

## Prevalence and Molecular Analyses of Hemotrophic *Mycoplasma* spp. (Hemoplasmas) Detected in Sika Deer (*Cervus nippon yesoensis*) in Japan

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**ABSTRACT.** Hemotropic mycoplasmas (hemoplasmas) are cell-wall deficient, epierythrocytic bacteria that cause infectious anemia in several mammalian species. The prevalence of hemoplasma species was examined by screening and species-specific PCR using blood samples collected from 51 sika deer in Hokkaido, Japan. Molecular analyses were performed for the 16S rRNA, 23S rRNA and RNase P RNA (*rnpB*) gene sequences. A total of 23/51 (45%) deer DNA samples were positive for hemoplasmas in the screening PCR. Using species-specific PCR, 12 and 17 samples were positive for 'Candidatus Mycoplasma haemocervae' and 'Candidatus M. erythrocervae', respectively. Sequencing and phylogenetic trees of those three genes indicate that the 'Candidatus M. haemocervae' and 'Candidatus M. erythrocervae' detected in Japanese deer are potentially different species from the cervine hemoplasma found in deer from America and Brazil.

**KEY WORDS:** *Cervus nippon yesoensis*, hemoplasma, Japan, phylogeny.

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Hemotropic mycoplasmas or hemoplasmas (formerly *Haemobartonella* and *Eperythrozoon* spp.) are cell-wall deficient, epierythrocytic bacteria that have a worldwide distribution and cause infectious anemia in several mammalian species [7]. To date, all attempts to cultivate these organisms *in vitro* have failed [11], and the sensitivity and specificity of microscopic examination of blood smears are low [6]. PCR-based assays have been used to detect and diagnose hemoplasma infection [7].

A few reports exist regarding hemoplasma infections in the family *Cervidae*. Stoffregen revealed that at least two species of hemoplasma are found in reindeer (*Rangifer tarandus*) [12]. Sequences from one species were closely related to *Mycoplasma ovis*, and the other species, which was most closely related to *Mycoplasma haemofelis* and *Mycoplasma haemocanis*, was named 'Candidatus Mycoplasma haemotarandirangiferis'. Variant strains of *Mycoplasma ovis* (*M. ovis*-like sp.) were detected in Dwarf Brocket deer (*Mazama nana*), Red Brocket deer (*Mazama americana*) and Marsh deer (*Blastocerus dichotomus*) in Brazil [3] and in White-tailed deer (*Odocoileus virginianus*) in America [1]. In addition, three species including an *M. ovis*-like sp. were

found in Pampas deer (*O. bezoarticus*) and Marsh deer in Brazil and were deemed to be distinct based on 16S and 23S rRNA gene sequences [2]. A recent study has shown that *M. ovis*-like sp. and other two novel hemotropic *Mycoplasma* species were found in White-tailed deer in America based on 16S rRNA and RNase P RNA (*rnpB*) genes [5]. On the other hand, 2 distinct hemoplasma species have been identified in sika deer (*Cervus nippon centralis*) in Japan [16]. Although there is no *rnpB* gene sequence available for 'Candidatus M. erythrocervae', the report called these 2 pathogens 'Candidatus M. haemocervae' and 'Candidatus M. erythrocervae' based on sequence results of their 16S rRNA and *rnpB* genes [16]. In short, there is no report using those 3 genes for the comparison of phylogenetic position of various hemoplasma strains which were found in the family *Cervidae*.

Hokkaido sika deer (*Cervus nippon yesoensis*) is the largest subspecies of sika deer that inhabits Hokkaido, the northern island of Japan. Hemoplasma infection in Hokkaido sika deer is poorly characterized. Thus, the aim of this study was to determine the prevalence and phylogenetic positions of hemoplasma in Hokkaido sika deer in Japan using 16S rRNA, 23S rRNA and *rnpB* genes.

### MATERIALS AND METHODS

**Samples:** EDTA-anticoagulated blood samples were collected from 51 sika deer (*Cervus nippon yesoensis*) that were maintained in captivity for the purpose of meat processing in the Kushiro District (Hokkaido, Japan) in June and July 2011. DNA was extracted from 200  $\mu$ l of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), eluted with 200  $\mu$ l of buffer AE according to the

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Table 1. Prevalence of hemoplasma infected deer

PCR result (n=51)	No. of deer (%)
Screening PCR	
Positive	23 (45.1)
Negative	28 (54.9)
Species-specific PCR	
' <i>C. Mycoplasma haemocervae</i> '	12 (23.5)
' <i>C. Mycoplasma erythroceruae</i> '	17 (33.3)
Dual infection	6 (11.8)

manufacturer's instructions, and stored at  $-30^{\circ}\text{C}$  until use.

**Screening and species-specific PCR:** We screened all DNA samples for the presence of hemoplasma using a previously described protocol [13]. The F2/R2 primer set amplifies the 16S rRNA gene of most hemoplasma species, including *M. haemofelis*, *M. haemocanis*, '*Candidatus M. haemominutum*' and '*Candidatus M. haemopurvum*' [4]. A 20- $\mu\text{l}$  reaction mixture for the screening PCR contained 2.5  $\mu\text{l}$  of 10X buffer, 2.5  $\mu\text{l}$  of 2 mM dNTP, 1  $\mu\text{l}$  of 50 mM  $\text{MgCl}_2$ , 0.75 U of Taq polymerase (Invitrogen, Foster City, CA, U.S.A.), 1.0  $\mu\text{l}$  of each primer, 11.35  $\mu\text{l}$  of distilled water and 0.5  $\mu\text{l}$  of DNA template. Cycling conditions were as follows: initial denaturation at  $95^{\circ}\text{C}$  for 5 min; 35 cycles of denaturation at  $95^{\circ}\text{C}$  for 30 sec, annealing at  $60^{\circ}\text{C}$  for 30 sec, extension at  $72^{\circ}\text{C}$  for 90 sec; and a final extension at  $72^{\circ}\text{C}$  for 5 min and cooling to  $4^{\circ}\text{C}$ . All amplicons were electrophoresed on a 2.0% agarose gel in TBE buffer and visualized under UV light.

To distinguish between '*Candidatus M. haemocervae*' and '*Candidatus M. erythroceruae*', a species-specific PCR method was developed for the 16S rRNA gene. Primers for the species-specific PCR were designed based on the 16S rRNA gene sequences of '*Candidatus M. haemocervae*' (AB558899) and '*Candidatus M. erythroceruae*' (AB558897 and AB558898). The primer sets were CMhc F (5'-CCGC-GAGTAGGATAGCAGCC-3') and R2 for '*Candidatus M. haemocervae*', and CMec F (5'-GCAAGGGGTTCCGC-GTAAA-3') and R2 for '*Candidatus M. erythroceruae*', respectively. PCR conditions were as previously described, except that the extension times were 60 sec. To evaluate their specificity, the method was initially tested using DNA samples from deer confirmed to be positive for '*Candidatus M. haemocervae*' and '*Candidatus M. erythroceruae*' in this study. The specific PCR was performed for all deer samples which were positive in the screening PCR that targeted the 16S rRNA gene as mentioned above.

**PCR assays for 16S rRNA, 23S rRNA and rnpB genes:** Sequences of the full 16S rRNA gene were then assessed in several randomly selected samples that tested positive in the screening PCR. To determine the longer 16S rRNA gene sequence of hemoplasma, 2 PCRs were performed using the following 2 primer sets: fD1 and R2, and F2 and Rp2 [17]. PCR conditions were as previously described, except that the annealing temperatures were  $58^{\circ}\text{C}$  for fD1/R2 and  $52^{\circ}\text{C}$  for F2/Rp2.

Based on the 23S rRNA genes of deer hemoplas-

mas, including *M. ovis*-like sp. (HQ197750, HQ197751 and HQ197752), Deerhemo F (5'-AAAGAGTGCG-TAACAGCTCAC-3') and Deerhemo R (5'-TCACGCCG-GAATTCTTACTT-3') were newly designed. To determine the 23S rRNA gene sequence of hemoplasma, 2 PCRs were performed using the following 2 primer sets: 23S Fw and Deerhemo R, and Deerhemo F and 23S Rv [3]. PCR conditions were as previously described, except that the annealing temperatures were  $55^{\circ}\text{C}$ .

The *rnpB* gene was amplified using a primer set described previously: 80F1 and 290R1 [9]. PCR conditions were as previously described, except that the annealing temperature was  $50^{\circ}\text{C}$ .

**Sequence and phylogenetic analyses:** To determine nucleotide sequences of the positive samples, PCR products were purified using a Qiaquick PCR purification kit (QIAGEN) or a QIAquick Gel Extraction Kit (QIAGEN). Direct sequence analysis was performed using BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.). Nucleotide sequence results were checked using the BLAST search program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) for comparison with other known sequences and aligned using CLUSTAL W software (<http://www.genome.jp/tools/clustalw/>). Phylogenetic trees were created in MEGA software version 5.05 [14] by the neighbor-joining method [10]. Tree stability was estimated by bootstrap analysis for 1,000 replications.

**Nucleotide accession numbers:** Hemoplasma sequences from deer in this study were registered in GenBank with the following accession numbers: for 16S rRNA gene: KF306246 (No. 12), KF306247 (No. 13), KF306248 (No. 16), KF306249 (No. 33), KF306250 (No. 34) and KF306251 (No. 49) and for 23S rRNA gene: KF306252 (No. 16), KF306253 (No. 33) and KF306254 (No. 49) and *rnpB* gene: AB836744 (No. 13), AB836745 (No. 16), AB836746 (No. 33), AB836747 (No. 34) and AB836748 (No. 49).

## RESULTS

**Prevalence of hemoplasma infection:** A total of 23/51 (45%) deer DNA samples were positive for hemoplasmas in the screening PCR. The species-specific PCR was performed using DNA samples from deer which were confirmed as '*Candidatus M. haemocervae*' and '*Candidatus M. erythroceruae*' by sequence analysis in this study. Two PCRs were used to amplify each gene, and 439 and 363 bp amplicons were observed in '*Candidatus M. haemocervae*' and '*Candidatus M. erythroceruae*' positive samples, respectively (Fig. 1). When the species-specific PCR was performed to screen PCR-positive samples, 12 and 17 samples were found to be positive for '*Candidatus M. haemocervae*' and '*Candidatus M. erythroceruae*', respectively. Six samples were found to be dual infections (Table 1).

**Molecular characterization of hemoplasmas:** The complete 16S rRNA gene sequences were successfully determined for the 6 randomly chosen hemoplasma-positive samples. Among cervine hemoplasma isolates, the 16S rRNA gene sequences obtained from deer Nos. 12, 16, 33 and 34

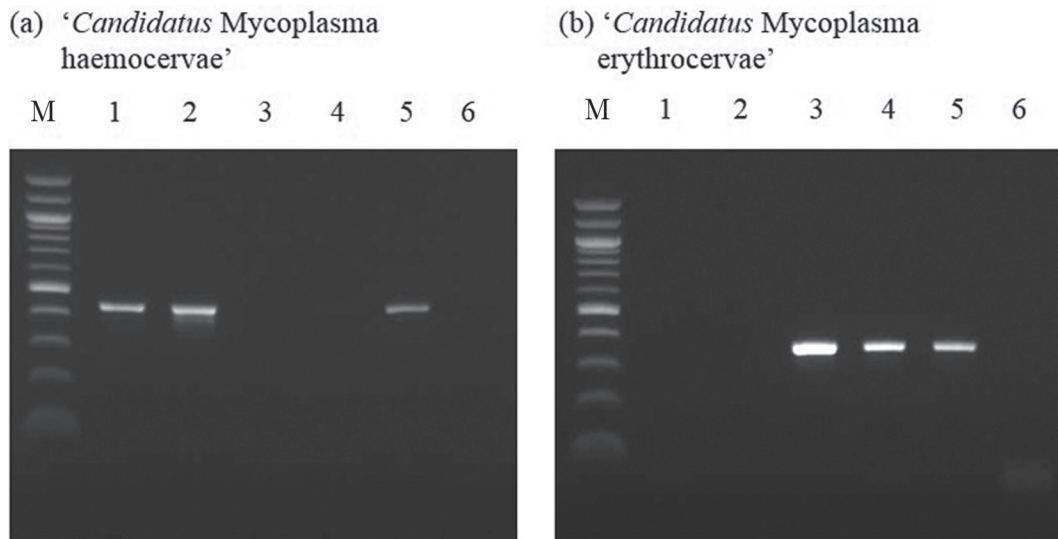


Fig. 1. Specificity of the species-specific PCR for (a) '*Candidatus Mycoplasma haemocervae*' and (b) '*Candidatus Mycoplasma erythroceruae*'. Lanes 1 and 2: '*Candidatus M. haemocervae*'; lanes 3 and 4: '*Candidatus M. erythroceruae*'; lane 5: dual infection; lane 6: negative control (DW); lane M: molecular size marker (100 bp).

Table 2. Sequence identities (%) among (a) 16S rRNA, (b) 23S rRNA and (c) *rnpB* genes of '*Candidatus Mycoplasma haemocervae*' (isolate 16) and '*Candidatus M. erythroceruae*' (isolate 49) detected in Hokkaido sika deer. Isolates 16 and 49 are one representative sequence of '*Candidatus M. haemocervae*' and '*Candidatus M. erythroceruae*'. Genbank accession numbers of each hemoplasma detected in the other family *Cervidae* are in parenthesis

Sample No.	<i>M. ovis</i> -like sp.	<i>Mycoplasma</i> sp.	<i>Mycoplasma</i> sp.	<i>Mycoplasma</i> sp.	<i>Mycoplasma</i> sp.
	Deer-Fawn	B62-2	B88-3	Group B	Group C
(a) 16S rRNA	(FJ824847)	(HQ634379)	(HQ634380)	(KC512403)	(KC512402)
Isolate 16	98.32	95.91	95.1	96.3	95.77
Isolate 49	95.92	97.75	93.58	97.79	97.8
(b) 23S rRNA	(HQ197752)	(HQ634381)	(HQ634382)	NA	NA
Isolate 16	97.61	93.08	90.83	-	-
Isolate 49	92.64	95.7	90.83	-	-
(c) <i>rnpB</i> gene	(JQ610624)*	NA	NA	(JQ610626)	(JQ610628)
Isolate 16	92.73	-	-	92.78	88.89
Isolate 49	92.59	-	-	91.84	92.59

NA; Not available. \*; *Mycoplasma* sp. isolate from White-tailed deer in America (Group A) (5).

were most closely related to '*Candidatus M. haemocervae*' (AB558899) with a percent identity of 99.86 to 99.32%. Isolate No. 16 which was representative sequence of '*Candidatus M. haemocervae*' matched with a *M. ovis*-like sp. from White-tailed deer in America (FJ824847) with a percent identity of 98.32%. Another sequences obtained from deer Nos. 13 and 49 were most closely related to '*Candidatus M. erythroceruae*' (AB558898) with a percent identity of 99.79 to 99.71%. Isolate No. 49 which was representative sequence of '*Candidatus M. erythroceruae*' matched 97.75% with a *Mycoplasma* sp. (HQ634379) detected from Marsh deer in Brazil, 97.79% with a *Mycoplasma* sp. (KC512403) and 97.80% with a *Mycoplasma* sp. (KC512402) (Table 2).

The 23S rRNA genes from sample Nos. 16, 33 and 49 were sequenced. Sequence result from No. 16 showed the

closest relationship to *M. ovis*-like sp. from White-tailed deer in Brazil (HQ197752) with a percent identity of 97.61%. Hemoplasma species in deer No. 49 were most closely related to *Mycoplasma* sp. (HQ634381) of Marsh deer in Brazil with a percent identity of 95.70% (Table 2).

The *rnpB* gene sequences of Nos. 16, 33 and 34 showed 99.46% identity to '*C. M. haemocervae*' (AB561882). Sequence result from No. 16 showed only 92.73% identity to a *Mycoplasma* sp. (JQ610624), and No. 49 also showed only 92.59% identity to its best match, *Mycoplasma* sp. (JQ610624 and JQ610628) recently described in a White-tailed deer from America [5] (Table 2).

The 16S rRNA gene-based phylogenetic tree revealed that '*Candidatus M. haemocervae*' from Hokkaido sika deer Nos. 12, 16, 33 and 34 was on a cluster separate from the *M.*

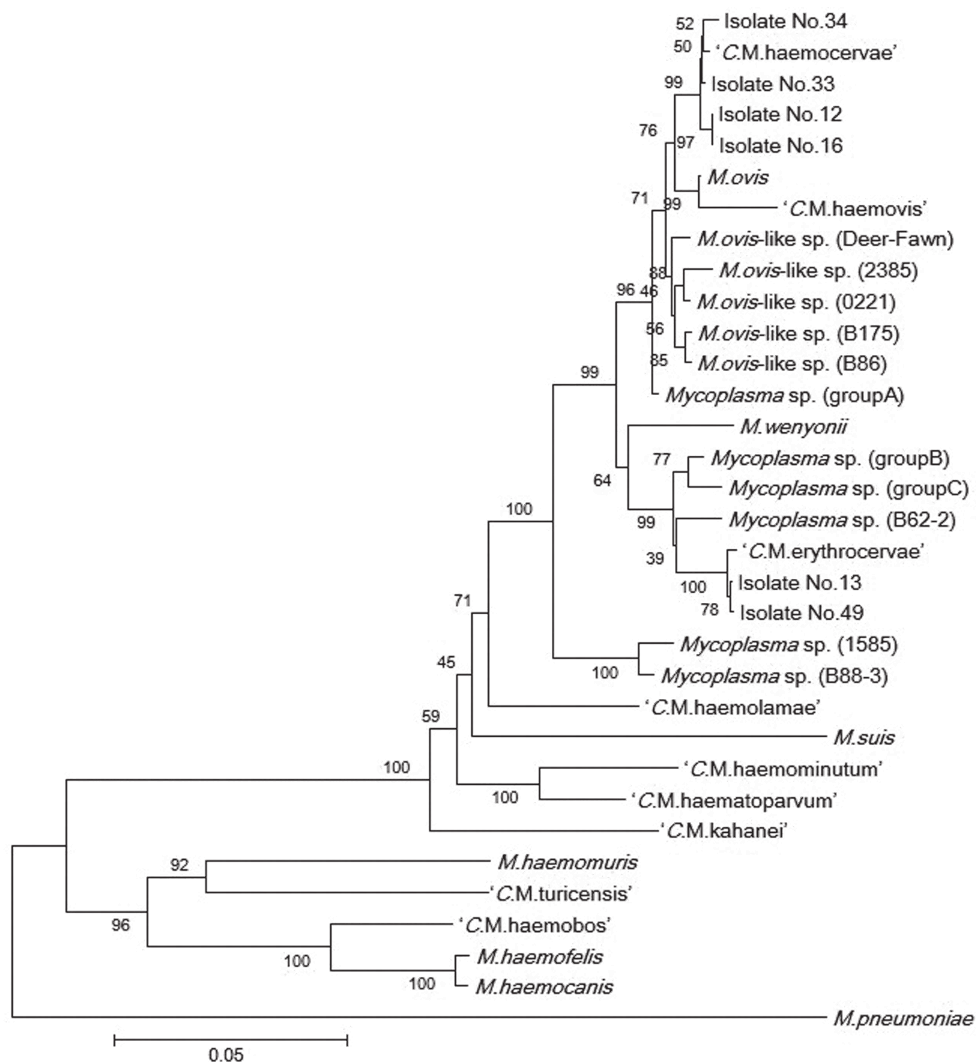


Fig. 2. Phylogenetic relationship of deer hemoplasma isolates (Nos. 12, 13, 16, 33, 34 and 49) and the other hemoplasma species based on 16S rRNA gene using a neighbor-joining method. *Mycoplasma ovis*-like sp. isolates B86, B175, Deer-Fawn, 0221, 2385 (HQ634377; HQ634378; FJ824847; HQ197746; HQ197748), *M. ovis* (JF931135), 'Candidatus *M. haemovis*' (JF931131), 'Candidatus *M. haemocervae*' (AB558899), *M. wenyonii* (AY946266), *Mycoplasma* sp. isolates B62-2, 1585, B88-3, group A, group B, group C (HQ634379, HQ197747, HQ634380, KC512404, KC512403 and KC512402), 'Candidatus *M. erythroceruae*' (AB558898), 'Candidatus *M. haemominutum*' (AY150980), 'Candidatus *M. haematoparvum*' (AY383241), *M. haemomuris* (U82963), 'Candidatus *M. turicensis*' (DQ825454), 'Candidatus *M. haemobos*' (EU367965), *M. haemofelis* (U88563), *M. haemocanis* (AY150973), *M. suis* (EU603330), 'Candidatus *M. haemolamae*' (NR074478) and 'Candidatus *M. kahanei*' (AF338269) are shown. *M. pneumoniae* (M29061) was used as an outgroup.

*ovis*-like spp. in deer from Brazil and America, and 'Candidatus *M. erythroceruae*' from deer Nos. 13 and 49 was also on a cluster separate from the unclassified hemoplasma (HQ634379) from Marsh deer (Fig. 2). The 23S rRNA gene-based phylogenetic tree revealed a similar result, wherein hemoplasmas from Nos. 16 and 33 and from No. 49 were separated from the *M. ovis*-like spp. and the unclassified *Mycoplasma* sp. (HQ634381) from Marsh deer, respectively (Fig. 3). The *rnpB* phylogenetic tree showed that hemoplasma from Nos. 16, 33 and 34 formed a separate cluster

from *Mycoplasma* sp. (JQ610624) which are closely related to a *M. ovis*-like spp. (Fig. 4).

## DISCUSSION

In the present study, hemoplasma species were detected from 45% (23/51) of the blood samples from sika deer in Hokkaido, Japan, by screening PCR. A past study revealed that hemoplasma infections were found in only 9% (13/147) of free-ranging sika deer in Iwate prefecture in the Tohoku



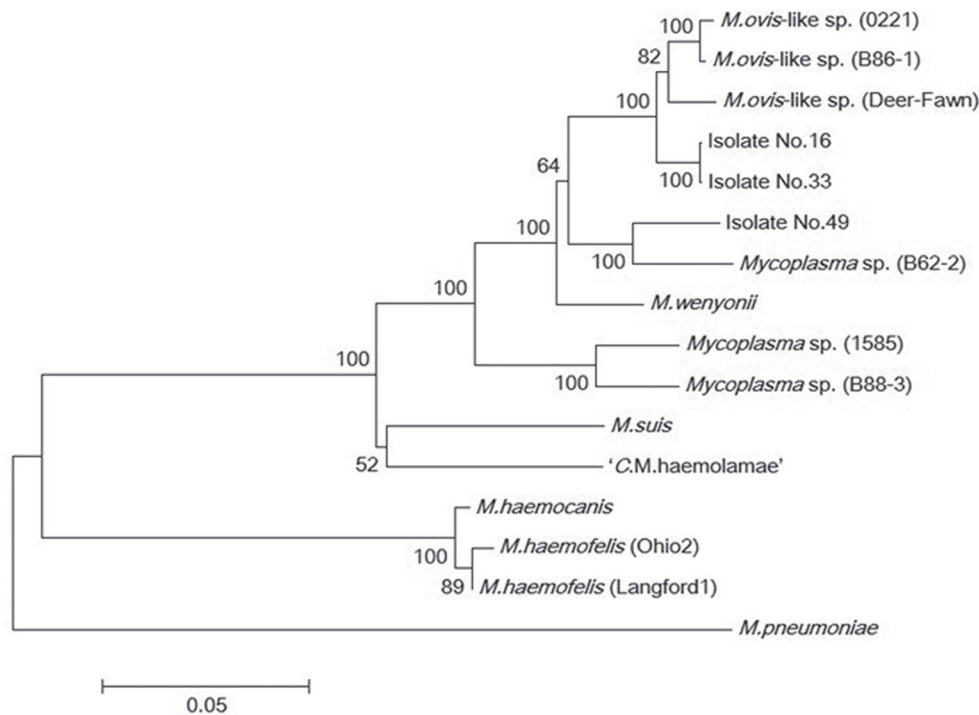


Fig. 3. Phylogenetic relationship of deer hemoplasma isolates (Nos. 16, 33 and 49) and the other hemoplasma species based on 23S rRNA gene using a neighbor-joining method. *Mycoplasma ovis*-like sp. isolates 0221, B86-1, Deer-Fawn (HQ197751; HQ634383; HQ197752), *Mycoplasma* sp. isolates B62-2, 1585, B88-3 (HQ634381; HQ197750; HQ634382), *M. wenyonii* (NR076982), *M. suis* (NC015153), *M. haemocanis* (NR076944), *M. haemofelis* isolates Ohio2, Langford1 (NC017520; NC014970) and ‘*Candidatus M. haemolamae*’ (NR076983) are shown. *M. pneumoniae* (NR077056) was used as an outgroup.

region of Honshu island, Japan [16]. However, hemoplasma infections were found in 89.0% (65/73) of white-tailed deer in America [5] and 87.1% (27/31) of Brazilian deer [3], both groups of which were maintained in captivity or confined area. Given that the sika deer in the present study had also been maintained in captivity, it seems that the high prevalence of hemoplasma infections is attributable to situations in which animals are in close contact with one another.

As a result of the comparison of phylogenetic position of various hemoplasma strains which were found in the family *Cervidae* using 16S, 23S rRNA and *rnpB* genes, ‘*Candidatus M. haemocervae*’ obtained from Hokkaido sika deer was closely related to a previously reported species which are so-called *M. ovis*-like spp. on 16S rRNA (FJ824847) and 23S rRNA (HQ634381) gene sequences, while low identity was shown on *rnpB* gene between those species. In addition, ‘*Candidatus M. erythrocervae*’ was closely related to a *Mycoplasma* sp. (HQ634379) detected from Marsh deer in Brazil on 16S rRNA gene, while homology of 23S rRNA gene sequence between those two species seemed to be low because the threshold of the 23S rRNA gene sequence for identification between different bacterial species was 1.15% [8]. Moreover, phylogenetic trees based on the three genes also indicate that ‘*Candidatus M. haemocervae*’ and ‘*Candidatus M. erythrocervae*’ are independent from other cervine

hemoplasma found in America and Brazil.

The phylogeny of hemoplasma species has been investigated based on the *rnpB* gene sequences, a more suitable target for phylogenetic discrimination of closely related taxa when compared with 16S rRNA sequences [9]. The RNA polymerase beta subunit (*rpoB*) and the 16S-23S rRNA intergenic transcribed spacer (ITS) region were found to be reliable and useful taxonomic tools for species differentiation within the family *Mycoplasmataceae* [15]. Further research is required to compare those genes among unclassified hemoplasma in the family *Cervidae*, including ‘*Candidatus M. haemocervae*’, ‘*Candidatus M. erythrocervae*’, *M. ovis*-like sp. and other hemoplasma of free-ranging deer in America and Brazil.

In this study, hemoplasma infections are common in Hokkaido sika deer. However, a further study using free-ranging Hokkaido sika deer is necessary, because animals using in this study were maintained in captivity. Lower percent identities and divergent phylogenetic position support the notion that ‘*Candidatus M. haemocervae*’ and ‘*Candidatus M. erythrocervae*’ are potentially different species from the other cervine hemoplasma found in America and Brazil. Further investigation is needed to clarify phylogenetic position, molecular characterization and pathogenicity of deer hemoplasma in Japan.

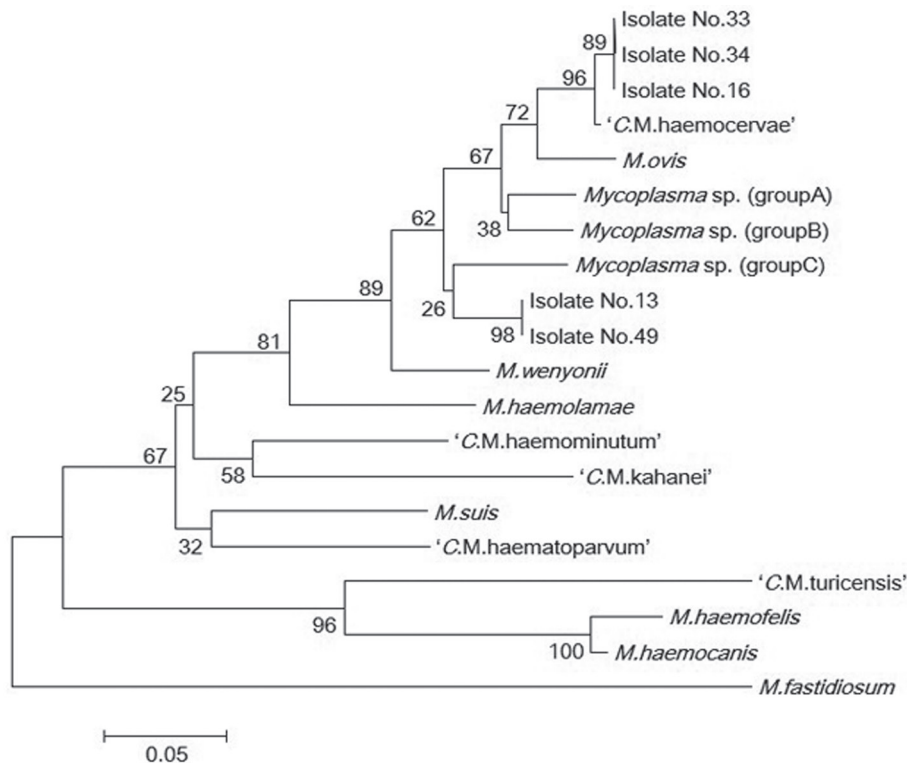


Fig. 4. Phylogenetic relationship of deer hemoplasma isolates (Nos. 13, 16, 33, 34 and 49) and the other hemoplasma species based on *rnpB* gene using a neighbor-joining method. 'Candidate Mycoplasma haemocervae' (AB561882), *Mycoplasma* sp. isolates group A, group B, group C (JQ610624, JQ610626 and JQ610618), *M. ovis* (EU078612), *M. wenyonii* (EU078610), *M. haemolamae* (EU078613), 'Candidate *M. haemominutum*' (EU078614), 'Candidate *M. haematoparvum*' (EU078616), *M. suis* (EU078611), 'Candidate *M. turicensis*' (EF212002), *M. haemofelis* (EU078617), *M. haemocanis* (EU078618) and 'Candidate *M. kahanei*' (EU078615) are shown. *M. fastidiosum* (EU078609) was used as an outgroup.

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