The genus *Anaplasma* currently recognizes 6 distinct species: *A. phagocytophilum*, *A. platys*, *A. marginale*, *A. centrale*, *A. ovis* and *A. bovis*. The reclassification was mainly based on the phylogenetic information derived from 16S rRNA (complete representation of all member species) and heat-shock operon or groEL (only selected member species) gene data sequences [6]. A recent study identified a potentially novel *Anaplasma* sp. in Japan (herein provisionally referred to as *Anaplasma* sp. Japan), which revealed phylogenetic divergence in the 16S rRNA, gltA and groEL genes from any recognized *Anaplasma* spp. [21]. However, the previous studies present paucity of information on whether the use of secondary structures was employed in their phylogenetic analyses, which is known to produce better tree resolution [15]. In addition, the widely accepted 16S rRNA gene-based phylogenies are sometimes inconsistent [16], which is probably due to the propensity of the 16S rRNA gene to recombination/horizontal or lateral gene transfer phenomenon [1]. Therefore, other genes like the gltA [12] and groEL [11, 13] can be alternatively used to clarify phylogenetic relationships.

*A. bovis* infects circulating monocytes [5]. This particular species has been mainly analyzed only using the 16S rRNA gene [6]. Previous phylogenetic analyses of the genus *Anaplasma* using the groEL gene did not include yet *A. bovis* [6, 21] due to the unavailability of the data sequence during the time of analyses. The present study generally aimed to molecularly characterize and analyze *A. bovis* based on gltA and groEL genes and to infer phylogenetic relationships within the genus *Anaplasma* using individual and multi-locus approach (including the 16S rRNA gene). Phylogenetic analyses were performed with or without the consideration of secondary structures, using maximum likelihood (ML) and Bayesian Inference (BI) methods.

Blood sample from a feral raccoon (*Procyon lotor*) [18] in Hokkaido, herein referred to as R499, was used. The sample was previously tested to be 16S rRNA-positive for *A. bovis* (1,387 bp; GenBank accession number GU937020) and was stored at −30°C. The DNA was extracted and stored as previously described [21]. The designing of primers, determination of the partial gltA and groEL sequences of *A. bovis* by PCR, genome walking and DNA sequencing strategies were performed as described previously [21]. Primers used in the present study are shown in Table 1. The negative control used was double distilled water. Instead of using an *A. bovis* DNA, the positive control used was *A. platys*.

The gltA and groEL sequences were translated into deduced amino acids (dAA) and were manually trimmed to include only the sequence of interest (generally from the start to stop codon). Percent identities were computed as previously described [21]. Multiple sequence alignments (MSA) were performed as suggested by Hall [7] or by using PROMALS3D [15], which considers secondary structures for protein coding genes. Subsequent analyses with and without using the secondary structure information were performed usingraxmGUI [19] by general time reversible (GTR) model. Analyses by ML with prior best model testing using MEGA 5 [20] and by BI using MrBayes 3.2 [17] were also employed. For the protein coding genes, ML analyses were performed using MEGA 5 with prior best model test-
Table 1. Oligonucleotide sequences of primers used in this study

| Primer name | Oligonucleotide (5’→3’) | Reference |
|-------------|-------------------------|-----------|
| PCR amplification for a partial gltA sequence | | |
| CSTF2 | ATGR*TAGAAAAW*GCTGT TTTC | [21] |
| HG1085R | ACTATACC*K*GAGTA AAAGTC | [10] |
| Flb | GATCATGAR*CAR*AATGC TTTC | [9] |
| AnaCS1085R1 | ACTATACC*K*GAGTAA AAAR*TC | This study |
| PCR amplification for a partial groEL sequence | | |
| EEgro1F | GAGTT CGACGTTAAGAA GTTCA | [3] |
| Anagro712R | CCCGCA TCAA AACTGC ATACC | [21] |
| Anagro122F | AAATACGGT W*GTCA CGG | This study |
| Anagro649R | CTTT CCTRC*ACATTAT ACAAG | This study |
| Genome walker gene-specific primers (gltA) | | |
| ABgl-46R1 | ATATCG AGCTGCTCCCGC ATTTA GCAAGTA | This study |
| ABgl-1R2 | GTTGAGGCCACC ACATTCT ACTGTAGATGTA | This study |
| ABgl-307F1 | CAATA TACGGTGCA TCTAGGA AATAGC | This study |
| ABgl-338F2 | TGGCTGCTGAA AGTGTTGGA AAGAG | This study |
| Genome walker gene-specific primers (groEL) | | |
| ABgr-361F2 | CACGTGAC TTCCAGCATAGA AAGTAG | This study |
| ABgr-1010GRF3 | ACAGTGC ATCTTCCAGCATAGA AAGTAG | This study |
| ABgr-1132F5 | TGGCTGCTGAA AGTGTTGGA AAGAG | This study |

*Degenerate primers: R=A or G, W=A or T, K=G or T

In the 16S rRNA phylogenetic analyses, 2 subclades were seen: (1) a subclade containing *A. marginale*, *A. centrale* and *A. ovis* and (2) a subclade containing *A. phagocytophilum*, *A. platys*, *A. bovis* and *Anaplasma* sp. Japan (Fig. 1). *A. bovis* also frequently formed a cluster with *Anaplasma* sp. Japan. In the gltA phylogenetic analyses (Fig. 2), topologies revealed the 2 subclades observed in the 16S rRNA trees. In the groEL phylogenetic analyses, some positions within the genus *Anaplasma* changed depending on whether nucleotide or dAA sequences were used, but the 2 subclades were still frequently observed (Fig. 3). On the other hand, trees generated from the supermatrix also revealed the 2 subclades (Fig. 4).

*A. bovis* consistently formed a cluster with *A. phagocytophilum*, *A. platys* and *Anaplasma* sp. Japan in the 16S rRNA phylogenetic trees. This finding varied from the tree results of Dumler et al. [6], in which their 16S rRNA phylogenetic analysis placed *A. bovis* closer to *A. centrale* and *A. ovis*, but was similar to that of Ooshiro et al. [14] and Doan et al. [4], in which *A. bovis* formed a cluster with *A. phagocytophilum* and *A. platys*.

For the gltA and groEL gene phylogenetic analyses, the subclade groupings of the different taxa appear to be consistent when protein secondary structures were considered in the MSA construction, than when nucleotide sequences were used. The groEL-based trees generated in the present study also varied from those of Dumler et al. [6] as sequences of *A. bovis*, *A. ovis*, *A. centrale* and *A. platys* were not yet
included in their analyses. The groEL sequences of *A. centrale*, *A. ovis* [13] and *A. platys* [12] were only determined at a later time. Dumler *et al.* [6] pointed out the ambiguities among *Anaplasma* spp. and the arbitrary position of *A. bovis* within the *Anaplasma* species clade in the various phylogenetic analyses they performed.

Comparing the single gene or the multi-loci phylogenetic trees, the consistently observed result was the formation of the 2 subclades when secondary structures were considered. Moreover, the resulting topologies corroborated with our
Fig. 2. Phylogenetic trees based on gltA with consideration of the protein secondary structures. Analyses were performed by the Bayesian method (Jones-Taylor-Thornton model) employed in MrBayes 3.2 [16]. Values in the nodes represent posterior probability values expressed in percent. *Rickettsia prowazekii* was set as the outgroup.

Fig. 3. Phylogenetic trees based on groEL genes with consideration of the protein secondary structures. Analyses were performed by the Bayesian method (Jones-Taylor-Thornton model) employed in MrBayes 3.2 [16]. Values in the nodes represent posterior probability values expressed in percent. *Rickettsia prowazekii* was set as the outgroup.
previous findings [20], in which the _Anaplasma_ sp. Japan was found to be a potentially novel species. The absence of statistical evidence of recombination (using PHI test) and the subsequent result of the phylogenetic network analysis (by NeighborNet method) on the concatenated alignment also supported the reliability of the tree results. PHI tests are used to test MSAs for the presence of recombination, which can obscure the results of phylogenetic analyses [2].

The present study documented the first molecular analyses of _A. bovis_ based on complete _groEL_ and _gltA_ gene sequences and inferred phylogenetic relationships within the genus _Anaplasma_ with the inclusion of new sequence data. Results clarified the phylogenetic position of _A. bovis_ and established the existence of 2 subclades within the genus _Anaplasma_. This information can serve as a guide to future phylogenetic studies using the same genus.

ACKNOWLEDGMENTS. This study was supported by a grant for Research on Emerging and Re-emerging Infectious Diseases (H21-Shinkou-Ippan-06) from the Ministry of Health, Labor and Welfare, Japan. The authors also thank Mr. Fujisawa, T., Mr. Angeles, J. M.a. M., Dr. Hakimi, H. and Ms. Ybañez, R. H. D for their technical assistance.

REFERENCES
1. Brayton, K. A., Palmer, G. H., Lundgren, A., Yi, J. and Barbet, A. F. 2002. Antigenic variation of _Anaplasma marginale_ msp2 occurs by combinatorial gene conversion. _Mol. Microbiol._ 43: 1151–1159. [Medline] [CrossRef]
2. Bruen, T. C., Philippe, H. and Bryant, D. 2006. A Simple and robust statistical test for detecting the presence of recombination. _Genetics_ 172: 2665–2681. [Medline] [CrossRef]
3. Chae, J. S., Foley, J. E., Dumler, J. S. and Madigan, J. E. 2000. Comparison of the nucleotide sequences of 16S rRNA, 444 Ep-ank, and _groESL_ heat shock operon genes in naturally occurring _Ehrlichia equi_ and human granulocytic ehrlichiosis agent isolates from Northern California. _J. Clin. Microbiol._ 38: 1364–1369. [Medline]
4. Doan, H. T., Noh, J. H., Choe, S. E., Yoo, M. S., Kim, Y. H., Reddy, K. E., Van Quyen, D., Nguyen, L. T., Nguyen, T. T., Kweon, C. H., Jung, S. C., Chang, K. Y. and Kang, S. W. 2013. Molecular detection and phylogenetic analysis of _Anaplasma bovis_ from _Haemaphysalis longicornis_ feeding on grazing cattle in Korea. _Vet. Parasitol._ 196: 478–481. [Medline] [CrossRef]
5. Donatien, A. and Lestoquard, F. 1936. _Rickettsia bovis_, novelle espece pathogene pour le boeuf. _Bull. Soc. Pathol. Exot._ 29: 1057–1061.
6. Dumler, J. S., Barbet, A. F., Bekker, C. P., Dasch, G. A., Palmer, G. H., Ray, S. C., Rikihisa, Y. and Rurangirwa, F. R. 2001. Reorganization of genera in the families _Rickettsiaceae_ and _Anaplasmataceae_ in the order _Rickettsiales_: unification of some species of _Ehrlichia with Anaplasma_, _Cowdria with Ehrlichia_ and _Ehrlichia with Neorickettsia_, descriptions of six new species combinations and designation of _Ehrlichia equi_ and ‘HGE agent’ as subjective synonyms of _Ehrlichia phagocytophila_. _Int. J. Syst. Evol. Microbiol._ 51: 2145–2165. [Medline] [CrossRef]
7. Hall, B. G. 2011. Phylogenetic Trees Made Easy: A How-To Manual, 4th ed., Sinauer Associates, Sunderland.
8. Huson, D. H. and Bryant, D. 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* **23**: 254–267. [Medline] [CrossRef]

9. Huson, D. H., Richter, D. C., Rausch, C., Dezulian, T., Franz, M. and Rupp, R. 2007. Dendroscope: an interactive viewer for large phylogenetic trees. *BMC Bioinformatics* **8**: 460. [Medline] [CrossRef]

10. Inokuma, H., Brouqui, P., Drancourt, M. and Raoult, D. 2001. Citrate synthase gene sequence: a new tool for phylogenetic analysis and identification of *Ehrlichia*. *J. Clin. Microbiol.* **39**: 3031–3039. [Medline] [CrossRef]

11. Inokuma, H., Terada, Y., Kamio, T., Raoult, D. and Brouqui, P. 2001. Analysis of the 16S rRNA gene sequences of *Anaplasma centrale* and its phylogenetic relatedness to other *Ehrlichia*. *Clin. Diag. Lab. Immunol.* **8**: 241–244.

12. Inokuma, H., Fujii, K., Okuda, M., Onishi, T., Beaufils, J. P., Raoult, D. and Brouqui, P. 2002. Determination of the nucleotide sequences of heat shock operon (groESL) and the citrate synthase gene (gltA) of *Anaplasma (Ehrlichia) platys* for phylogenetic and diagnostic studies. *Clin. Diag. Lab. Immunol.* **9**: 1132–1136. [Medline] [CrossRef]

13. Lew, A. E., Gale, K. R., Minchin, C. M., Shkap, V. and de Waal, D. T. 2003. Phylogenetic analysis of the erythrocytic *Anaplasmata* species based on 16S rDNA and GroEL (HSP60) sequences of *A. marginale*, *A. centrale*, and *A. ovis* and the specific detection of *A. centrale* vaccine strain. *Vet. Microbiol.* **92**: 145–160. [Medline] [CrossRef]

14. Ooshiro, M., Zakimi, S., Matsukawa, Y., Katagiri, Y. and Inokuma, H. 2008. Detection of *Anaplasma bovis* and *Anaplasma phagocytophilum* from cattle on Yonaguni Island, Okinawa, Japan. *Vet. Parasitol.* **154**: 360–364. [Medline] [CrossRef]

15. Pei, J., Kim, B. H. and Grishin, N. V. 2008. PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Res.* **36**: 2295–2300. [Medline] [CrossRef]

16. Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. and Huelsenbeck, J. P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **61**: 539–542. [Medline] [CrossRef]

17. Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. and Huelsenbeck, J. P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **61**: 539–542. [Medline] [CrossRef]

18. Sashika, M., Abe, G., Matsumoto, K. and Inokuma, H. 2011. Molecular survey of *Anaplasma* and *Ehrlichia* infections of feral raccoons (*Procyon lotor*) in Hokkaido, Japan. *Vector Borne Zoonotic Dis.* **11**: 349–354. [Medline] [CrossRef]

19. Silvestro, D. and Michalak, I. 2012. raxmlGUI: a graphical front-end for RAxML. *Org. Divers. Evol.* **12**: 335–337.

20. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731–2739. [Medline] [CrossRef]

21. Ybañez, A. P., Matsumoto, K., Kishimoto, T. and Inokuma, H. 2012. Molecular analyses of a potentially novel *Anaplasma* species closely related to *Anaplasma phagocytophilum* detected in sika deer (*Cervus nippon yesoensis*) in Japan. *Vet. Microbiol.* **157**: 232–236. [Medline] [CrossRef]