Anaplasma is a tick-borne infectious disease of cattle, sheep, horse, and other domestic ruminants. Among the *Anaplasma* spp., *A. phagocytophilum* (formerly *Ehrlichia phagocytophila* and *E. equi*) is a Gram-negative bacterium that has been recognized as the causative agent of tick-borne fever in cattle, sheep, goats, and horses in the United States and Europe (1,2). In 1990, in the United States, *A. phagocytophilum* was identified as a potential illness-causing agent in humans causing the human granulocytic anaplasmosis (HGA) (3). In Europe, the first case of HGA was reported in 1997 (4). The Roe and red deer are recognized as reservoir hosts for *A. phagocytophilum* in Europe. Furthermore, *A. phagocytophilum* has been isolated from wild boars in Austria and the Czech Republic (5). We have previously shown *A. phagocytophilum* infection via *Ixodes persulcatus* and *Ixodes ovatus* in Japan using PCR targeted to the p44/msp2 paralogs, which encode a 44-kDa surface protein and the 16S rDNA of *A. phagocytophilum* (6). The 16S rDNA sequences isolated from *I. ovatus* and *I. persulcatus* in Japan showed a higher similarity to that of *A. phagocytophilum* isolated from a patient in the United States (7,8). *A. phagocytophilum* was also detected in the Sika deer in Shimane Prefecture and Hokkaido (9). These *A. phagocytophilum* sequences were similar to those detected in cattle on Yonaguni Island, one of the southern islands of Japan (10), and in cattle and the Sika deer in Hokkaido, the northernmost island of Japan (11). However, on the basis of our knowledge, we assumed that *A. phagocytophilum* detected from the ixodid ticks and from the animals in Japan were phylogenetically different, and the exact reservoir hosts for *A. phagocytophilum* have not been clearly determined in Japan. The aim of this study was to determine the prevalence and geographical distribution of *A. phagocytophilum* in wild deer and boars in Japan.

The study was conducted in 56 wild boars (*Sus scrofa*) and 32 Sika deer (*Cervus nippon*) captured in the hunting seasons of 2006 and 2007 in the region from Hokkaido to Kagoshima Prefecture in Kyushu, a southern island of Japan (Fig. 1). Blood and spleen samples of the animals were obtained on field by the hunters. The frozen blood and/or spleen were disrupted for 30 s using Tissue-Lyser (Qiagen, Haan, Germany) with 3-mm tungsten carbide beads, and the total DNA was extracted using QuickGene-800 nucleic-acid isolation system (Fuji Film, Tokyo, Japan). To detect the specific DNA of the *Anaplasma* spp., in the first step of PCR, a 16S rDNA was amplified with forward primer EC12A (5′-TACTGCCAGACTAGTGGAG-186) and reverse primer AP-R (5′-ATCTGCCAGACTAGTGGAGAAT-3′) specific to the sequences of *Anaplasma bovis*, *A. phagocytophilum*, and *Anaplasma platys*, and Acom (5′-TACTGCCAGACTAGTGGAGAATG-3′) specific to the sequences for *Anaplasma centrale*, *Anaplasma ovis*, and *Anaplasma marginale*, and reverse primer AP-R (5′-TTGCAACCTATTGTAGTC-3′) were used as the inner primer sets; these primers ampli-
fied approximately 600 base pairs (bp) of DNA. Longer (>1,300 bp) 16S rDNA gene sequences were determined using DNA from 16S rDNA-positive samples as the template DNA. These sequences were amplified using the forward primer ER5-3 and reverse primer ER-R1 in the first step of PCR (12), and forward primer EC9 and reverse primer EC12A in the nested PCR.

For DNA sequencing analysis, the nested PCR amplicon was purified using a Microcon-PCR purification column (Millipore, Bedford, Mass., USA) and was then subjected to DNA cycle-sequencing analysis using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, Calif., USA) and an ABI 3130-Avant genetic analyzer (Applied Biosystems).

The sequences determined were deposited to DDBJ/EMBL/GenBank under accession numbers AB588965–AB588978. A phylogenetic tree was constructed on the basis of the alignment of the 16S rDNA sequences (600 bp) and previously published sequences using Clustal W algorithm and the sequence analysis software MegAlign (DNASTAR Inc., Madison, Wis., USA).

Anaplasma spp. were identified on the basis of the 16S rDNA sequences (600 bp). The infection rates of 16S rDNA-positive samples were determined for sequences detected in other animals (deer and boars) from Ohita and Kumamoto Prefectures in Kyushu. For DNA sequencing analysis, the nested PCR amplicon was subjected to DNA cycle-sequencing analysis using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, Calif., USA) and an ABI 3130-Avant genetic analyzer (Applied Biosystems).

Fig. 1. Anaplasma isolated from two deer and two boars from the wild in Japan. Fig. 2. Anaplasma infected deer and boars detected in Japan. D and B indicate deer and boars, respectively. Numbers indicate number of positive animals/number of tested animals. * indicates dual infection. Each deer sampled in Hokkaido and Iwate Prefecture was dually infected with A. phagocytophilum and A. bovis, or with A. bovis and A. centrale. Furthermore, two deer in Nagano Prefecture was dually infected with A. bovis and A. centrale.

In Japan, A. phagocytophilum was detected in the Sika deer in Hokkaido, Nagano Prefecture in Honshu, the main island, Tokushima Prefecture in Shikoku Island; and Kumamoto Prefectures in Kyushu (Fig. 1, left). A. phagocytophilum was also detected in the boars found in Ohita and Kumamoto Prefectures in Kyushu. The phylogenetic tree (Fig. 2), constructed on the basis of the 16S-rDNA sequence (600 bp) showed that the A. phagocytophilum sequences detected from deer and boars formed clusters along with the sequence clusters of A. phagocytophilum previously found in deer and cattle in Japan and dogs in South Africa (AY570540) in Fig. 2 (Cluster III). The clusters of A. phagocytophilum sequences obtained from the study animals (deer and boars) were found to be 99–100% identical to those obtained for sequences detected in other animals (deer and cattle) in Japan and were 99.3–99.8% identical to the cluster obtained for sequences detected in dog in South Africa. However, these branched from those detected from a patient in the United States, horse in Sweden, I. ovatus in Japan and I. persulcatus in Russia (Cluster IV). Similar findings were obtained using longer 16S rDNA sequences (1,300 bp, data not shown). The p44/msp2 gene family of A. phagocytophilum is a multigene family comprising hypervariable and conserved regions at the 5’ and 3’ ends (13). The strain HZ isolated from the HGA patient in the United States had 113 p44/msp2 loci in its genome (14). The high sensitivity and specificity of nested PCR aided in targeting the p44/msp2 genes of A. phagocytophilum. Although, Kawahara et al. (9) had obtained p44/msp2 paralogs from deer, the entire p44/msp2 sequences of A. phagocytophilum obtained from the animal samples from Japan constituted a separate cluster from those of A. phagocytophilum detected from I. persulcatus and I.
Fig. 2. Phylogenetic tree of *Anaplasma* spp. detected from wild boars and deer on the basis of 16S rDNA sequences. The phylogenetic tree was constructed using the alignment of 16S rDNA sequences using Clustal W algorithm followed by the neighbor-joining method with 1,000 bootstrap resamplings. The sequences obtained from boars and deer are indicated with “B” and “D”, respectively. (I) and (II) indicate clusters composed of sequences identified as *A. bovis* from wild boars and Sika deer, respectively. (III) and (IV) indicate clusters composed of sequences derived from *A. phagocytophilum*-related species and *A. phagocytophilum*, respectively. (V) and (VI) indicate clusters composed of sequences derived from *A. centrale* detected in Japan and foreign countries, respectively. Sequence accession numbers are in parentheses.

From the sequences obtained in the present study, we identified *A. centrale* and *A. bovis* (Fig. 1, center and right, respectively). *A. centrale* was identified as the only causative agent of anaplasmosis with mild anemia among cattle in Japan (15). The *A. centrale* sequences detected in deer (10 sequences) and boars (2 sequences) were identical to those detected in cattle in Aomori Prefecture (AF283007) (15), and the Sika deer in Shimane Prefecture in Japan (AB211164, Cluster V in Fig. 2) (9). This is the first study to report the detection of *A. centrale* gene sequences in wild boars. However, since the samples were collected by hunters on field, the possibility of cross contamination from deer samples was not ruled out. Further studies are required to confirm these results. The branching of the clusters of sequences detected in Europe and Africa (Cluster VI) from that of the sequences detected in Japan (similarity of 98.5–98.6%) indicated the genetic differences between the pathogen found in Japan and those in other countries. Classification of this pathogen remains a problem, and further investigation is required.

In this study, *A. bovis* sequences were detected in deer...
and boars (Fig. 1, right). *A. bovis* were previously detected in the Sika deer, cattle, and the vector ticks, *Haemaphysalis longicornis* and *Haemaphysalis megaspinosa* (9,11,16,17). *A. bovis* infections are mostly observed in cattle in Africa, and this strain is phylogenetically more closely related to *A. phagocytophilum* than to *A. centrale* (2,15). Most of these sequences were distributed in two clusters: cluster of sequences detected in deer (Cluster II, Fig. 2) and cluster of sequences detected in boars (Cluster I). This finding suggests a potential specific relationship between some genotypes of *A. bovis* and the reservoir animal species.

Our study revealed the presence of *A. phagocytophilum*-related species, *A. bovis*, and *A. centrale* in deer and wild boars in Japan. Further studies are required to confirm the animal reservoir hosts of *A. phagocytophilum* and for genetic characterization of *A. phagocytophilum* and *A. centrale* in Japan to understand the spread of diseases associated with these pathogens.

Acknowledgments

We are grateful to Dainihon Ryouyu for providing tissue and blood samples of wild boars and deer. We would like to thank Professors Hisashi Inokuma, Obihoro University of Agriculture and Norio Ohashi, University of Shizuoka, Japan for providing *A. platys* DNA and for valuable discussion.

This study was supported in part by a Grant-in-Aid for Scientific Research B (No. 20406011) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest

None to declare.

REFERENCES


