single-step PCR using primer pairs p3709-p4257 and p3761-p4183 (Table 1) (30). The initial amplification consisted of 40 cycles, each cycle consisting of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C. For the nested PCR amplification, 1 µl of the product from the first amplification was used for amplification with specific primers in a 25-µl reaction mixture; the amplification consisted of 40 cycles, each of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C.

**DNA sequencing and data analysis.** Sequences of every PCR amplicon of 16S rRNA genes were determined. All sequences shown in this study had the primer region sequences removed before analysis. Longer (>1,300-bp) 16S rRNA gene sequences were determined for seven representative sequences (ES34P-L from deer on Hokkaido; SS15E-L, SS24B-L, SS33P-L, and SS40C-L from deer in Shimane; TS37 from a tick in Shimane; and NR07 from a tick in Nara) and were compared to sequences of known *Anaplasma* and *Ehrlichia* spp. CLUSTAL W (version 1.6.5) and TreeView (version 1.6.5) on the DDBJ website and GENETYX-WIN (version 7.0.6; Software Development Co., Tokyo, Japan) were used for the sequence analysis and phylogram construction.

Deduced amino acid sequences of 16 newly obtained *p44* sequences from Japanese deer were aligned with those from all *A. phagocytophilum* sequences

available at GenBank (all sequences listed below are accession numbers from GenBank). To simplify the comparison, eighteen *p44* genes of *A. phagocytophilum* strain HZ isolated from a human patient in the United States were used to obtain the consensus amino acid sequence designated the P44 USA HZ consensus: P44-1 (ORF00017), P44-2 (ORF01470), P44-3 (ORF01414), P44-4 (ORF01447), P44-5 (ORF00136), P44-6 (ORF01412), P44-7 (ORF01405), P44-11 (ORF00218), P44-12 (ORF00076), P44-14 (ORF00039), P44-15 (ORF00054), P44-16 (ORF00095), P44-18E (ORF00047), P44-23 (ORF00083), P44-24 (ORF00135), P44-32 (ORF00113), P44-36 (ORF01462), and P44-45 (ORF00413). Fifteen *p44* genes of the United Kingdom strains described by Casey et al. (12) were used to obtain the consensus amino acid sequence designated the P44 UK consensus: FGA1 (AY176532), FGA4 (AY176534), FGB11 (AY176564), Lep1 (AY176565), Leph4 (AY176567), NW2 (AY176568), NWB2 (AY176581), NWT1 (AY176582), AB1 (AY176512), Cairn1 (AY176513), Cairn2 (AY176514), Cairn3 (AY176515), Cow1 (AY176529), Cow2 (AY176530), and Cow3 (AY176531). Sequence alignments and analyses of *p44* genes were performed using MegAlign and MapDraw programs (DNASStar, Inc., Madison, WI).

The *groESL* sequence (about 1,300 bp) of TS37 was obtained by a nested PCR with primers HS1a (5'-AITGGGCTGGTAITGAAAT) and HS6a (5'-CCICCI GGIACIAIACCTTC) in the first PCR, primers HS43 (5'-ATWGCWAARGAA GCATAGTC) and HSVR (5'-CTCAACAGCAGCTCTAGTAGC), and primers based on the sequencing result of the PCR product amplified with primer pair HS43-HSVR in the nested reactions (32).

**Nucleotide sequence accession numbers.** The 16S rRNA gene sequences of ES34P-L, SS33P-L, SS40C-L, SS24B-L, SS15E-L, TS37, and NR07 have been deposited in the GenBank data library under accession numbers AB196720, AB196721, AB211164, AB211163, AB211162, AB074459, and AB196475, respectively. The *groESL* sequence of TS37 has been deposited in the GenBank data library under accession number AB074462. Sixteen different *p44* sequences have been deposited in the GenBank data library under accession numbers DQ020144 to DQ020159.

#### RESULTS

Deer from two Japanese islands were tested for infection with *Anaplasma* and *Ehrlichia* species by PCR-based analysis of 16S rRNA genes. PCR products were sequenced to determine the pathogen identity. As shown in Table 2, 26% (33/126) of deer tested were infected with *Anaplasma* and/or *Ehrlichia* species: 14% (11/79) of deer (*Cervus nippon yesoensis*) from Hokkaido Island and 47% (22/47) of deer (*Cervus nippon nippon*) from Shimane, Honshu Island. Nineteen percent (24/126) of the deer were infected with *A. phagocytophilum*. Twelve percent (15/126) of the deer were infected with *Anaplasma*

TABLE 2. Detection of 16S rRNA sequences of *Anaplasma* and *Ehrlichia* spp. from deer in Japan

Species or result	No. of specimens		Total no.
	Hokkaido	Shimane	
<i>A. phagocytophilum</i> positive	8	16	24
<i>A. centrale</i> positive	1	14	15
<i>A. bovis</i> positive	5	6	11
<i>Ehrlichia</i> sp. (TS37) positive	0	3	3
Total positive	14	39	53
PCR-positive samples <sup>a</sup>	11	22	33
No. of deer tested	79	47	126

<sup>a</sup> Any species, including multiple PCR-positive species.

*centrale*. Nine percent (11/126) of deer were infected with *Anaplasma bovis*. Two percent (3/126) of deer were infected with an *Ehrlichia* sp. *E. muris*, *Ehrlichia* HF strain, and '*Candidatus Neohrlichia mikurensis*' were not detected in these deer specimens. One of 68 of *H. longicornis* ticks from the deer in Shimane Prefecture was infected with the *Ehrlichia* sp. Twelve percent (3/25) of *H. longicornis* ticks in Nara Prefecture were infected with *A. bovis* and *A. centrale*.

To define the pathogen identity, almost full-length 16S rRNA sequences were determined for seven representative strains from deer and from ticks. The phylogenetic analysis used 1,332-bp sequences of all seven new strains determined in this study and previously sequenced *Anaplasma* and *Ehrlichia* spp. (Fig. 1). Of the Hokkaido deer, 10% (8/79) were infected with *A. phagocytophilum*, and the 16S rRNA sequences (579 bp) of the amplicons from all eight infected deer were 99.9 to 100% identical to each other. The longer representative sequence (ES34P-L; 1,338 bp) was most closely related (99.3% identical; 10 bp differed of 1,338 bp compared) to *Anaplasma* sp. SA1076 from a dog in South Africa (GenBank accession no. AY570539) (21). This sequence was 98.7% identical (1,321 of 1,338 bp compared) to the *A. phagocytophilum* strain 'HGE agent' (where HGE is a designation for human granulocytic ehrlichiosis) CAHU-HGE2 from a human patient in northern California (GenBank accession no.

AF093789). Of the Shimane deer, 34% (16/47) were infected with *A. phagocytophilum*, and the 16S rRNA sequences (579 bp) of the amplicons from all 16 infected deer were 99.9 to 100% identical to each other. The longer 16S rRNA sequence (SS33P-L; 1,399 bp) from the representative specimen was most closely related (99.4% identical; 1,373 of 1,381 bp compared) to *Anaplasma* sp. SA1076 from a dog in South Africa (GenBank accession no. AY570539). It also was the next most closely related (98.4% identical; 21 bp differed of 1,402 bp compared) sequence to that of *A. phagocytophilum* strain LGE from a llama (*Llama llama*) and llama-associated ticks (*Ixodes pacificus*) (GenBank accession no. AF241532) (4). The sequence identity between ES34P-L from the Hokkaido deer and SS33P-L from the Shimane deer was 99.6% (Fig. 1; Table 3), implying geographic segregation of strains between the two islands.

Using the *p44*-specific primer pairs shown in Table 1 (30) for PCR, followed by sequencing of the amplicons, *p44* sequences of approximately 370 bp including primer regions (330 to 410 bp) were obtained from deer blood specimens. We obtained nine, four, and three different *p44* paralog sequences from deer SS14, SS33, and SS40, respectively. The sequences of the primer regions were removed, and the deduced P44 amino acid sequences were compared. These sequences were characterized by the central hypervariable regions flanked by N- and C-terminal-conserved regions (30). The P44 sequences from Japanese deer samples contained the identical amino acids C, C, W, and P found in the P44 USA HZ and P44 UK *A. phagocytophilum* consensus amino acid sequences (Fig. 2) (12, 30). However, all *p44* genes of Japanese samples had relatively shorter regions, delineated by two conserved cysteines (~12 amino acid residues), in the hypervariable region than those of the P44 USA HZ stains available at GenBank and the P44 UK strains (~25 to 30 amino acid residues). When 16 deduced amino acid sequences from Japanese deer were compared, ~63% (10/16) of Japanese deer P44 sequences had >50% identity, and 37% (6/16) had 50 to 30% identity. Phylogenetic analyses showed that the 16 sequences clustered with P44 se-

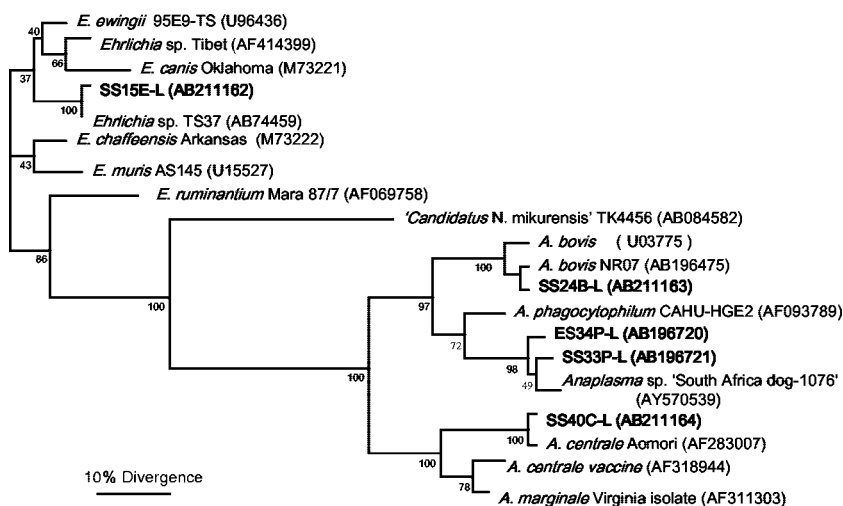


FIG. 1. Phylogram of the five new sequences from deer and other members of the family *Anaplasmataceae* based on 16S rRNA gene sequence comparison (1,332 bp). GenBank accession numbers are shown in parentheses. Numbers above internal nodes indicate the percentages of 1,000 bootstrap replicates that supported the branch.



TABLE 3. *Anaplasma* and *Ehrlichia* infection of 33 deer in Japan as determined by PCR, followed by sequencing

Deer identification no. <sup>a</sup>	<i>A. phagocytophilum</i>		<i>A. centrale</i>	<i>A. bovis</i>	<i>Ehrlichia</i> sp. (TS37)
	SS33 genotype	ES34 genotype			
ES11				+	
ES28				+	
ES34		+			
ES35		+			
ES40		+			
ES43		+			
ES51		+			
ES55				+	
ES56		+	+	+	
ES57		+			
ES60		+		+	
SS05	+				
SS07	+				
SS12	+		+		
SS13	+				
SS14	+				
SS15			+	+	+
SS16	+				
SS17			+		
SS20	+		+		
SS23	+		+		
SS24	+		+	+	
SS30	+		+		+
SS31	+			+	
SS32	+		+	+	
SS33	+				+
SS37			+		
SS38			+		
SS39			+	+	
SS40	+		+	+	
SS42	+				
SS43			+		
SS46	+		+		
Total	16	8	15	11	3

<sup>a</sup> ES11 to ES60, *Cervus nippon yesoensis* from Hokkaido Island; and SS01 to SS46, *Cervus nippon nippon* from Shimane in Honshu Island. Samples from ES11 was collected in 1991, ES28 to ES60 were collected in 1989, SS05 to SS24 were collected in 2001, and SS30 to SS46 were collected in 2002.

quences of *A. phagocytophilum* from the P44 USA HZ and the P44 UK consensus sequences (Fig. 3). These P44 sequences constituted a separate cluster from the Msp2 sequences of *A. phagocytophilum* and from the Msp1, Msp2, Msp3, and Msp5 sequences of *A. marginale* (Fig. 3).

The 16S rRNA sequences from *A. centrale* (426 bp) from 1 Hokkaido deer, 14 Shimane deer, and two *Hemaphysalis longicornis* ticks removed from deer in Nara Prefecture on Honshu Island, Japan, in 2004 were 99.9 to 100% identical to each other, despite the geographic separation (two separate islands) and up to 13 years of time span between collections (1989 on Hokkaido and 2001 and 2002 in Shimane). The longer representative sequence (SS40C-L; 1,361 bp) was obtained from one of the Shimane deer; it was 99.9% identical to that of *A. centrale* (GenBank accession no. AF283007) previously detected in the cattle from Aomori Prefecture on northern Honshu Island, Japan (22).

Furthermore, the 16S rRNA sequences (550 bp) of *A. bovis* from five Hokkaido deer, six Shimane deer, and one *Hemaphysalis longicornis* tick removed from deer in Nara Prefecture were 99.9 to 100% identical, despite geographic separation and up to 13 years separating collections (1989 and 1991 on Hokkaido and 2001 and 2002 in Shimane). The longer sequence (SS24B-L; 1,344 bp) from one representative specimen was 99.9% identical to strain NR07 (1,391 bp) that was detected in the *H. longicornis* tick in Nara Prefecture on Honshu Island, Japan, in 2004 and 99.7% identical (1,448 bp were compared) to that of the *A. bovis* strain from South Africa (GenBank accession no. U03775; strain name unavailable) (unpublished data).

Of the 22 *Anaplasma* species-positive specimens, three specimens, all from Shimane Prefecture, were coinfecting with a novel *Ehrlichia* sp. of the identical 16S rRNA gene sequence (475 bp). The almost full-length 16S rRNA gene sequence (1,447 bp) from the *Ehrlichia* sp. (SS15E-L; 1,365 bp) was 99.9% identical to that of *Ehrlichia* sp. TS37 (GenBank accession no. AB074459) that was detected in a single tick out of 68 *H. longicornis* ticks collected in the Shimane Prefecture in 1999 (the TS37 result was presented at the 101st General Meeting of American Society for Microbiology, Orlando, Fla., 20 to 24 May 2001). It was 98.7% identical to that of *Ehrlichia* sp. strain Tibet from *Boophilus microplus* ticks (GenBank accession no. AF414399) (62), 98.3% identical to *Ehrlichia ewingii* strain 95E9-TS (GenBank accession no. U96436) (19), and 98.2% identical to *E. chaffeensis* strain Arkansas (GenBank accession no. AF416764). To confirm the novel nature of the *Ehrlichia* sp. found from the deer and one tick, the 1,316-bp *groESL* sequence of TS37 was obtained. This sequence was 90.2, 90.2, 89.2, 90.6, 89.8, and 88.37% identical to that of *E. ewingii*, *E. chaffeensis*, *E. canis*, *Ehrlichia* sp. Anan, *Ehrlichia* sp. HF565,



FIG. 2. Comparison between the consensus-deduced amino acid sequences of *p44* genes from the 16 Japanese deer (SS) obtained in this study with consensus sequences of *p44* paralogs from strains from the United States (USA HZ) and the United Kingdom (UK) (11, 27). Underlined letters indicate the absolutely conserved amino acids from Japan, the United States, and the United Kingdom within the hypervariable region. Dashes show sequence gaps. The region delineated by two cysteines (underlined with dots) is shorter in Japanese strains than in strains from the United States and the United Kingdom.

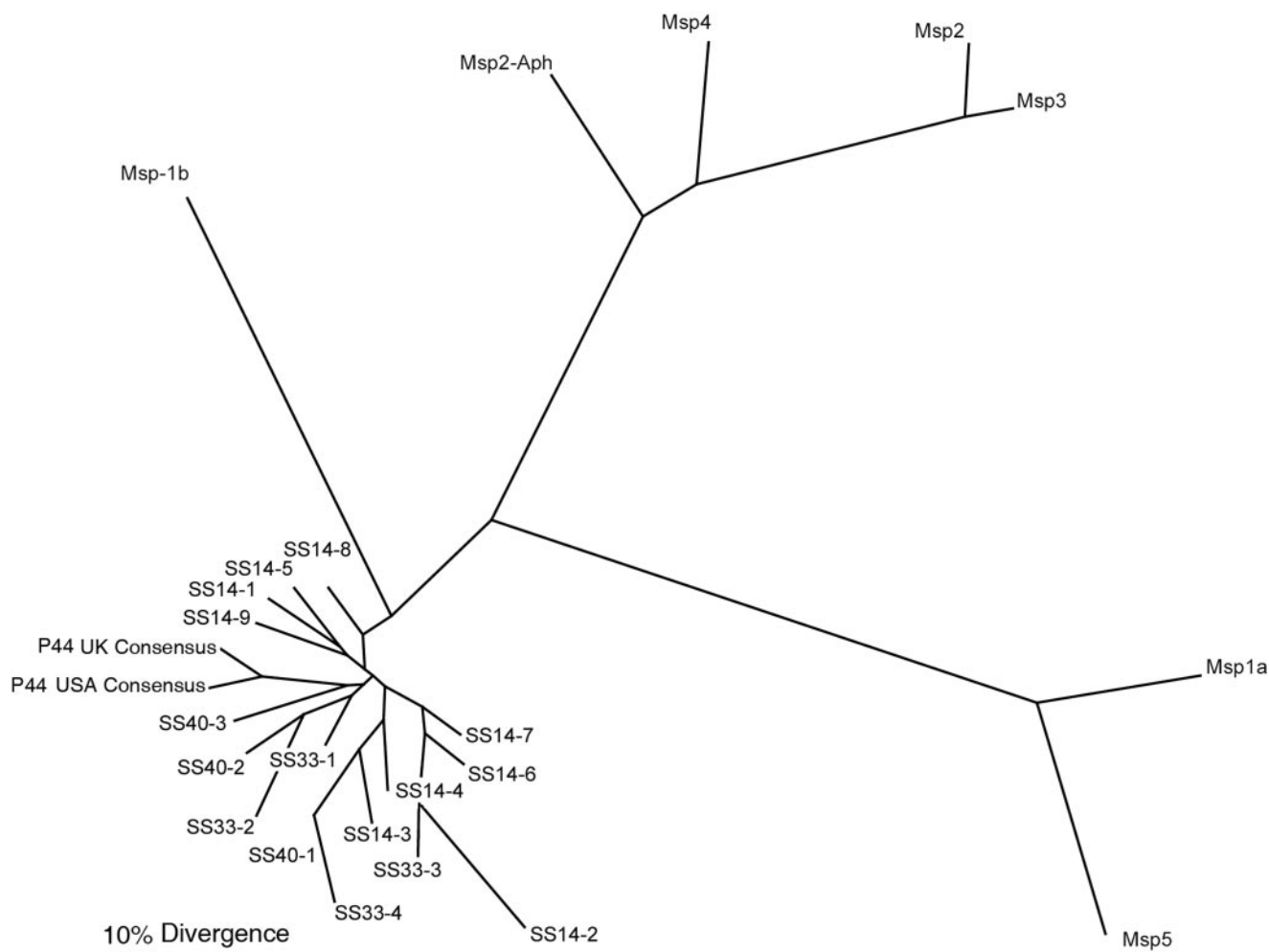


FIG. 3. Phylogram of deduced amino acid sequences of *p44* genes from three deer specimens compared to consensus sequences of *p44* paralogs from strains from the United States and the United Kingdom and sequences of Msp2 *A. phagocytophilum* (Msp2-Aph; GenBank no. AY541007 for strain NY-31) and sequences of St. Maries strains of *Anaplasma marginale*. GenBank accession numbers for sequences determined in this work are SS14-1 (DQ020144), SS14-2 (DQ020145), SS14-3 (DQ020146), SS14-4 (DQ020147), SS14-5 (DQ020148), SS14-6 (DQ020149), SS14-7 (DQ020150), SS14-8 (DQ020151), SS14-9 (DQ020152), SS33-1 (DQ020153), SS33-2 (DQ020154), SS33-3 (DQ020155), SS33-4 (DQ020156), SS40-1 (DQ020157), SS40-2 (DQ020158), and SS40-3 (DQ020159). The GenBank accession number for the whole genome sequence of *Anaplasma marginale* strain St. Maries is CP000030. The accession numbers for strain St. Maries are Msp1a (AM530), Msp1b (AM133), Msp2 (AM1144), Msp3 (AM1063), Msp4 (AM090), and Msp5 (AM236).

and *E. muris*, respectively. The deduced GroEL 402-amino-acid sequence of strain TS37 (GenBank accession no. AB074462) was 97.5, 97.3, 97.3, 97.0, 86.6, and 56.7% identical to that of HF565, *E. muris*, *E. ewingii*, *E. chaffeensis*, *A. phagocytophilum*, and *N. sennetsu*, respectively. A phylogram based on GroEL amino acid sequences showed that strain TS37 fell between *E. ewingii* and *E. canis*. The lengths of the intergenic space between the *groES* and *groEL* genes of TS37 were 96 bp, in agreement with the sequence-based phylogram (54).

Of 33 infected deer, 14 (42%) deer had concurrent infections with one or more *Ehrlichia* and *Anaplasma* species, where 8 deer were coinfecting with two species and 6 deer were coinfecting with three different species (Table 3). Thirty-three percent (11/33) of deer had concurrent infections with more than two species of *Anaplasma* and four deer had concurrent infections with three *Anaplasma* species. The three *Ehrlichia*-

infected deer had concurrent infections with one of three *Anaplasma* species.

## DISCUSSION

Findings from the present study show that the wild deer residing in two islands in Japan are naturally infected with four *Anaplasma* and *Ehrlichia* spp. and suggest that they may play a role in the enzootic maintenance of *Anaplasma* and *Ehrlichia* spp. in the region. White-tailed deer (*Odocoileus virginianus*) in the United States are known to be infected with *A. phagocytophilum* (3, 6, 31). Wild deer (*Cervus elaphus*, *Capreolus capreolus*, and *Odocoileus virginianus*) infected with *A. phagocytophilum* have also been reported in Europe (8, 33, 40, 45). Thus, Japan is the third geographic region where a high prevalence of infection of wild deer with *A. phagocytophilum* is found. What

is unique about *A. phagocytophilum* in Japan is that the 16S rRNA gene sequences were divergent from any previously reported *A. phagocytophilum* sequences from deer or other mammals in Europe or the United States or from ticks from Asia. The 16S rRNA gene sequence of *A. phagocytophilum* was found in ticks from China and Korea (11, 27). *A. phagocytophilum* 16S rRNA gene sequences from Hokkaido and Shimane deer were 99.3% and 98.9% identical, respectively, to the Korean tick strain (GenBank accession no. AF470699; 926 bp) and were 98.5% and 98.0% identical, respectively, to the Chinese tick strain (GenBank accession no. AY079425; 919 bp). Surprisingly, the bacterium closest to *A. phagocytophilum* from Japanese deer, as determined by 16S rRNA gene sequence comparison, was *Anaplasma* sp. SA1076 from a dog in South Africa (21). Therefore, it is possible that this *A. phagocytophilum* strain infects domestic dogs in Japan.

The *p44* gene of *A. phagocytophilum* encodes the immunodominant major outer membrane protein P44 (66), and multiple *p44* homologs have been found in every *A. phagocytophilum* strain in the United States and England examined so far. Although the hypervariable regions are quite diverse, phylogenetic analyses of these *p44* sequences are possible (12, 30, 66). To further define Japanese *A. phagocytophilum* strains, *p44* gene sequences were determined. Sequences of 16 distinct *p44* genes were obtained from three infected Japanese deer. The *p44* locus consists of a central hypervariable region and 5' and 3' conserved regions (30, 66). The Japanese deer *p44* genes were quite unique compared to all known *p44* sequences, but retained the conserved *p44* group-specific deduced amino acids observed within the hypervariable region of all sequenced *p44* genes. Thus, the P44 major surface protein structure of the *A. phagocytophilum* strain from Japanese deer had a unique feature compared with those of *A. phagocytophilum* strains found in the United States and England. This *p44* sequence difference also may explain why serological testing using the recombinant P44 protein of an *A. phagocytophilum* strain from the United States (55) could not detect most of the infected deer in Japan (data not shown).

In Japan, *A. phagocytophilum* has not been detected in ticks. However, *A. phagocytophilum* was found in *Ixodes persulcatus* from China (11) and in *Hemaphysalis longicornis* ticks from Korea (27). *I. persulcatus* is present on Hokkaido but has not been found in Shimane Prefecture (65). *H. longicornis* ticks are found on Honshu Island in Japan (65), but this tick has not been noted on Hokkaido. Thus, on Hokkaido and Honshu Islands, different species of *I. persulcatus* and *H. longicornis* ticks, respectively, may serve as vectors for *A. phagocytophilum* transmission between mammals.

To our knowledge, the present work is the first to document infection of deer with *A. centrale* or *A. bovis*, although the seroprevalence of *Anaplasma marginale* among deer in the United States and Mexico has been previously reported (26, 35); infection with an *A. marginale*-*A. ovis*-like agent in roe deer (*Capreolus capreolus*) in Spain has been previously reported as well (14, 40). The sequence of 16S rRNA from deer in Shimane Prefecture was 99.9% identical to that of *A. centrale* from cattle in Aomori, Japan (22); 98.6% identical to that of *A. marginale* from cattle in the United States (strain Virginia; GenBank accession no. AF309866) (unpublished

data); and 98.5% identical to that of *A. centrale* from cattle in Europe (GenBank accession no. AF318944) (5). Thus, wild deer may serve as the reservoir for economically important anaplasmosis of cattle caused by several *Anaplasma* species in the United States, Europe, and Japan. The *Rhipicephalus simus* tick is considered to be a vector of *A. centrale* in Africa (48), but in other geographic regions, a vector tick species has not been identified. Hokkaido and Shimane regions are not known to be inhabited by *Rhipicephalus* sp. ticks (65). In the present study, we found that the *H. longicornis* tick is the potential vector of *A. bovis* and *A. centrale*. Furthermore, *A. bovis* from an *H. longicornis* tick collected in Korea was reported (27).

White-tailed deer (*Odocoileus virginianus*) in the United States are known to be infected with *Ehrlichia chaffeensis*, the agent of human monocytic ehrlichiosis, and with *Ehrlichia ewingii*, the agent of human granulocytic ehrlichiosis (3, 31, 63). In the present study, the sequence (SS15E-L; 1,332 bp) detected in the deer from Shimane Prefecture was 99.9% identical to that of *Ehrlichia* sp. strain TS37 from *H. longicornis* ticks from Shimane Prefecture. This result suggests that *H. longicornis* ticks serve as vectors for the mammalian transmission of *Ehrlichia* sp. strain TS37.

Both 16S rRNA and *groEL* sequences of TS37 were distinct from any known *Ehrlichia* species and phylogenetically close to those of *E. muris*, *E. ewingii*, and *E. chaffeensis*. We propose to name this *Ehrlichia* species '*Candidatus Ehrlichia shimanensis*.'

All three *Ehrlichia*-positive deer were coinfecting with *Anaplasma* species. Similar coinfections of deer with *Ehrlichia* and *Anaplasma* species, including *E. ewingii*, *E. chaffeensis*, or the WTD agent (an *Anaplasma* sp.) in Missouri or *E. chaffeensis*, *A. phagocytophilum*, or the WTD agent in Georgia were previously reported (3, 31). The WTD agent was not detected in Japanese deer in the present study. The 16S rRNA gene sequences from Japanese deer had only very low levels of identity (97%) to those of the WTD agent.

Several deer were infected with three *Anaplasma* species, namely *A. centrale*, *A. phagocytophilum*, and *A. bovis*; these agents infect erythrocytes, granulocytes, and monocytes, respectively (Table 3). To our knowledge, this is the first report of concurrent infection of any animal with three *Anaplasma* species. The results suggest the absence of cross protection among these *Anaplasma* species, illustrate the potential difficulty in diagnosing the deer infection by stained blood smear and/or serological test, and support the usefulness of molecular diagnosis of *Anaplasma* and *Ehrlichia* infection.

Serum specimens are a more convenient source than whole-blood specimens for retrospective analyses of infection, since many well-preserved archival and clinical specimens are available. Although less DNA from obligatory intracellular bacteria can be recovered from serum than from whole blood, previous studies have indicated the utility of human and deer serum specimens for the nested-PCR amplification of *A. phagocytophilum* DNA (36). The present study showed that serum from deer blood is a good source for DNA from *Ehrlichia* and *Anaplasma* species.

This present study suggests that enzootic cycles of several *Ehrlichia* and *Anaplasma* species between ticks and wild deer are established in Japan.



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