NOTE *Bacteriology*

Two Genotypes among '*Candidatus* Mycoplasma haemobos' Strains Based on the 16S-23S rRNA Intergenic Spacer Sequences

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ABSTRACT. '*Candidatus* Mycoplasma haemobos', sometimes causative of bovine infectious anemia at various extents, has been demonstrated throughout the world. Here, we show two distinct types of '*Ca.* M. haemobos' are distributed among cattle in Japan, by examining the primary and secondary structures of the 16S-23S rRNA intergenic spacer region that has been shown to be a stable genetic marker for mycoplasma species. Our results may explain differences in severity of anemic condition as well as provide a genetic marker for an epidemiological study of bovine hemoplasma infections.
KEY WORDS: hemoplasma, mycoplasma, rRNA.

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'*Candidatus* Mycoplasma haemobos (synonym of '*Ca.* M. haemobovis')' is a trivial name of hemotropic mycoplasma that may cause anemia at various degrees in cattle [11, 12, 16, 18]. Reason of different severity in anemic condition caused by '*Ca*. M. haemobos' infection has yet been unknown. Although this alleged species has provisionally been identified by only nucleotide sequence of the 16S rRNA gene because of uncultivable trait [10, 17, 20], genetic variation in this species remained unexplored. In our previous study, the 16S-23S rRNA intergenic spacer (ITS) region of hemoplasma was found to be a useful tool for determination of taxonomic status of this particular species [15] as well as other mycoplasma species [2, 4–6]. Here, we show the '*Ca.* M. haemobos' strains are divided into two distinct types according to the ITS sequences.

EDTA-anticoagulated blood samples were collected from 25 Japanese black cattle bred on an experimental farm at Omyojin (latitude 39.7N and longitude 141.1E) of Iwate University during October 2011 to March 2012. Total DNA was extracted from 200 μl of the whole blood samples by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, eluting into 200 μl of buffer AE, and stored at −20°C until examination in the PCR assay. No clinical symptom was reported on the cattle examined, despite infection by *Bovine leukemia virus*.

Hemoplasma infection was found in all the 25 cattle diagnosed by real-time PCR by using forward primer Hemo-F1, 5′-ATATTCCTACGGGAAGCAGC-3′, equivalent to nucleotide numbers 328 to 347 of *M. wenyonii* and reverse primer Hemo-R1, 5′-ACCGCAGCTGCTGGCACATA-3′, equivalent to nucleotide numbers 503 to 522 of *M. wenyonii* as described previously [13]. Real-time PCR was performed in a SmartCycler instrument (Cepheid, Sunnyvale, CA, U.S.A.) with SYBR Premix Ex *Taq*(Code #RR041A, TaKaRa Bio., Otsu, Japan). The reaction mixture contained 1 μl of each primer (10 pmol/μl), 12.5 μl of 2X premix reaction buffer and water to volume of 23 μl. Finally, 2 μl of DNA samples as templates were added to this mixture. Amplification was carried out 40 cycles of denaturation at 95°C for 5 sec, renaturation at 57°C for 20 sec and elongation at 72°C for 15 sec, after the initial denaturation at 94°C for 30 sec. After real-time PCR, melting experiment was performed from 60 to 95°C at 0.2°C/sec with smooth curve setting averaging one point. Melting peaks were visualized by plotting the first derivative against the melting temperature as described previously [7].

Of all the cattle examined, eight cattle were found infected with '*Ca*. M. haemobos' alone based on the 16S rRNA gene analysis, and they were subjected to analysis of the ITS region by end-point PCR. Briefly, PCR amplification was carried out at 94°C for 30 sec, 55°C for 2 min and 72°C for 2 min for 30 cycles using forward (5′-GTTCCCAGGTCTTGTACACA-3′) and reverse (5′-CAGTACTTGTTCACTATCGGTA-3′) primers as described previously [1]. The PCR products were then fractionated on horizontal, submerged 1.0% SeaKem ME agarose gels (FMC Bioproducts, Rockland, ME, U.S.A.) in TAE (40 mM Tris, pH8.0, 5 mM sodium acetate and 1 mM disodium ethylenediaminetetra cetate) buffer at 50 volts for 60 min. After electrophoresis, the gels were stained in ethidium bromide solution (0.4 μg/ml) for

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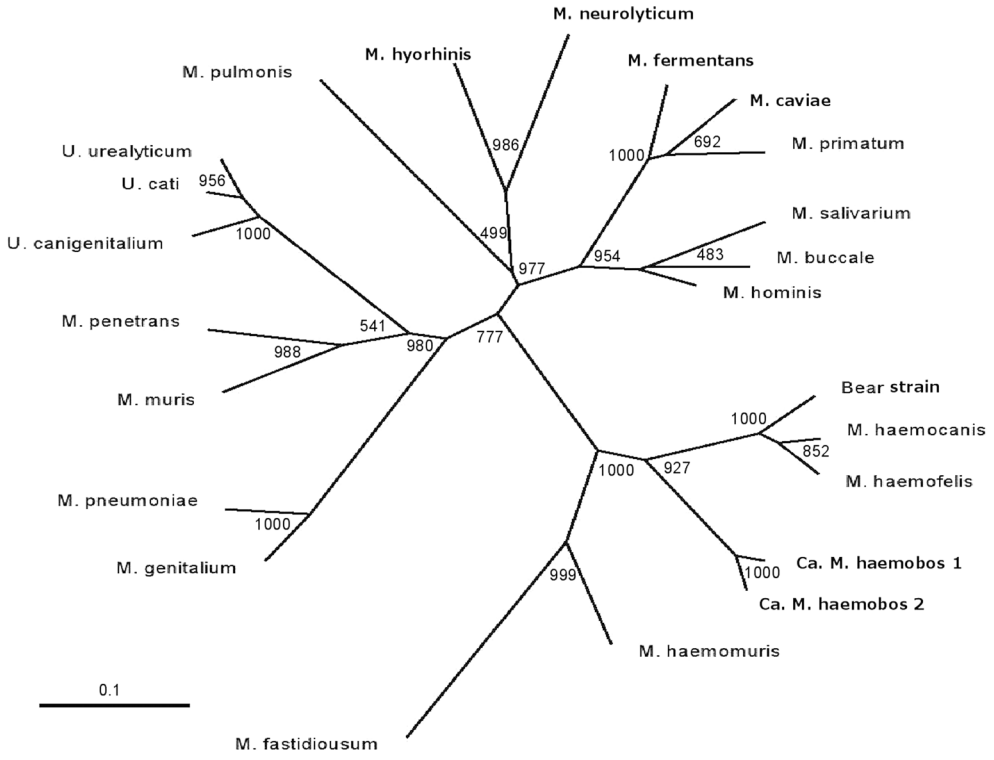

Fig. 1. Unrooted phylogenetic tree based on the ITS comparison, suggesting a monophyletic relationship among hemoplasmas and *M. fastidiosum*. Following nucleotide sequences obtained from the DNA databases are shown with an accession number in parenthesis. They are *M. salivarium* PG20 (X58558), *M. pulmonis* m53 (X58554), *M. neurolyticum* Sabin Type A (X58552), *M. buccale* CH20247 (D89504), *M. primatum* HRC292 (D89509), *M. caviae* G122 (D89505), *M. hominis* PG21 (X58559), *M. fermentans* PG18 (X58553), *M. hyorhinis* BTS-7 (X58555), *M. penetrans* GTU54 (D89508), *M. muris* RIII4 (D89507), *M. pneumoniae* Mac (D14528), *M. genitalium* G37 (D14526), *M. fastidiosum* ATCC33229 (AY781782), *Ureaplasma urealyticum* T960 (X58561), *U, cati* F2 (D636859, *U. canigenitalium* D6P-C (D63684), *M. haemomuris* Shizuoka (AB080799), *M. haemofelis* Gandai2 (AB638408), Bear hemoplasma strain (AB725596), '*Ca*. M. haemobos' type1 Gandai1 (AB638407) and '*Ca*. M. haemobos' type 2 B2.20 (AB740010). Scale bar indicates the estimated evolutionary distance that was computed with CLUSTAL W [19] using neighbor-joining method [14]. Numbers in the relevant branches refer to the values of boot-strap probability of 1,000 replications.

15 min and visualized under UV transilluminator. DNA in a clearly visible band was extracted by using NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and was subjected to direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The GenBank/EMBL/DDBJ accession numbers for ITS sequences of '*Ca.* M. haemobos' strains used in this study are AB740009 through AB740016.

The nucleotide sequences of ITS regions of the '*Ca*. M. haemobos' strains were compared with authentic mycoplasma species in phylogenetic analysis. In the present study, we examined the phylogenetic relatedness among 23 mycoplasma species including not only 3 ureaplasmas but also 6 hemoplasmas, *M. haemomuris*, *M. haemofelis*, '*Ca.* M. haemominutum', bear strain and 2 types of '*Ca*. M. haemobos' (Fig. 1). In addition to our previous illustration of ITS from '*Ca*. M. haemobos' type 1 [15], the present analysis revealed existence of another type of ITS among '*Ca*. M. haemobos' strains. Alignment of nucleotide sequences of ITS regions defined so far indicated 94% similarity between these two genotypes (Table 1). Of the eight strains, six were identical to the '*Ca*. M. haemobos' type 1, and the remaining two strains showed an identical but were distinct from type 1 sequence. Although we examined only Japanese black cattle without clinical symptom, this variation can be used for an epidemiological marker of '*Ca*. M. haemobos' infections in cattle population, since nucleotide sequences in ITS region have been conserved within a mycoplasma species or subspecies [2, 6, 8].

The secondary structures of the ITS were predicted according to the algorithm of Zuker and Stiegler [21]. Five stem-loop domains were allocated in ITS of '*Ca*. M. haemobos' (Fig. 2). Domains III and V were well conservative, despite several deletions in others domains. Secondary structures in ITS region have sometime provided a key character to distinguish closely related species of mycoplasmas [3, 6, 7, 9]. In the present study, ITS regions of the two genotypes of '*Ca*. M. haemobos' showed characteristic features of myco-

Table 1. Similarity matrix showing nucleotide sequence homology among the ITS regions of hemoplasmas. Nucleotide sequences of ITS regions have been determined on only four species. Numbers indicate homology percentage between two ITS sequences.

	M. haemocanis	*M. haemofelis*	Bear strain	*Ca.* M. haemobos 1	*Ca.* M. haemobos 2	*M. haemomuris*
M. haemocanis	100					
M. haemofelis	95	100				
Bear strain	93	93	100			
Ca. M. haemobos 1	80	81	81	100		
Ca. M. haemobos 2	82	82	83	94	100	
M. haemomuris	44	44	44	43	44	100

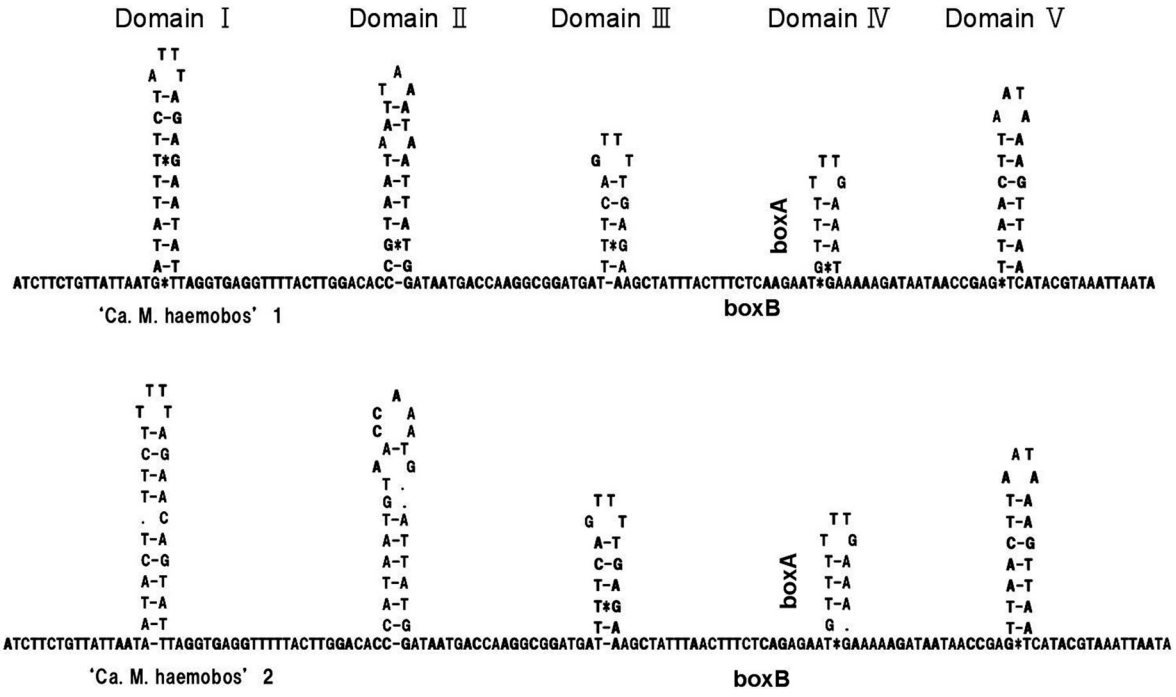

Fig. 2. Hypothetical secondary structures for the ITS regions of '*Ca*. M. haemobos' type 1 (top) and '*Ca*. M. haemobos' type 2 (bottom). Canonical Franklin-Watson-Crick base-pairing is hyphened, and a wobble base-pairing tolerated in the secondary structure is shown by an asterisk. The boxA was a part of the stem region of domain IV, and boxB was located between domains III and IV.

plasmas, lacking spacer tRNA genes and having boxA and boxB motifs [15].

In conclusion, 2 genotypes of '*Ca*. M. haemobos' were demonstrated by analyzing the primary and secondary structures of ITS regions. This may provide a clue to elucidate differences in severity of anemia in cattle, though virulence of these 2 genotypes remained unexplored. These genotypes can also be used for a genetic marker for bovine hemoplasma infections.

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