buffer), before being stained with a PE- or APC-conjugated anti-mouse IFN-γ mAb (clone XMG1.2; eBioscience) or isotype control Ab (eBioscience) for 45 min at 4 °C. Cells were washed, first with permeabilization buffer, then with FACS buffer, before being applied to a FACSCalibur or FACSAria flow cytometer (BD). Analyses were performed using FlowJo software (Ashland, OR).

Cytological analysis. Fifty thousand cells were subjected to a cytospin (Thermo Shandon). For morphological analysis, the cytospin preparations were fixed with methanol and visualized with May-Grünwald-Giemsa staining. For intracellular IFN-γ staining, the cytospin preparations were fixed with 2% paraformaldehyde for 10 min, treated with 50 mM NH_4Cl/PBS for 15 min, and blocked with an anti-mouse FcγR mAb (clone 2.4G2) in permeabilization buffer for 15 min. Slides were stained with an APC-conjugated anti-mouse IFN-γ mAb (clone XMG1.2) or isotype control Ab (eBioscience) for 45 min, washed 3 times with permeabilization buffer, and then washed 3 times with PBS. Nuclei were visualized with propidium iodide staining. Samples were viewed and photographed with a Carl Zeiss LSM510 confocal laser scanning microscope.

Histology. For histological analysis, the tissues from GAS-infected mice were fixed in 10% formalin/PBS. The paraffin-embedded sections were stained with hematoxylin and eosin.

***In vitro* culture of γIMCs and BMPCs**. Purified $CD11b^+$ $CD11c^-$ $F4/80^{low}$ $Ly\text{-}6G^+$ γIMCs and $CD11b^+$ $CD11c^{low}$ $F4/80^+$ $Ly\text{-}6G^{low}$ BMPCs from GAS-infected (monensin-untreated) mice were cultured at 0.5×10^6 cells per ml in medium containing RPMI 1640 (Wako) with 10% FBS (Nichirei), 100 U ml^{-1} penicillin, 10 μg ml^{-1} streptomycin, 2 mM glutamine, 25 mM HEPES, and 50 μM 2-ME, with or without 10–50 ng ml^{-1} recombinant mouse GM-CSF, G-CSF, M-CSF, or IL-5 (R&D Systems), in the presence or absence of 1 μg ml^{-1} control rat IgG or R4-6A2, for 2–6 days. On days 2 and 4, 50% of the medium was replaced with fresh medium, or the cells were collected for May-Grünwald-Giemsa staining and flow cytometry analysis. In some experiments, the cells were cultured at 0.5×10^6 cells ml^{-1} with 25 μg ml^{-1} erythromycin and NIH34 (MOI 100) in 10% FBS/phenol red-free RPMI medium, supplemented with 10 ng ml^{-1} GM-CSF, in the presence of 1 μg ml^{-1} control rat IgG or R4-6A2 for 24 h. The levels of IFN-γ and NO in the culture supernatants were measured by an instant ELISA kit (eBioscience) and a Griess reagent (Wako), respectively, according to the manufacturer's instructions.

***In vitro* culture of Eos and MDSCs**. For obtaining Eos, naïve bone marrow cells were cultured in 10% FBS/RPMI medium supplemented with stem cell factor (PeproTech) and FLT3-ligand (PeproTech) for 4 days, and then with medium containing recombinant mouse IL-5 (R&D Systems). On day 12, the cells were collected for May-Grünwald-Giemsa staining and flow cytometry analysis[46]. To isolate bone marrow-derived MDSCs, naïve bone marrow cells were cultured at 1.0×10^6 cells per ml in 10% FBS/RPMI medium, supplemented with 40 ng ml^{-1} GM-CSF. On day 4, the cells were collected for FACS analysis of $CD11b^+$ $CD11c^-$ $Ly\text{-}6C^+$ $Ly\text{-}6G^{low}$ MDSCs[9].

Ag-specific T-cell proliferation and IFN-γ production. The Ag-specific proliferation of $CD8^+$ T cells was evaluated using OT-I OVA-specific MHC Class I-restricted TCR transgenic mice. Varying amounts of purified $CD11b^+$ $CD11c^-$ $F4/80^{low}$ $Ly\text{-}6G^+$ γIMCs from infected C57BL/6 mice at 2 days post-infection, or bone marrow-derived $F4/80^+$ $Ly\text{-}6C^+$ $Ly\text{-}6G^{low}$ MDSCs, were added to 2.0×10^5 naïve splenocytes from OT-I mice in medium containing RPMI 1640 with 10% FBS in a U-bottom 96-well plate. These co-cultures were stimulated with antigenic $OVA_{357-364}$ peptides (10 μM) for 4 days. Proliferation of OT-I cells in triplicate was estimated by the incorporation of [^{3}H] thymidine (1 μCi (0.0037 MBq) per well), added at 18 h before cell harvest. The level of IFN-γ in the culture supernatants was measured by an instant ELISA kit (eBioscience), according to the manufacturer's instructions.

Adoptive transfer of γIMCs. $CD11b^+$ $CD11c^-$ $F4/80^{low}$ $Ly\text{-}6G^+$ γIMCs in splenocytes from *Ifng*$^{+/+}$ mice infected with NIH34 (3.0×10^7 CFU) for 48 h were isolated with a FACSAria flow cytometer. Recipient mice were i.v. administered with purified $CD11b^+$ $CD11c^-$ $F4/80^{low}$ $Ly\text{-}6G^+$ γIMCs (3.0×10^6 cells), and i.p. inoculated with NIH34 (1.0×10^7 CFU to 5.0×10^7 CFU) on the same day.

Measurement of bacterial loads. At 24 h post-infection, 20 μl of peripheral blood was removed from the tail vein by phlebotomy. The blood was diluted at 1:10–1:1000 with PBS and spread on a Columbia agar plate containing 5% sheep blood (BD). To determine the number of NIH34 in peripheral blood, the plates were incubated for 20 h at 37 °C in a 5% CO_2 atmosphere, and the colonies were counted. The number of NIH34 was compared statistically using the Mann–Whitney *U*-test.

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Author contributions

T.M., M.A., and T.I. designed and performed the experiments. T.M. and M.A. analysed the data. T.M., M.A., T.I., M.O., H.W. and K.K. wrote the manuscript.

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Short Communication

Detection and Characterization of *p44/msp2* Transcript Variants of *Anaplasma phagocytophilum* from Naturally Infected Ticks and Wild Deer in Japan

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SUMMARY: *Anaplasma phagocytophilum* is an obligate intracellular bacterium and causes a febrile illness in humans and livestock. In nature, this bacterium is sustained in a tick-mammal cycle. Several *p44/msp2*-related genes are expressed from a single expression locus by gene conversion. In this study, we obtained 119 cDNA sequences of *p44/msp2* transcripts from *A. phagocytophilum* in 6 *Haemaphysalis* ticks and 3 wild sika deer (*Cervus nippon*) in Japan. These 119 sequences were classified into 36 different variant sequences based on their similarities. The 36 cDNA sequences were phylogenetically grouped into 2 major clusters—tick- and deer-associated. The tick-associated sequences were further classified into 4 distinct subclusters, suggesting that *A. phagocytophilum* in ticks seems to selectively express specific *p44/msp2* transcripts, such as the transcripts in the 4 subclusters that were closely related to previously identified *p44/msp2* genes. The deer-associated sequences were also grouped into 4 subclusters, but these transcripts were probably more diverse than the transcripts derived from ticks. This might be due to the relatively nonselective expression of *p44/msp2* in deer or the strain differences in *A. phagocytophilum* from ticks and deer in separate geographic regions or both. Thus, this study may contribute to the understanding of *A. phagocytophilum p44/msp2* expression in nature in Japan.

Anaplasma phagocytophilum is an obligate intracellular bacterium and a causative agent of human granulocytic anaplasmosis (HGA) and also infects granulocytes in sheep, horses, dogs, cattle, and deer (1,2). In nature, the organism is sustained in a life cycle alternating between tick vectors and mammalian hosts. This bacterium possesses a *p44/msp2* multigene family encoding multiple 44-kDa major outer membrane proteins (3,4). The 113 *p44/msp2* paralogous genes, including pseudogenes, are distributed throughout the genome and contain a hypervariable region flanked by 5′- and 3′- end conserved regions. *A. phagocytophilum* generates antigenic variation by producing of a variety of P44 surface proteins due to a unidirectional gene-conversion mechanism in which the *p44/msp2* copies are recombined into a single expression site in the genome to avoid the immune defense system (5–7). Previous studies have analyzed the structure of the *p44/msp2* expression site in *A. phagocytophilum* strains obtained from infections in different animal species (8). Recently, we characterized the structure of *A. phagocytophilum p44/msp2* expression sites from naturally infected ticks in Japan (9), and identified the *p44/msp2* cassettes within the expression sites, which are probably transcribed. In this study, we successfully detected and characterized *p44/msp2* transcript variants of *A. phagocytophilum* from *Haemaphysalis* ticks and wild sika deer (*Cervus nippon*).

A total of 171 ticks (*Haemaphysalis megaspinosa*, 51; *H. flava*, 10; *H. formosensis*, 9; and *H. kitaokai*, 2 in Wakayama Prefecture; *H. formosensis*, 68; *H. longicornis*, 23; *Amblyomma testudinarium*, 5; and *H. yeni*, 3 in the Goto islands of Nagasaki Prefecture) were collected by flagging in 2009. Total RNA was extracted from the salivary gland of each tick using RNAspin Mini Kit (GE Healthcare Life Sciences, Uppsala,

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Sweden). Wild deer were obtained by hunting in Shizuoka Prefecture in 2010–2011, as described elsewhere. Total RNA was extracted from the spleens of the deer using TRIzol Reagent (Invitrogen, Carlsbad, Calif., USA). After RNA preparation, all the RNA samples were treated with 5 units of recombinant DNase I (Takara Bio, Inc., Otsu, Japan) for 20 min at 37°C and reverse transcribed to synthesize cDNA by using the ReverTra Ace-α-RT-PCR kit (Toyobo Biochemicals, Osaka, Japan). The reverse transcription (RT) reaction was performed for 20 min at 42°C according to the manufacturer's protocol. To characterize *p44/msp2* transcripts, cDNA prepared from the salivary glands of ticks and the spleens of deer were amplified by nested polymerase chain reaction (PCR) and first-step PCR alone, respectively. The primer pairs used for the *p44/msp2* amplification were p3726 and p4257 for the first-step PCR (approximately 450–500 bp) and p3761 and p4183 for the second-step PCR (approximately 400 bp), as described previously (10). To confirm the quality of RNA preparation and to eliminate DNA contamination in the RNA preparations, RT-PCR was performed with or without reverse transcriptase, which targets the tick 18S rRNA or sika deer mitochondria (mt)-16S rRNA, as well as the *p44/msp2* mRNA. The primers Tick18S-63f (forward: 5′-CGA AAC CGC GAA TGG CTCA) and Tick18S-567r (reverse: 5′-GGC TGC TGG CAC CAG ACT), which amplify a 500-bp fragment of tick 18S rRNA, and primers Sika-16Sf (forward: 5′-GGA TAC AAC CTT AAC TAG AG) and Sika-16Sr (reverse: 5′-GAG AAC AAG TGA TTA TGC TAC), which amplify a 490-bp fragment of sika deer mt-16S rRNA, were newly designed in this study. By using nested RT-PCR targeting the *A. phagocytophilum p44/msp2* mRNA, we successfully detected 400-bp amplicons from 6 *Haemaphysalis* ticks (*H. formosensis*, 3; *H. megaspinosa*, 2; and *H. longicornis*, 1) out of 171 ticks. Additionally, 450-bp amplicons of *p44/msp2* cDNA were detected from total RNA prepared from 3 fresh deer spleens by single RT-PCR (shown in Fig. 1 as representatives). All *p44/msp2* cDNA amplicons obtained were gel-purified and sequenced into the pCR2.1 vector using the TA Cloning Kit (Invitrogen), and the recombinant plasmids were introduced into *Escherichia coli* DH5α (Toyobo Biochemicals). The recombinant *p44/msp2* cDNA clones were randomly selected, and the inserted cDNAs of the respective clones were all sequenced. A total of 119 *p44/msp2* cDNA clone sequences (74 from ticks and 45 from deer) were obtained and aligned by Clustal X software. The recombinant cDNA clones with identical sequences were determined and grouped. Thus, we eventually found 36 different *p44/msp2* cDNA clone sequences with distinctive base sequences.

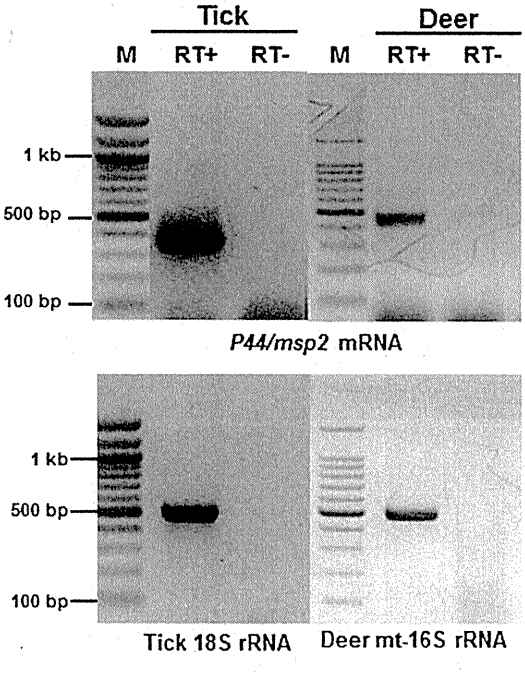

Fig. 1. RT-PCR detection of *p44/msp2* transcripts from *A. phagocytophilum* in tick salivary gland and deer spleen. RT-PCR targeting *p44/msp2*, tick 18S rRNA, and sika deer mitochondria (mt)-16S rRNA were performed with (RT+) or without (RT−) reverse transcriptase to detect *p44/msp2* transcripts, to confirm the qualities of RNA preparation, and to eliminate DNA contamination in the RNA preparation. Amplified product sizes are approximately 400 bp and 450 bp for *p44/msp2* mRNA from ticks and sika deer, respectively, and 500 bp and 490 bp for tick 18S rRNA and sika deer mt-16S rRNA, respectively. The figure shows one example of positive samples from ticks and deer. M, 100-bp size marker.

A phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap resamplings based on 335-bp to 398-bp *p44/msp2* cDNA clone sequences of *A. phagocytophilum* from ticks and sika deer in this study (Fig. 2). The *p44/msp2* cDNA sequences in the tree were clearly separated into 2 large clusters (A from ticks and B from sika deer in Fig. 2), and the similarities between these 2 clusters were relatively low (3.8–36.0%). Most of the cDNA sequences in cluster A (ticks) were further grouped into 4 distinctive subclusters (a1 to a4 in Fig. 2), and the similarities among those subclusters were 14.2–66.8%. The tick-associated cDNA sequences within each subcluster, except for a sequence of Tick35-12-mRNA-Goto-Hform in subcluster a1, were very similar or identical to each other (similarities: 99.5–100% in a1, 99.2–100% in a2, 99.4–100% in a3, and 99.5–100% in a4). Additionally, the tick-associated cDNA sequences were not obviously segregated into tick species as well as the individual ticks. In cluster B (sika deer), *p44/msp2* cDNA sequences could also be grouped into 4 subclusters (b1 to b4 in Fig. 2), and the similarities among the subclusters were 22.7–99.7%. Some of the deer-associated cDNA sequences were very similar or identical to each other, i.e., (i) 16 cDNA sequences within subcluster b2, except for a sequence of Sika29-4-mRNA-Fuji (similarities, 98.2–100%); (ii) 13 cDNA sequences, including Sika7-1-mRNA-Izu and Sika7-22-mRNA-Izu, within subcluster b3 (similarities, 99.7–100%); and (iii) 4 cDNA sequences, including Sika7-2-mRNA-Izu and Sika7-18-mRNA-Izu, within subcluster b3 (similarities, 99.4–100%). However, the deer-associated *p44/msp2* cDNA sequences were likely to be more diverse than the tick-associated cDNA sequences, as described above. In addition, those deer-associated cDNA sequences were not segregated into the 3 deer individuals.

By BLASTN search, the closest relatives for each of the 36 different *p44/msp2* cDNA sequences in this study were identified (Table 1). The tick-associated *p44/msp2*

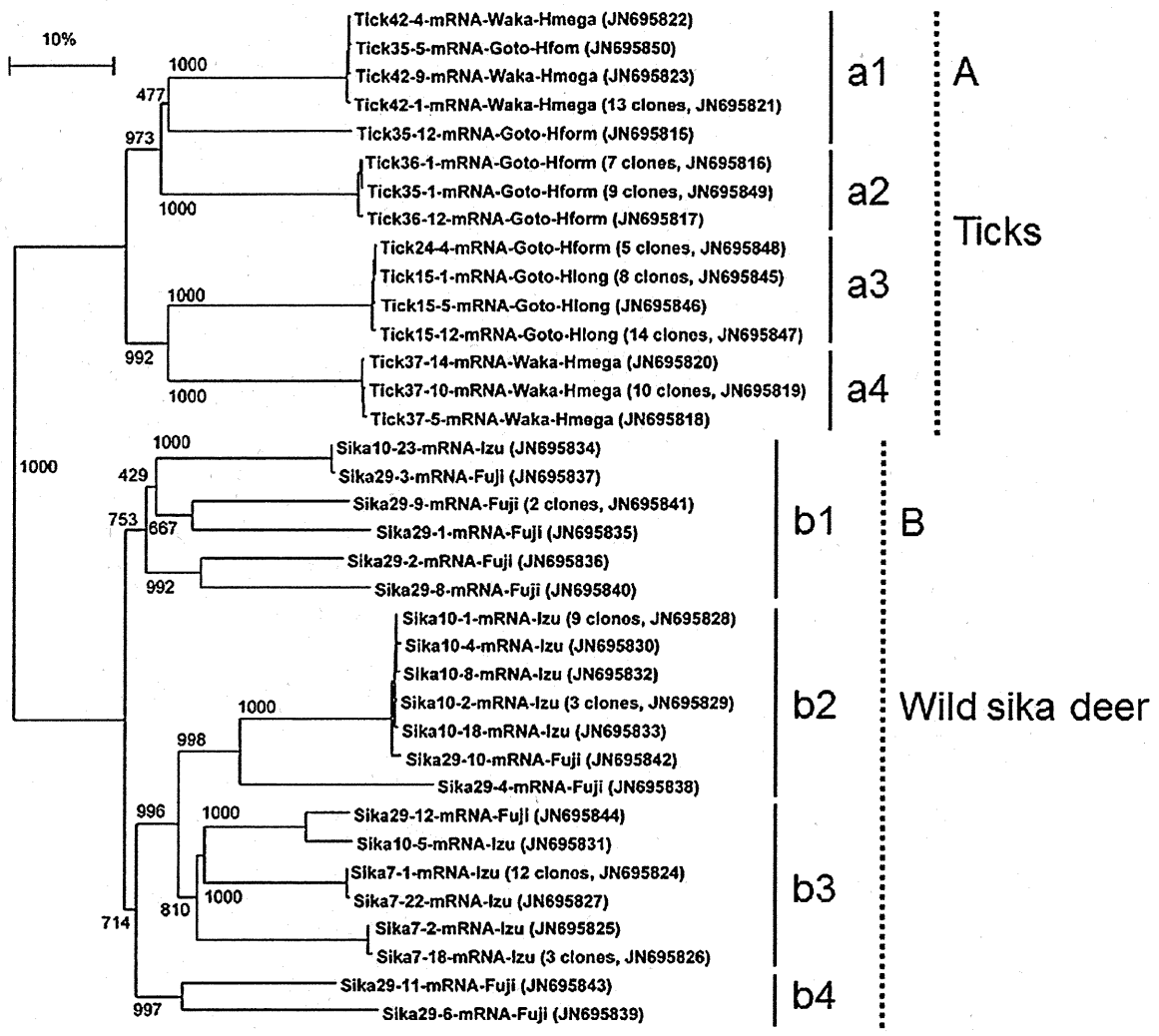

Fig. 2. Phylogenetic classification of *p44/msp2* cDNA clone sequences from *A. phagocytophilum* in tick salivary glands and wild sika deer. The tree was constructed based on 335-bp to 398-bp *p44/msp2* cDNA clone sequences by the neighbor-joining method with 1,000 bootstraps resamplings. The *p44/msp2* cDNA sequences of *A. phagocytophilum* were clearly separated into two clusters; A for ticks and B for wild sika deer. The cDNA sequence members in each cluster could be further grouped into 4 subclusters; a1 to a4 for cluster A (ticks) and b1 to b4 for cluster B (wild sika deer). The number of *p44/msp2* cDNA clones with identical sequences and/or accession numbers are shown in parentheses. A horizontal bar indicates 10% sequence divergence.

cDNA sequences in subclusters a2 and a3 were very similar or identical to Tick-162-10L-Iwate-ff-Ip (accession no. FJ600590) and Tick-162-12L-Iwate-ff-Ip (FJ600596), respectively (similarities, 98.3–100%) that were found in *p44/msp2* expression locus of *A. phagocytophilum* in *Ixodes persulcatus* ticks in our previous study (9). The cDNA sequences in subclusters a1 and a4 were also very similar or identical to *p44-35E/p44-62* and *p44-20* genes, respectively (similarities, 99.5–100%), which were located on the *A. phagocytophilum* genome from US human isolates HZ (CP000235). In deer-associated *p44/msp2* cDNA sequences, BLASTN search showed that, unlike tick-associated cDNA sequences, several subclusters from deer included multiple closest relatives of previously identified *p44/msp2* multigenes from Japanese sika deer, as described by Kawahara et al. (11), with much lower similarities (Table 1). Indeed, the *p44/msp2* cDNA sequences in subcluster b1 had the 3 closest relatives of SS14-3, SS14-7, and SS14-9, and the similarities were 68.4–73.0%. The cDNA sequences in subcluster b2 included the 2 closest relatives of SS14-1 and SS14-9, and the similarities were 74.2–91.5%. The closest relative for cDNA sequences in subcluster b3 was SS14-3 (similarities, 70.0–71.7%). The cDNA sequences in subcluster b4 had the 2 closest relatives of SS14-3 and SS14-5, and the similarities were 72.3–73.0%. Thus, the results suggest that *p44/msp2* transcript variants of *A. phagocytophilum* detected in this study seem to be newly identified *p44/msp2* repertories.

In Japan, *A. phagocytophilum* has been identified by PCR amplification in naturally infected ticks of *I. persulcatus, I. ovatus*, and *H. megaspinosa* (10,12–14), and in naturally infected mammals such as wild deer, wild boars, and cattle (11,13,15–17). In this study, we demonstrated for the first time that *H. longicornis* and *H. formosensis* ticks are naturally infected with *A. phagocytophilum*, suggesting additional potential vectors. Furthermore, we successfully characterized, for the first time, *p44/msp2* transcripts of *A. phagocytophilum* from salivary glands of naturally infected ticks and from spleens of wild sika deer in Japan. The

Table 1. Characterization of *p44/msp2* transcript variants from *A. phagocytophilum* in *Haemaphysalis* ticks and wild sika deer

p44/msp2 cDNA clone	Tick/deer species	Subcluster in Fig. 2	Closest relative	Similarity (%)/ accession no.
Tick15-1-mRNA-Goto-Hlong (8 clones)[1)]	*H. longicornis*	a3	Tick-162-12L-Iwate-ff-Ip	391/392 (99.7) FJ600596
Tick15-5-mRNA-Goto-Hlong	ibid	a3	Tick-162-12L-Iwate-ff-Ip	390/392 (99.5) FJ600596
Tick15-12-mRNA-Goto-Hlong (14 clones)	ibid	a3	Tick-162-12L-Iwate-ff-Ip	390/392 (99.5) FJ600596
Tick24-4-mRNA-Goto-Hform (5 clones)	*H. formosensis*	a3	Tick-162-12L-Iwate-ff-Ip	417/417 (100) FJ600596
Tick35-1-mRNA-Goto-Hform (9 clones)	ibid	a2	Tick-162-10L-Iwate-ff-Ip	394/395 (99.7) FJ600590
Tick35-5-mRNA-Goto-Hform	ibid	a1	*p44-35E*	379/380 (99.7) CP000235
Tick35-12-mRNA-Goto-Hform	ibid	a1	*p44-62*	392/392 (100) CP000235
Tick36-1-mRNA-Goto-Hform (7 clones)	ibid	a2	Tick-162-10L-Iwate-ff-Ip	395/395 (100) FJ600590
Tick36-12-mRNA-Goto-Hform	ibid	a2	Tick-162-10L-Iwate-ff-Ip	460/468 (98.3) FJ600590
Tick37-5-mRNA-Waka-Hmega	*H. megaspinosa*	a4	*p44-20*	373/374 (99.7) CP000235
Tick37-10-mRNA-Waka-Hmega (10 clones)	ibid	a4	*p44-20*	374/374 (100) CP000235
Tick37-14-mRNA-Waka-Hmega	ibid	a4	*p44-20*	373/374 (99.7) CP000235
Tick42-1-mRNA-Waka-Hmega (13 clones)	ibid	a1	*p44-35E*	378/380 (99.5) CP000235
Tick42-4-mRNA-Waka-Hmega	ibid	a1	*p44-35E*	379/380 (99.7) CP000235
Tick42-9-mRNA-Waka-Hmega	ibid	a1	*p44-35E*	379/380 (99.7) CP000235
Sika7-1-mRNA-Izu (12 clones)	*Cervus nippon*	b3	SS14-3	315/444 (70.9) DQ020151
Sika7-2-mRNA-Izu	ibid	b3	SS14-3	312/443 (70.5) DQ020150
Sika7-18-mRNA-Izu (3 clones)	ibid	b3	SS14-3	311/444 (70.0) DQ020151
Sika7-22-mRNA-Izu	ibid	b3	SS14-3	314/444 (70.7) DQ020151
Sika10-1-mRNA-Izu (9 clones)	ibid	b2	SS14-1	391/426 (91.8) DQ020151
Sika10-2-mRNA-Izu (3 clones)	ibid	b2	SS14-1	390/426 (91.5) DQ020144
Sika10-4-mRNA-Izu	ibid	b2	SS14-1	390/426 (91.5) DQ020151
Sika10-5-mRNA-Izu	ibid	b3	SS14-3	304/435 (69.9) DQ020146
Sika10-8-mRNA-Izu	ibid	b2	SS14-1	390/426 (91.5) DQ020144
Sika10-18-mRNA-Izu	ibid	b2	SS14-1	388/425 (91.3) DQ020144
Sika10-23-mRNA-Izu	ibid	b1	SS14-3	319/453 (70.5) DQ020144
Sika29-1-mRNA-Fuji	ibid	b1	SS14-7	290/424 (68.4) DQ020150
Sika29-2-mRNA-Fuji	ibid	b1	SS14-9	314/438 (71.6) DQ020151
Sika29-3-mRNA-Fuji	ibid	b1	SS14-3	339/465 (73.0) DQ020146
Sika29-4-mRNA-Fuji	ibid	b2	SS14-9	316/426 (74.2) DQ020152
Sika29-6-mRNA-Fuji	ibid	b4	SS14-3	357/489 (73.0) DQ020146
Sika29-8-mRNA-Fuji	ibid	b1	SS14-9	325/459 (70.9) DQ020146
Sika29-9-mRNA-Fuji (2 clones)	ibid	b1	SS14-3	342/483 (70.9) DQ020146
Sika29-10-mRNA-Fuji	ibid	b2	SS14-1	386/426 (90.5) DQ020144
Sika29-11-mRNA-Fuji	ibid	b4	SS14-5	297/411 (72.3) DQ020146
Sika29-12-mRNA-Fuji	ibid	b3	SS14-3	312/435 (71.7) DQ020146

[1)]: The number of identical cDNA clones obtained in this study are shown in parenthesis.

p44/msp2 transcripts obtained in this study were phylogenetically quite distinguishable between tick- and deer-associated ones. Only specific *p44/msp2* mRNA species such as the distinctive subclusters a1 to a4 shown in Fig. 2 seem to be transcribed selectively from the multiple *p44/msp2* repertories of *A. phagocytophilum* existing in the salivary glands of *Haemaphysalis* ticks, but such selective expression of the *p44/msp2* multigene family in wild sika deer does not appear to be strictly regulated. Furthermore, because mammalian host specificity of *A. phagocytophilum* and the *p44/msp2* sequence diversity has been reported (2), the differences between the sequences from ticks and deer might also be due to the strain variation of *A. phagocytophilum* in separate geographic regions in Japan. Thus, our study provides significant information for better understanding of *p44/msp2* expression of *A. phagocytophilum* in different life environments between naturally infected ticks and wild deer.

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Conflict of interest None to declare.

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NOTE *Bacteriology*

Detection of the New *Ehrlichia* Species Closely Related to *Ehrlichia ewingii* from *Haemaphysalis longicornis* in Yonaguni Island, Okinawa, Japan

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ABSTRACT. We collected a total of 206 *Haemaphysalis longicornis* ticks by flagging in pastures in Yonaguni Island, Okinawa, Japan, in April 2008. Four of the 206 tick DNA samples tested were positive in a polymerase chain reaction (PCR) screening for the 16SrRNA gene of Anaplasmataceae. Partial sequences of 4 PCR products were identical to each other. Longer sequences of the 16SrRNA gene were successfully determined in 2 of the 4 tick samples, and the obtained 1,392 bp and 1,300 bp sequences revealed high similarity to the 16SrRNA gene sequences of the validated *Ehrlichia* species, including *Ehrlichia ewingii, E. chaffeensis*, and *E. canis* (98.3–98.6%). We also sequenced 1,304 bp of the *groEL* gene from the 2 tick samples, and found that these had the highest similarity to sequences of *E. ewingii* (94.0–94.4%) in the validated ehrlichial species. Based on the 16SrRNA and *groEL* gene sequences, the ehrlichial agents detected in this study were similar to the *Ehrlichia* species detected in Asia and may compose a new *Ehrlichia* species with other *Ehrlichia* species detected in Asia.

KEY WORDS: 16SrRNA, *Ehrlichia*, *groEL*, *Haemaphysalis longicornis*.

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Ehrlichia and *Anaplasma* species are Gram-negative, obligate intracellular bacteria, and are reported as pathogens of emerging diseases [2, 4]. Bacteria in the genera *Ehrlichia/Anaplasma* are transmitted by blood-sucking arthropods, including ticks, fleas, and lice, the reservoirs of which are wild mammals such as rodents and deer [5, 15]. Recently, *Ehrlichia/Anaplasma* species have been detected in mammals and ticks in Japan [6, 11]. In Yonaguni Island, which is the westernmost island of Japan and lies between the East China Sea and the Pacific Ocean (Fig. 1), *Anaplasma phagocytophilum* and *A. bovis* were detected in peripheral blood samples of grazing cattle in 2006 [11]. However, no survey of ticks in Yonaguni Island for *Ehrlichia/Anaplasma* species has been performed, and the relationship between ticks and *Ehlrichia/Anaplasma* species in Yonaguni Island is unclear. The present study aimed to reveal how ticks were involved in the transmission of *Ehlrichia/Anaplasma* species by using DNA-based tools to test for the presence of *Ehrlichia/Anaplasma* species in ticks in Yonaguni Island.

We used flagging methods to collect a total of 206 ticks from 6 grazing pastures on Yonaguni Island in April 2008. Following tick identification, we extracted DNA from each tick using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Tick DNA samples were stored at –30°C until use.

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Tick DNA samples were screened by PCR for 345 bp of the 16SrRNA gene of Anaplasmataceae using the primer pair EHR16SD and EHR16SR [12]. Sequences of positive PCR products were determined through direct sequencing by BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, U.S.A.). Obtained sequences were compared to known sequences using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Tick DNA samples positive for *Ehrlichia/Anaplasma* species were used in PCR and sequencing of nearly the full length of the 16SrRNA gene and part of the *groEL* gene. The 16rRNA gene was amplified with primer pairs fD1 and EHR16SR, as well as EHR16SD and Rp2, which amplify the 16SrRNA gene of Anaplasmataceae bacteria [7]. Nested PCR was used to amplify approximately 1,300 bp of the *groEL* gene of *Ehrlichia* species. Primer pairs HS1a and HS6a as well as HS43 and HSVR were used in the 1st and 2nd PCRs, respectively [10]. Sequencing and BLAST search of PCR products were performed as described above. Phylogenetic trees based on 16SrRNA and *groEL* genes were constructed using the ClustalW software program [14] and MEGA4 software [13] by the neighbor-joining method. The 16SrRNA gene sequences of Yonaguni138 and Yonaguni206 were submitted to GenBank with accession numbers HQ697588 and HQ697589, respectively, and the *groEL* gene sequences of Yonaguni138 and Yonaguni206 were submitted with accession numbers HQ697590 and HQ697591, respectively.

All ticks, comprising 31 females and 175 nymphs, were

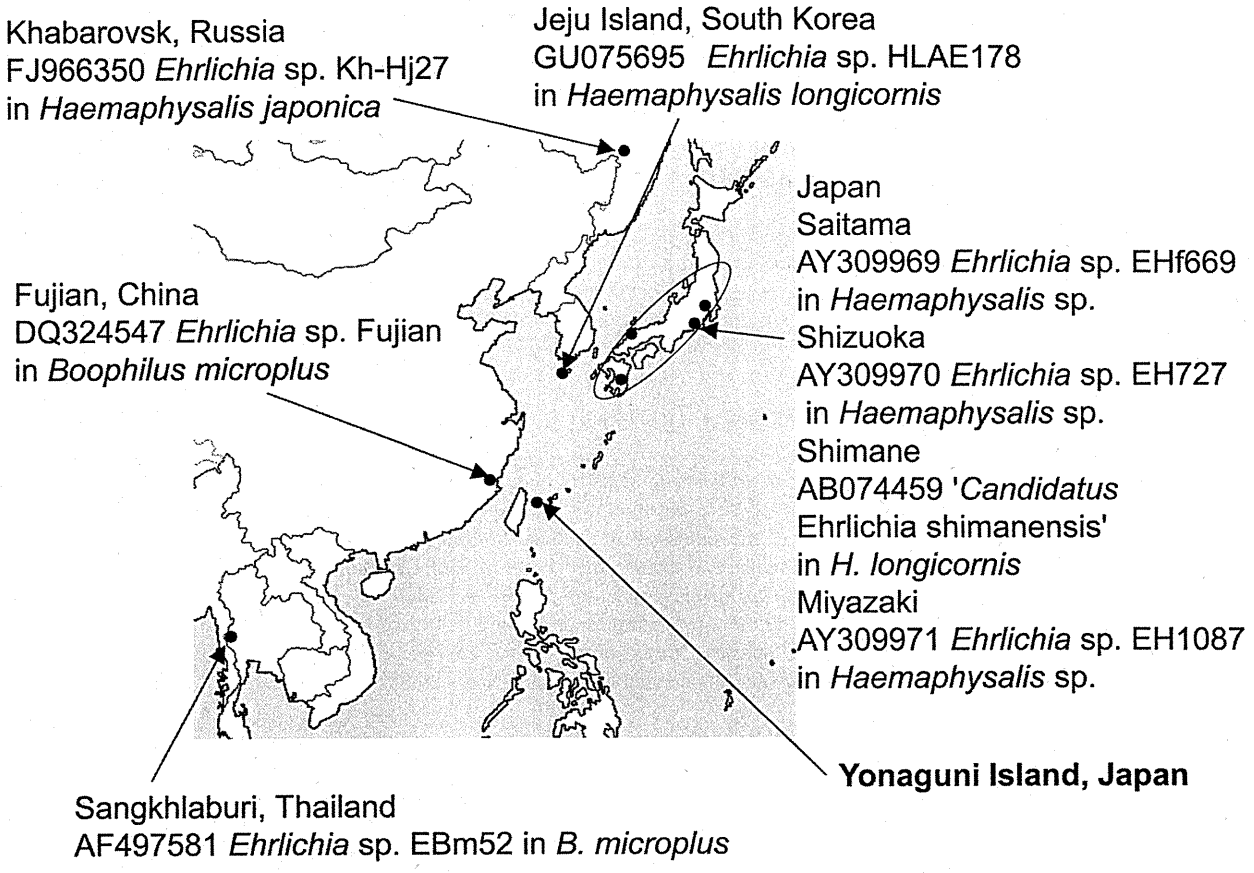

Fig. 1. Geographical relationship of Yonaguni Island with Asian countries. The ehrlichial agents close to *Ehrlichia* species detected in this study, including GenBank accession numbers, tick species and country name, were shown in this figure.

identified as *Haemaphysalis longicornis*, which may be caused by that all ticks were collected from grazing pastures. Two females (numbers 138 and 206) and 2 nymphs of 206 tick DNA samples tested were positive in the PCR screening (1.9%). Sequences (305 bp) of 4 positive PCR products were identical and showed high similarity to the 16SrRNA gene in *Ehrlichia* species. We successfully sequenced the 1,392 bp and 1,300 bp sequences using 16SrRNA primers (numbers 138 and 206, respectively) and 1,304 bp sequences using *groEL* primers in 2 of the female samples. The BLAST search for the obtained sequences using 16SrRNA primers showed the highest similarity (99.8–99.9%) to the 16SrRNA gene of *Ehrlichia* sp. HLAE178 detected from *H. longicornis* in South Korea. The ehrlichial species with the highest similarity to sequences from Japan was *Ehrlichia* sp. EH727 detected in *Haemaphysalis* sp. in Shizuoka Prefecture (99.1%). In the validated ehrlichial species, the 16SrRNA gene sequences of *E. chaffeensis*, *E. canis*, and *E. ewingii* had relatively high similarity to the obtained sequences (98.3–98.6%). The 1,304 bp *groEL* sequences obtained from 2 female ticks in this study showed the highest similarity to that of *Ehrlichia* sp. Kh-Hj27 (94.2–94.4%), which was detected in *Haemaphysalis japonica* in Khabarovsk, Russia. Our obtained sequences had the highest similarity to the *groEL* genes of *Candidatus E. shimanensis* (91.8%) and *E. ewingii* (94.0–94.4%) in the Japanese ehrlichial agents and the validated species, respectively. *E. ewingii* is a pathogenic agent to humans and dogs, and infection with *E. ewingii* causes fever, headache, thrombocytopenia, and leukopenia [1, 2]. The ehrlichial species detected in *H. longicornis* from pastures on Yonaguni Island may interact with cattle. Further study of the pathogenesis and epidemiology of *Ehrlichia* agents is needed.

Sequences of 16SrRNA and *groEL* genes obtained in this study have relatively low similarity to the validated *Ehrlichia* species and made unique clades with other *Ehrlichia* species, including *Ehrlichia* sp. EBm52, *Ehrlichia* sp. HLAE178, and *Ehrlichia* sp. Kh-Hj27, which were detected in ticks in Thailand, South Korea, and the Russian Far East, respectively (Figs. 1, 2 and 3). These *Ehrlichia* species detected in Asia may represent a new *Ehrlichia* species. Of *Ehrlichia* species shown in figure 1, *Ehrlichia* sp. HLAE178 and '*Candidatus* Ehrlichia shimanensis' were detected in *H. longicornis*, and the distribution of the ehrlchial species close to Yonaguni 138 and 206 were included in that of *H. longicornis*. Further study including isolation of the ehrlichial agent and more genetic analysis is needed.

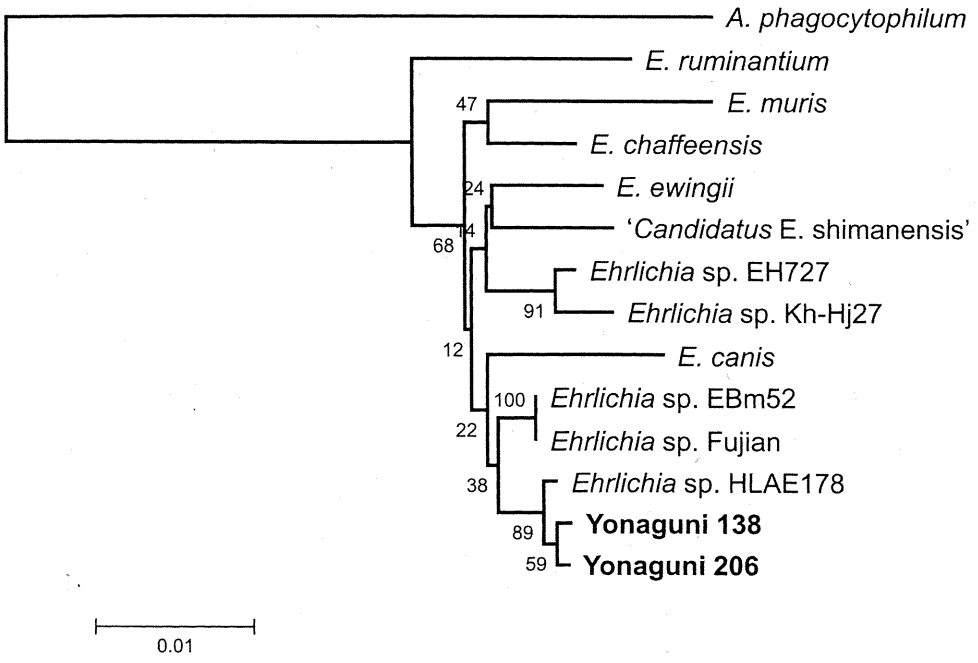

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method based on sequences of the 16SrRNA gene. Yonaguni 138 and Yonaguni 206 were obtained from *H. longicornis* in this study. Numbers at branch nodes indicate bootstrap values. GenBank accession numbers for the sequences used to construct the trees are as follows: *E. canis* (M73221), *E. chaffeensis* (M73222), *E. ewingii* (U96436), *E. muris* (U15527), *E. ruminantium* (U03776), *Candidatus* E. shimanensis (AB074459), *Ehrlichia* sp. EBm52 (AF497581), *Ehrlichia* sp. EH727 (Y309970), *Ehrlichia* sp. Fujian (DQ324547), *Ehrlichia* sp. HLAE178 (GU075695), *Ehrlichia* sp. Kh-Hj27 (FJ966350), and *A. phagocytophilum* (CP000235).

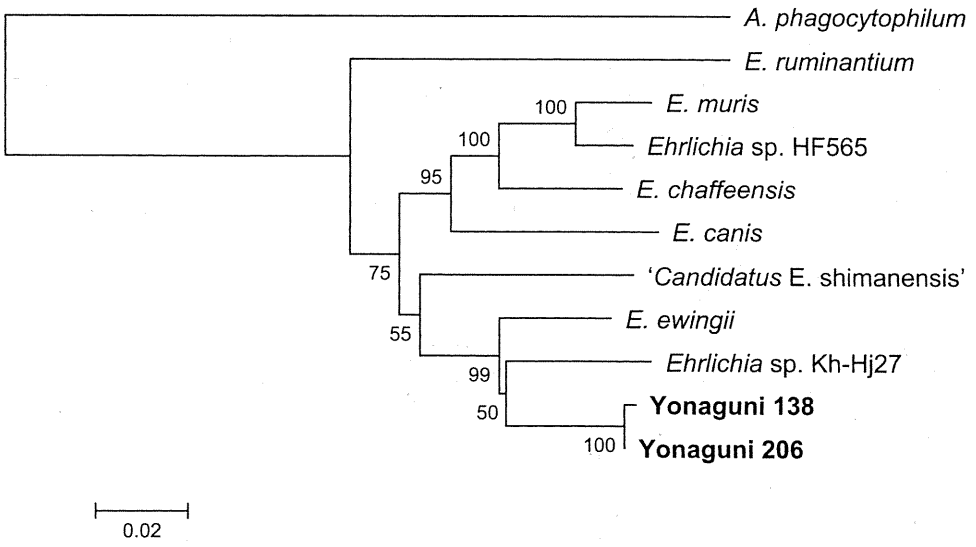

Fig. 3. Neighbor-joining tree based on the sequences of *groEL* gene. Yonaguni 138 and Yonaguni 206 were obtained from *H. longicornis* in the present study. Numbers at branch nodes indicate bootstrap values. GenBank accession numbers for the sequences used to construct the trees are as follows: *E. canis* (U96731), *E. chaffeensis* (L10917), *E. ewingii* (AF195273), *E. muris* (AF210459), *E. ruminantium* (DQ647014), *Candidatus* E. shimanensis (AB074462), *Ehrlichia* sp. HF565 (AB032712), *Ehrlichia* sp. Kh-Hj27 (FJ966349), and *A. phagocytophilum* (CP000235).

An examination of blood samples from cattle on Yonaguni Island in 2006 showed a high prevalence of *A. phagocytophilum* and *A. bovis* [11]. However, *Anaplasma* species were not detected in *H. longicornis* in the present study. The grazing pastures in this study were apart from the farm surveyed previously. In South Korea, *A. phagocytophilum* and *A. bovis* were detected in *H. longicornis* [8], which suggests that *H. longicornis* can harbor these *Anaplasma* species. Moreover, *A. phagocytophilum* was detected in the rodent, *Apodemus agrarius* [3, 9]. The prevalence rate of *A. phagocytophilum* in *H. longicornis* was relatively low (0–2.4 %) [3, 7, 9], however, that in *A. agrarius* was high (5.6–23.6 %). The distribution of rodents in Yonaguni Island was unclear, and the rodents were not tested in this study. The absence of *A. phagocytophilum* and *A. bovis* in this study may indicate that reservoirs of *A. phagocytophilum* and *A. bovis* were not located in the studied pastures and/or that the number of tested ticks was insufficient to detect *Anaplasma* species.

Prevalence of *Ehrlichia* species of *H. longicornis* in this study was 1.1% (2 of 175) in nymphs and 6.5% (2 of 31) in adults, which may indicate that larvae and/or nymphs of *H. longicornis* become possessed of ehrlichial agents when they take blood from the hosts on the pasture on Yonaguni Island. However, the nature of the reservoir for *Ehrlichia* species on Yonaguni Island is not clear. Further study to reveal infection rates in mammals, including rodents, in pastures on Yonaguni Island is needed.

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Original article

Molecular detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in *Ixodes ricinus* ticks from Istanbul metropolitan area and rural Trakya (Thrace) region of north-western Turkey

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ABSTRACT

We demonstrated the presence of the agent of human granulocytic anaplasmosis (HGA), *Anaplasma phagocytophilum*, and the agent of Lyme borreliosis, *Borrelia burgdorferi* sensu lato, in north-western Turkey. A total of 241 questing *Ixodes ricinus* adult ticks were sampled by flagging from recreational parks of the Asiatic and European sides of the heavily populated Istanbul metropolitan area and rural forests of Kirklareli located in the Thrace region in 2008. Both tick-borne pathogens were detected and identified by PCR and DNA sequencing analysis. *A. phagocytophilum* infection rates were 2.7% in Istanbul and 17.5% in the Kirklareli area. *B. burgdorferi* sensu lato infection rates were 38.7% in Istanbul and 11.4% in the Kirklareli area. Only 3 of 241 ticks were coinfected with *A. phagocytophilum* and *B. burgdorferi* sensu lato.

Introduction

Lyme borreliosis (LB) is the most prevalent tick-borne zoonotic disease and is caused by *Borrelia burgdorferi* sensu lato genospecies in Europe, North America, and Far-East Asia (Gern, 2008; Steere, 2001, 2006). *B. burgdorferi* sensu lato is currently classified into 19 species including non-validated species (Masuzawa, 2004; Margos et al., 2009). In Europe, the vector tick *Ixodes ricinus* can transmit 6 genospecies, *B. burgdorferi* sensu stricto, *B. garinii* (Type 20047 including *B. bavariensis*), *B. afzelii*, *B. lusitaniae*, *B. spielmanii*, and *B. valaisiana* (Fingerle et al., 2008; Margos et al., 2009; Wang et al., 1999; Wilske, 2005). Of those 6 species, *B. burgdorferi*, *B. afzelii*, *B. garinii*, *B. lusitaniae*, and *B. spielmanii* are known to be pathogenic to humans (Collares-Pereira et al., 2004; Steere, 2006).

Another tick-borne disease with public health importance is human granulocytic anaplasmosis (HGA) which is caused by *Anaplasma phagocytophilum* (Dumler and Bakken, 1995; Dumler et al., 2001). It has been recognized as a pathogen for cattle, sheep, and goats in Europe (Blanco and Oteo, 2002). In 1992, *A. phagocytophilum* was first recognized as a human pathogen in the United States (Chen et al., 1994), and in Europe, the first case of HGA was described in 1997 (Petrovec et al., 1997). Coexistence of *A. phagocytophilum* with *B. burgdorferi* sensu lato is attributed to common vectors, *I. ricinus* in Europe, *I. scapularis* in North America, and *I. persulcatus* in Asia (Masuzawa et al., 2008; Parola et al., 2005; Stanczak et al., 2004). In Turkey, *A. phagocytophilum* infections in sheep, cattle, and in *I. ricinus* attached to humans in the Middle and Eastern Black Sea regions have been reported (Aktas et al., 2010; Gokce et al., 2008). However, no clinical cases of HGA have been reported in this country so far. Additionally, the geographical distribution and prevalence of *A. phagocytophilum* in questing ticks have not been determined in Turkey.

The aim of this study was to investigate the existence and infection rates of *A. phagocytophilum* and *B. burgdorferi* sensu lato in *I. ricinus* collected in the heavily populated Istanbul metropolitan area and in Kirklareli, a rural region which shares the same climatic conditions with Istanbul.

Materials and methods

Tick collection

Questing *I. ricinus* ticks were collected from the vegetation using a 1-m^2 flannel flag once a week during 3 weeks in June, 2008, at

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selected Asiatic and European sites including recreational parks of the heavily populated Istanbul metropolitan area and countryside of Kirklareli in north-western Turkey (Fig. 1). The sampling areas in Istanbul and Kirklareli have a temperate climate composed of 4 seasons. It is described with warm summers (10–29 °C for the Istanbul area and 22–24 °C for Kirklareli) and mild winters (4–15 °C for Istanbul and 5–7 °C for Kirklareli). The precipitation values were measured as an average of 33 mm for Istanbul and 800 mm for Kirklareli in summer and 107 mm for Istanbul and 1000 mm for Kirklareli in winter.

In Istanbul, the vegetation zones of our sampling sites from both Asiatic and European sections of the city can be classified into 2 groups: (i) scrub and forest as natural vegetation, grove, park and housing gardens made by human efforts; (ii) scrubs which occur as a result of destruction of forests are generally found together with forest. The main features of vegetation in our sampling sites of Istanbul were damp, mixed-leaf forests, and the dominant trees are oaks (*Quercus robur*, *Q. petraea*, and *Q. frainetto*) which are spread over a broad area. The features of vegetation in the Kirklareli rural area included deciduous forest of *Fraxinus ornus*, *Alnus glutinosa*, *Ulmus campestris*, and *Salix* sp. with dense canopy layer and leaf litter, at a maximum of 100 m inland.

DNA extraction

The tick samples were disrupted for 30 s using 3 mm tungsten carbide beads in 1.5 mL tubes of TissueLyser (Qiagen, Haan, Germany). Total DNA was isolated from whole tick tissues using QuickGene-800 Nucleic-acid Isolation System (Fuji Film, Tokyo, Japan). The quality of the extracted DNA was evaluated by PCR, targeting the tick ribosomal DNA internal transcribed spacer 2 (ITS2) with primer sets 5.8S F3/1 and 28S R1/1 according to the previously described method (Fukunaga et al., 2000).

Detection of A. phagocytophilum by PCR and sequencing

To detect specific DNA of *Anaplasma* species, 16S rRNA gene (*rrs*) and *p44/msp2* were amplified by PCR, and all amplified DNAs were sequenced to confirm the specificity of PCR-amplicons. *rrs* was amplified with forward primer EC9 and reverse primer EC12A (Kawahara et al., 2006) in the first-step PCR. For the nested PCR, mixtures of forward primer Abpp (5′-TACTGCCAGACTAGAGTCCGGGA-3′) specific to sequences of *Anaplasma bovis*, *A. phagocytophilum*, and *A. platys*, and another forward primer Acom (5′-TACTGCAGGACTAGAGTCCGGAA-3′) specific to sequences for *A. centrale*, *A. ovis*, and *A. marginale*, and reverse primer AP-R (5′-TTGCAACCTATTGTAGTC-3′) were used as inner primer sets, which amplified approximately 600 base pairs of DNA. For DNA sequencing analysis, the nested PCR amplicons purified with Microcon-PCR purification column (Millipore, Bedford, MA, USA) were subjected to DNA cycle-sequencing analysis using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with an ABI 3130-Avant Genetic Analyzer (Applied Biosystems).

To detect *p44/msp2* gene of *A. phagocytophilum*, PCR was conducted using primers p3726F and p4257R for the first step PCR and then p3761F and p4183R for nested PCR as described previously (Ohashi et al., 2005; Lin et al., 2002; Zhi et al., 1999). The nested-PCR products (approximately 300–400 bp) were cloned into the $_{p}$CR2.1 vector using TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and introduced into *E. coli* DH5a. For sequencing, the recombinant *E. coli* clones were randomly selected and the plasmid DNA in the colony was amplified using Illustra Templiphi DNA amplification kit (GE Healthcare, Buckinghamshire, UK) at 30 °C for 18 h according to the manufacturer's protocol (Masuzawa et al., 2008). Two to four clones for each amplicons were subjected to sequencing analysis.

Detection and genotyping of Borrelia spp.

For detection of *B. burgdorferi* sensu lato species, 5S-23S rDNA intergenic spacer (IGS) was amplified with primer set RIS1 and RIS2 (Postic et al., 1994), in the first-step PCR. For the nested PCR, inner primer sets RIS3 (5′-GGA GAG TAG GTT ATT GCC AGG-3′) and RIS4 (5′-GAC TCT TAT TAC TTT GAC C-3′) which amplify approximately 200 bp DNA were used. The PCR amplicon was subjected to DNA cycle-sequencing analysis.

Phylogenetic analyses

rrs and *p44/msp2* sequences determined in this study were deposited into DDBJ/EMBL/GenBank under accession numbers AB262330–AB262358 and AB604782–AB604784. A phylogenetic tree was constructed on the basis of alignment of *rrs* sequences by Clustal W algorithm using sequence analysis software, MegAlign (DNASTAR Inc., Madison, WI, USA), followed by the neighbour-joining method with 1000 bootstrap resampling. The plotted NJ tree was arranged with software NJ plot.

Results and discussion

Infection rates

DNAs extracted from 241 questing adult *I. ricinus* ticks were ITS2-PCR-positive (data not shown) and were subjected to PCR to detect *A. phagocytophilum* and *Borrelia burgdorferi* sensu lato.

The numbers of *rrs* and/or *p44/msp2* PCR-positive samples are shown in Table 1. In the Istanbul metropolitan area, a lower prevalence for *A. phagocytophilum* (2.7%) and a higher prevalence for *B. burgdorferi* sensu lato (38.7%) were observed. In 3 countryside sampling regions, Avcilar, Hamdibey, and Longoz in Kirklareli, located in north-western Thrace, a higher prevalence for *A. phagocytophilum* (17.5%) and a lower prevalence for *B. burgdorferi* sensu lato species (11.4%) in ticks were observed in comparison with those found in Istanbul. The differences are statistically significant between the 2 regions. Three of 241 ticks were coinfected with *A. phagocytophilum* and *B. lusitaniae*, *B. garinii* (20047 Type), or *B. afzelii*. In this study, *A. phagocytophilum*-specific DNA was detected by 2 PCR systems targeted to *rrs* and *p44/msp2*, respectively. PCR targeted to *rrs* could detect *A. phagocytophilum* and also other *Anaplasma* species. The genetic differences of pathogens could be revealed by a phylogenetic analysis of *rrs* genes. On the other hand, the *p44/msp2* multigene family which encoded 44-kDa major surface proteins (P44/Msp2) of *A. phagocytophilum* were composed of a hypervariable region and a conserved region on its 5' and 3' end (Lin et al., 2002). The strain HZ isolated from a patient in the United States has 113 *p44/msp2* loci in its genome (Hotopp et al., 2006). With respect to nature, the PCR targeted to *p44/msp2* genes showed a higher sensitivity than that of PCR targeted to *rrs*.

It has been reported that domestic animals such as sheep and cattle were infected with *A. phagocytophilum* at a rate of 10–15% in the eastern part of the Black Sea region (Gokce et al., 2008). Aktas et al. (2010) found a prevalence of 17.0% for *A. phagocytophilum* in pooled samples of *I. ricinus* removed from humans living in the eastern part of the Black Sea region. Those infection rates in *I. ricinus* are equivalent to that found in north-western Turkey in this study.

Rosef et al. (2009) reported that the prevalence of *A. phagocytophilum* infection in *I. ricinus* was significantly higher in localities with a high density of wild cervids, roe deer, and red deer, in contrast to a lower prevalence of *B. burgdorferi* sensu lato. Kurtenbach et al. (1998) showed that the reservoir competence of vertebrate

Table 1
Detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato from *Ixodes ricinus* sampled in north-western Turkey.

Province	Survey site	No. ticks	No. positive ticks (%)				
			Anaplasma [a]			*Borrelia*	
			p44	*rrs*	*p44+rrs* [b]	IGS	
Istanbul	Tasdelen (Asia)	24	0	1	1	10	
		75				2 (2.7)	29 (38.7)
	Zekeriyakoy (Europe)	51	1	1	1	19	
						p<0.01 [c]	p<0.01 [c]
Kirklareli (Europe)	Avcilar	62	10	9	12	4	
	Hamdibey	41	166 8	8	11	29 (17.5) 5	19 (11.4)
	Longoz	63	4	5	6	10	
Total		241	23	24	31	48	

[a] The number of ticks infected with *A. phagocytophilum* is shown in the table. Two unknown *Anaplasma* species detected (AB604784) are not included the number of positive ticks.
[b] Number of *p44*-PCR positive and/or *rrs*-PCR positive ticks.
[c] The difference in proportion is significant ($p < 0.01$) between 2 regions in the evaluation of Fisher's exact test.
Three out of 241 ticks were coinfected with *A. phagocytophilum* and *B. lusitaniae*, *B. garinii*, or *B. afzelii*.

species for *B. burgdorferi* sensu lato species could be elucidated by complement-mediated borreliacidal effects. *B. garinii*, *B. valaisiana*, and *B. afzelii* are sensitive to sera from ruminants, sheep, cattle, and deer, which play important roles as reservoir hosts for *A. phagocytophilum*. These findings approve the supposition that cervids are no reservoir hosts of *B. burgdorferi* sensu lato (Kurtenbach et al., 2002). In Kirklareli, our sampling areas included pastureland of cattle, goats, and sheep. Higher infection rates for *A. phagocytophilum* and lower infection rates for *B. burgdorferi* sensu lato in Kirklareli, and lower infection rates for *A. phagocytophilum* and higher infection rates for *B. burgdorferi* sensu lato in the Istanbul metropolitan area might be related to the differences in the density of ruminants in the 2 areas. In this study, 3 of 241 ticks were coinfected with *A. phagocytophilum* and *Borrelia* species. The low coexistence rate suggests that these pathogens have different reservoir hosts and that there is a varying complement-sensitivity of *Borrelia* to ruminants.

Identification of Borrelia burgdorferi sensu lato

The *B. burgdorferi* sensu lato genospecies identified in this study are shown in Table 2. *B. lusitaniae* infection in ticks dominated with 59.1% followed by *B. garinii*, *B. afzelii*, and *B. valaisiana*. *B. burgdorferi* sensu stricto has not been found in either heavily populated metropolitan areas or countryside of north-western Turkey in this survey. We previously obtained one isolate of *B. burgdorferi* sensu stricto from *I. ricinus* collected in the same sampling area, but the density of *B. burgdorferi* sensu stricto in the Turkey is low (Guner et al., 2003). This genospecies is mostly found in western Europe (Wang et al., 1999).

B. lusitaniae is the predominant species in countries of the Mediterranean basin, such as Portugal, Morocco, and Tunisia (De Michelis et al., 2000), and is associated with the lizards *Lacerta agilis* and *Podarcis muralis* in Germany (Richter and Matuschka, 2006) and *Psammodromus algirus* in Tunisia (Dsouli et al., 2006).

Fig. 1. Geographic location of tick sampling sites in the Istanbul metropolitan area and Kirklareli in the north-western part of Turkey. Tasdelen and Zekeriyakoy regions located in Istanbul metropolitan area are indicated by numbers 1 and 2, respectively. Avcilar, Hamdibey, and Longoz regions positioned in Kirklareli area in the Thrace region of north-western Turkey are shown as number 3, 4, and 5, respectively.

Table 2
Borrelia burgdorferi sensu lato species detected in *Ixodes ricinus* sampled in north-western Turkey.

Province	Survey site	No. of *B. burgdorferi* sensu lato species identified (%).			
		B. afzelii	*B. garinii*	*B. lusitaniae*	*B. valaisiana*
Istanbul	Tasdelen	0	3	6	1
	Zekeriyakoy	1	4	11	3
Kirklareli	Avcilar	0	0	3	1
	Hamdibey	3	2	0	0
	Longoz	1	1	8	0
Total		5(10.4)	10(20.8)	28(58.3)	5(10.4)

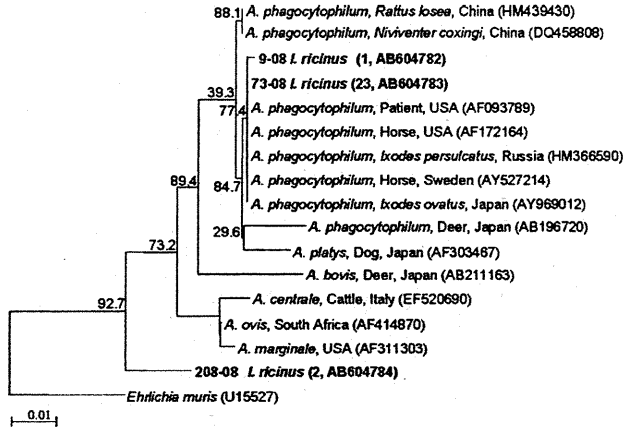

Fig. 2. Phylogenetic tree of *rrs* sequences of *A. phagocytophilum* in Turkey. The phylogenetic tree was constructed on the basis of alignment of *rrs* sequences by Clustal W algorithm followed by the neighbour-joining method with 1000 bootstrap resampling. The sequences obtained from Turkish ticks are indicated with bold face type. Sequence accession numbers are in parentheses. Bootstrap values are indicated on the nodes.

In Bulgaria, the neighbouring country, *B. afzelii* associated with wild rodents, was the predominant *Borrelia* species, while *B. lusitaniae* was rarely found (Christova et al., 2003). It may be speculated that this difference resulted from differences in major reservoir animals for *B. burgdorferi* sensu lato species between Turkey and Bulgaria.

Phylogenetic analysis on the basis of rrs sequence

To identify the *Anaplasma* species, partial *rrs* sequences were determined. A phylogenetic tree was prepared using these and previously reported sequences (Fig. 2). Twenty-three sequences (AB604783) of 24 positive samples (Table 1) were identical to those of *A. phagocytophilum* previously reported and the remaining one (AB604782) showed one base substitution. The other 2 sequences (AB604784) were distinct from those and also from those of other *Anaplasma* species. The phylogenetic analysis of *p44/msp2* sequences from Turkey (AB262330–AB262358), the United States, and the United Kingdom revealed that there was no significant relationship between these sequences and sampling sites (data not shown).

In conclusion, we demonstrated that *I. ricinus* ticks were infected with LB and HGA agents sampled in the Istanbul metropolitan area and Kirklareli, a countryside region in north-western Turkey. This study indicates the public health significance of both LB and HGA infections in the recreational parks of the Istanbul metropolitan area as well as in rural regions of north-western Turkey.

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FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Restriction of the growth of a nonpathogenic spotted fever group rickettsia

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Abstract

The growth kinetics of pathogenic and nonpathogenic rickettsiae were compared to elucidate the mechanism responsible for the pathogenicity of rickettsiae. Vero and HeLa cells derived from mammals were inoculated with a nonpathogenic species of spotted fever group rickettsia, *Rickettsia montanensis*, before being infected with the pathogenic species *Rickettsia japonica*. The mammalian cells became persistently infected with *R. montanensis* and produced low levels of rickettsiae. On the other hand, superinfection of the *R. montanensis*-infected cells with *R. japonica* resulted in increased yields of *R. montanensis* accompanied by *R. japonica* growth. Both rickettsiae also grew well in the *R. japonica*-infected cells subjected to superinfection with *R. montanensis*. Western blotting with an antibody to the autophagy-related protein LC3B found that autophagy was induced in the cells infected with *R. montanensis* alone. On the contrary, autophagy was restricted in the cells that were co-infected with *R. japonica*. Electron microscopy of the cells infected with *R. montanensis* alone demonstrated rickettsia particles being digested in intracytoplasmic vacuoles. Conversely, many freely growing rickettsiae were detected in the co-infected cells.

Introduction

Rickettsiae are the causative agents of spotted fever group (SFG) and typhus group rickettsioses. Many factors have been found to be important for the pathogenicity of rickettsiae.

As an obligate intracellular pathogen, rickettsiae adhere to and invade host cells to grow and express their pathogenicity. Rickettsial outer membrane proteins (rOmp) A and B have been demonstrated to play roles in these initial steps in various studies, including studies using recombinant *Escherichia coli* expressing these proteins on their surfaces (Li & Walker, 1998; Uchiyama, 2003; Uchiyama *et al.*, 2006; Chan *et al.*, 2009). After their invasion, rickettsiae are able to rapidly escape from phagosomes by secreting phospholipase D and hemolysin C, which lyse lipid membranes (Whitworth *et al.*, 2005).

Rickettsiae also activate NF-κB, which inhibits host cell apoptosis and hence enhances the yield of rickettsiae (Joshi *et al.*, 2004). Oxidative stress is one of the candidate mechanisms responsible for the injuries suffered by endothelial cells infected with rickettsiae. Endothelial cells that have been infected with SFG rickettsiae (SFGR) produce reactive oxygen species, which cause lipid peroxidative damage to the host cell membrane (Rydkina *et al.*, 2004; Sahni *et al.*, 2005).

From genomic analysis, it is also known that rickettsiae possess homologs of genes related to the pathogenicity of other microorganisms (Andersson *et al.*, 1998; Ogata *et al.*, 2001). For example, rickettsiae possess a homolog of the *virB* operon, which is known to be related to the Type IV secretion system (T4SS), containing a reduced number of genes. However, no genes for effectors of the T4SS have been identified in rickettsiae. Moreover, of the ten *Staphylococcus aureus* virulence factor genes (*capA–M*), which are involved in the biosynthesis of capsular polysaccharides, three of them have homologs in the rickettsia genome (Lin *et al.*, 1994). The rickettsia genome also

possesses *lpxA-D* genes, which are involved in the biosynthesis of lipid A, the active center of the endotoxin LPS. These factors might also act as virulence factors in rickettsiae.

Recently, the whole genome sequences of many rickettsial species including pathogenic and nonpathogenic species have been reported. A comparative study of these rickettsial genomes suggested that the inactivation of some genes by genome reduction abrogates host-induced rickettsial growth restriction. Indeed, Fournier *et al.* (2009) described a paradoxical relationship between smaller genome size and higher pathogenicity in rickettsiae.

A comparative study of avirulent and virulent strains of *Rickettsia rickettsii* revealed that the *relA/spoT* gene is essential for the growth restriction of rickettsiae in host cells (Clark *et al.*, 2011).

Autophagy is one of the innate defense systems against invading pathogens. Among such pathogens, *Listeria monocytogenes*, *Shigella*, and *Burkholderia pseudomallei* are known to be able to evade autophagy (Sasakawa, 2010). In the case of *Listeria*, recruitment of the Arp2/3 complex and Ena/VASP to the bacterial surface via the bacterial ActA protein disguises the bacteria from autophagic recognition (Yoshikawa *et al.*, 2009). *Shigella* evades autophagic recognition by secreting IcsB via the type III secretion system (TTSS) (Ogawa *et al.*, 2005). A factor secreted by *B. pseudomallei* TTSS, BopA protein, also plays a role in autophagy evasion (Cullinane *et al.*, 2008). Interestingly, the BopA protein shares 23% homology with IcsB, a factor secreted by *Shigella*.

Here, to establish the cellular basis of rickettsial pathogenicity, we conducted a comparative study of the growth of pathogenic and nonpathogenic rickettsiae in mammalian cells.

As a result, we suggest that autophagy is at least partially responsible for the growth restriction of nonpathogenic rickettsiae.

Materials and methods

Cells

Vero cells derived from the kidney of an African green monkey and HeLa cells derived from a human uterus were used as mammalian cells.

Rickettsial strains

Rickettsia japonica strain YH, which is the major causative agent of SFG rickettsioses in Japan and has been clinically isolated from patients with the condition (Uchida *et al.*, 1988, 1989, 1992; Uchiyama, 1999), was used as a pathogenic SFGR. On the other hand, *Rickettsia montanensis* strain Tick, which was first isolated from a tick (Bell *et al.*, 1963), was used as a nonpathogenic SFGR. These rickettsiae were propagated in Vero cells, partially purified as described previously, and then used for infection.

Growth kinetics of rickettsiae

Vero and HeLa cell monolayers in 12-well plates were inoculated with one of the rickettsiae strains at a multiplicity of infection (MOI) of 0.01 PFU per cell and incubated in Eagle's minimal essential medium containing 2% fetal bovine serum at 34 °C. The medium was changed every 3 days, and the yields of rickettsiae were plaque-assayed on Vero cell monolayers, as described previously (Uchida *et al.*, 1992). Then, some of the cells that had been infected with *R. montanensis* were also infected with *R. japonica* at the same MOI on day three of infection and treated in the same manner as the cells subjected to single infection. In addition, some of the *R. japonica*-infected cells were also infected with *R. montanensis* and cultured in the same manner. To assess the yields of each rickettsial species in the cells that had been co-infected with *R. montanensis* and *R. japonica*, the cell monolayers in the 12-well plates were inoculated with serially diluted rickettsial samples for 2 h and then overlaid with Leibovitz L-15 medium containing 0.5% methylcellulose and 2% fetal bovine serum. After being incubated at 34 °C for 4 days in a closed container, the monolayers were washed with PBS twice and then fixed with ethanol for 10 min, before being air-dried. Species-specific monoclonal antibodies and the VECTASTAIN Elite ABC Kit (Mouse IgG) (Vector Laboratories, Inc., CA) were used to immunostain the infectious centers of each rickettsia, according to the manufacturer's instructions.

Western blotting of the infected cells

Vero cells were inoculated with *R. montanensis* at an MOI of 0.01 PFU per cell and incubated at 34 °C, and the medium was changed every 3 days.

Some of the *R. montanensis*-infected cells were also co-infected with *R. japonica* at an MOI of 0.01 PFU per cell on day 3 of infection. The infected cells were solubilized every 3 days and analyzed by Western blotting using rabbit antibody to the autophagy-related protein LC3B (Cell Signaling Technology Japan, K.K. Tokyo, Japan). Rabbit monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology Japan, K.K.) was also used to detect the housekeeping protein as an internal control.

Vero cells were also inoculated with *R. japonica* and incubated in the same manner. Some of the *R. japonica*-

infected cells were also co-infected with *R. montanensis*. These cells were also analyzed by Western blotting.

Transmission electron microscopy

Confluent monolayers of Vero cells were inoculated with *R. montanensis* at an MOI of 0.01 PFU per cell. On day 7 of infection, the infected cells were washed with PBS and processed for electron microscopic observation, as described previously (Uchiyama & Uchida, 1988). Thin sections of the samples were then observed under a Hitachi H-7650 transmission electron microscope (TEM). After some of the *R. montanensis*-infected cells had been superinfected with *R. japonica* on day 3 of infection, they were processed on day 7 of infection and observed under a TEM. The numbers of rickettsiae in more than one hundred sections of *R. montanensis*-infected cells and *R. montanensis*- and *R. japonica*-co-infected cells were counted and compared.

Results

Growth of *R. montanensis* in Vero and HeLa cells

As shown in Fig. 1a, the nonpathogenic species *R. montanensis* infected the Vero cells in a persistent manner, resulting in constant, low-level rickettsiae production. On the other hand, when the *R. montanensis*-infected cells were superinfected with the pathogenic species *R. japonica* on day 3 of infection, the yield of *R. montanensis* increased and was accompanied by *R. japonica* growth (Fig. 1a). As shown in Fig. 1b, *R. montanensis* growth restriction was also observed in HeLa cells. After superinfection with *R. japonica*, the growth of *R. montanensis* was enhanced also in HeLa cells (Fig. 1b). Contrary to *R. montanensis*, the *R. japonica* grew well in both the Vero cells (Fig. 1a) and HeLa cells (Fig. 1b) subjected to single infection, eventually resulting in cell death.

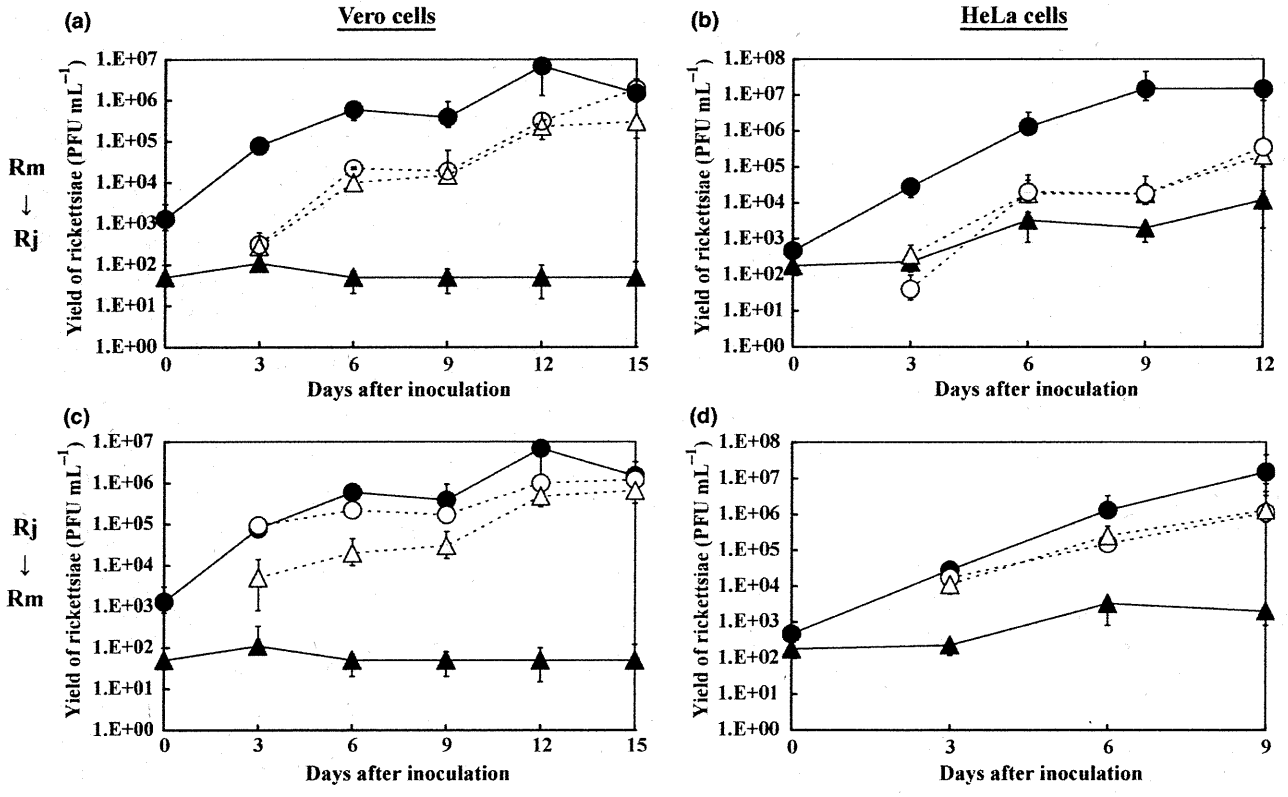

Fig. 1. Growth of *Rickettsia montanensis* (closed triangles) and *Rickettsia japonica* (closed circles) in (a) Vero and (b) HeLa cells subjected to single infection. The growth of *R. montanensis* (open triangles) and *R. japonica* (open circles) after the co-infection of *R. montanensis*-infected (a) Vero or (b) HeLa cells with *R. japonica* on day 3 of infection is also shown. Growth of *R. japonica* (open circles) and *R. montanensis* (open triangles) after the co-infection of *R. japonica*-infected (c) Vero or (d) HeLa cells with *R. montanensis* on day 3 of infection. The growth of *R. japonica* (closed circles) and *R. montanensis* (closed triangles) in (c) Vero and (d) HeLa cells subjected to single infection is also shown. All data are shown as the mean and standard error of three independent experiments.

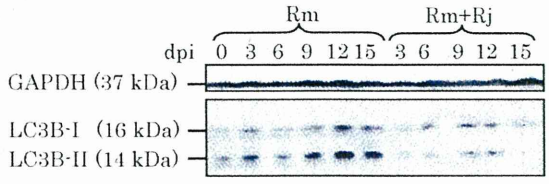

Fig. 2. Western blotting using an antibody to the autophagy-related protein LC3B of Vero cells at 7 days after their infection with *Rickettsia montanensis* and of *R. montanensis*-infected Vero cells at 4 days after their co-infection with *Rickettsia japonica*. The conversion of LC3B-I to LC3B-II is considered to be a marker of autophagy. The data shown are representative of three independent experiments.

After the superinfection of the *R. japonica*-infected cells with *R. montanensis*, rickettsiae grew well in both the Vero cells (Fig. 1c) and HeLa cells (Fig. 1d).

Production of the autophagy-related protein LC3B

The amount of LC3B gradually increased in the *R. montanensis*-infected Vero cells (Fig. 2). In addition, the majority of LC3B was converted from the 16-kDa LC3B-I to the 14-kDa LC3B-II. However, the amount of LC3B was much lower in the *R. japonica*-superinfected cells, and little LC3B-II was detected; i.e., at the very least, its concentration was much lower than that of LC3B-I. The LC3B expression levels of all of the samples were normalized to the amount of host GAPDH. Western blotting of the cells that were subjected to single infection with *R. japonica* and the *R. japonica*-infected cells that were co-infected with *R. montanensis* found that they displayed similar LC3B patterns to those of the *R. montanensis*-infected cells that were co-infected with *R. japonica* (data not shown).

Transmission electron microscopy of *R. montanensis*-infected and *R. japonica*-co-infected Vero cells

We found rickettsiae being digested in intracytoplasmic vacuoles when Vero cells were infected with the nonpathogenic species *R. montanensis*, as shown in Fig. 3a. As shown in the bottom left panel of Fig. 3a, many rickettsia particles were surrounded by a double or multilamellar structure, which is a representative finding of autophagosomes. Rickettsial particles were also seen outside of vacuoles, but this was rare. On the contrary, when the *R. montanensis*-infected cells were further infected with *R. japonica* on day 3 of infection, although a small number of rickettsiae were being digested inside vacuoles on day 7 of infection, many growing rickettsiae were detected in the cells (Fig. 3b).

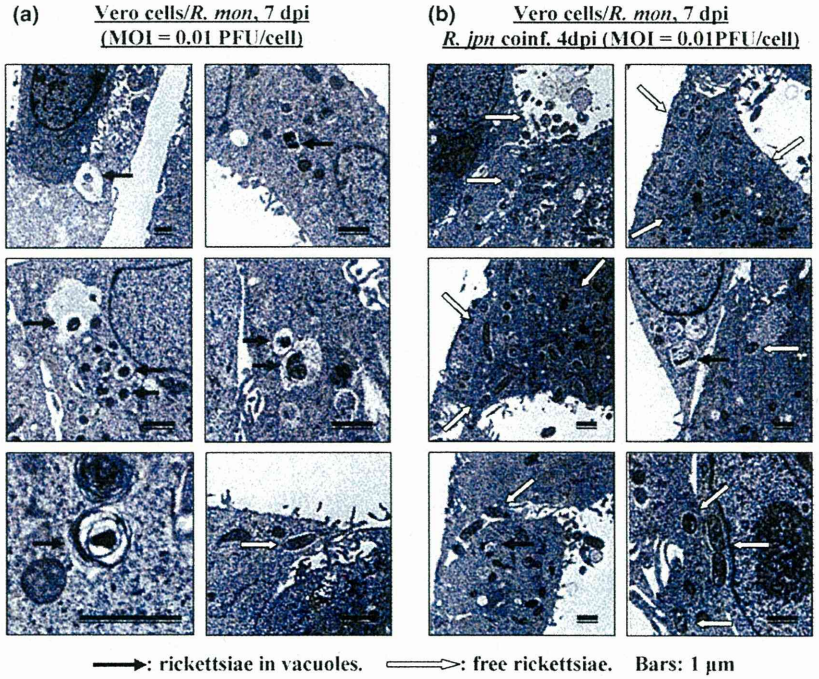

Fig. 3. Transmission electron microscopy of Vero cells infected with (a) *Rickettsia montanensis* alone (7 dpi) and (b) Vero cells infected with *R. montanensis* followed by *Rickettsia japonica* (4 days after their co-infection). Closed arrows = rickettsiae in intracytoplasmic vacuoles; open arrows = free rickettsiae. Scale bars = 1.0 μm. The presented figures are representative of three independent experiments.