Proposal for ‘Candidatus Mycoplasma haemomuris subsp. musculi’ in mice, and ‘Candidatus Mycoplasma haemomuris subsp. ratti’ in rats

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Mycoplasma haemomuris is causative of infectious anaemia or splenomegaly in rodents. We examined the nucleotide sequences of the non-ribosomal genes, mnpB and dnaK, in strains of the species M. haemomuris detected in small field mice and black rats. mnpB nucleotide sequences in strains of the species M. haemomuris isolated from small field mice and black rats had only 89% sequence similarity, suggesting their separation into two distinct subgroups. dnaK had a nucleotide sequence similarity of 84% between the subgroups. These results support the classification of M. haemomuris into two genetically distinct subgroups. Here we propose the establishment of these subgroups as ‘Candidatus Mycoplasma haemomuris subsp. musculi’, detected in small field mice (Apodemus argenteus), and ‘Candidatus Mycoplasma haemomuris subsp. ratti’, detected in black rats (Rattus rattus).

The group of bacterial species known as haemotropic mycoplasma or haemoplasma are pathogens that have been recognized as agents of infectious anaemia in various mammalian species (Messick, 2004). This group includes not only species formerly classified as Haemobartonella and Eperythrozoon, but also newly identified haemotropic species of the genus Mycoplasma. Haemoplasma strains have been identified in rodents, including mice, rats and hamsters, and have been classified primarily based on nucleotide sequences of the 16S rRNA or RNase P RNA (rnpB) genes (Peters et al., 2008; Tasker et al., 2003). The two haemotropic species of the genus Mycoplasma currently recognized in rodents, Mycoplasma haemomuris and Mycoplasma coccoides, were transferred from Haemobartonella muris (formerly Bartonella muris) and Eperythrozoon coccoides, respectively, based on their 16S rRNA gene sequences (Neimark et al., 2001, 2002, 2005; Rikihisa et al., 1997). Recent studies have suggested that M. haemomuris consists of two subgroups based on differences in the 16S–23S rRNA intergenic spacer (ITS) sequence (Sashida et al., 2013). In the present study, we further investigated nucleotide sequences of the non-ribosomal genes mnpB and dnaK to evaluate these two subgroups among strains of the species M. haemomuris.

Anti-coagulated blood or spleen homogenates were obtained from small field mice (Apodemus argenteus) and black rats (Rattus rattus) previously diagnosed with M. haemomuris infection, based on a 99% nucleotide sequence similarity of the 16S rRNA gene (Sashida et al., 2013). Details regarding the source of the samples examined in this study are provided in Table 1. Total DNA was extracted from 200 μl of the spleen or blood cells of these mice and rats using the QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer’s instructions.

Eight DNA samples extracted from small field mice and black rats were subjected to PCR to amplify the mnpB and dnaK. PCR was carried out with 50 μl reaction mixtures, each containing 2 μl of DNA solution, 24 μl of EmeraldAmp PCR Master Mix and water to a final volume of 50 μl. Primer sequences for PCR and sequencing were designed as described in previous reports (Hicks et al., 2014; Steer et al., 2011) and as shown in Table S1 (available in the online Supplementary Material). The mnpB was amplified using

The GenBank/EMBL/DDBJ accession numbers for mnpB and dnaK, appearing for the first time in this study, are AB973078 to AB973092.
Table 1. Details of the sources of the samples examined in this study

<table>
<thead>
<tr>
<th>Sample designation</th>
<th>Host</th>
<th>Place animal was trapped</th>
<th>Year of sampling</th>
<th>Sample conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shizuoka</td>
<td>Small field mouse</td>
<td>Shizuoka</td>
<td>1985</td>
<td>Spleen homogenate</td>
</tr>
<tr>
<td>S151-2</td>
<td>Small filed mouse</td>
<td>Fukushima</td>
<td>1985</td>
<td>Erythrocyte suspension</td>
</tr>
<tr>
<td>S152-2-4</td>
<td>Small field mouse</td>
<td>Fukushima</td>
<td>1986</td>
<td>Spleen homogenate</td>
</tr>
<tr>
<td>S152-5-7</td>
<td>Small field mouse</td>
<td>Fukushima</td>
<td>1986</td>
<td>Spleen homogenate</td>
</tr>
<tr>
<td>S159-11-13</td>
<td>Small filed mouse</td>
<td>Aomori</td>
<td>1988</td>
<td>Spleen homogenate</td>
</tr>
<tr>
<td>S154</td>
<td>Black rat</td>
<td>Fukushima</td>
<td>1987</td>
<td>Spleen homogenate</td>
</tr>
<tr>
<td>Ikemajima5-1</td>
<td>Black rat</td>
<td>Okinawa</td>
<td>2010</td>
<td>Whole blood</td>
</tr>
<tr>
<td>Ikemajima14-1</td>
<td>Black rat</td>
<td>Okinawa</td>
<td>2010</td>
<td>Whole blood</td>
</tr>
</tbody>
</table>

All samples were collected from animals killed under anaesthesia.

primers RNP-F and RNP-R, and the dnaK was amplified using primers DNK-F1 and DNK-R1. After initial denaturation at 94 °C for 30 min, the reaction cycle was carried out 30 times, with each cycle consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s in a thermal cycler. The PCR products were fractionated on horizontal, submerged 1.0% SeaKem ME agarose gels (FMC Bioproducts) in TAE buffer (40 mM Tris, pH 8.0, 5 mM sodium acetate and 1 mM disodium ethylene-diaminetetraacetate) at 100 V for 30 min. After electrophoresis, the gels were stained in GelRed solution (Biotium) for 15 min and visualized under a UV transilluminator. DNA in a clearly visible band was extracted using (model C-61 Cultra-Violet Products, San Gabriel, CA) a NucleoSpin Extract II kit (Macherey-Nagel) and was subjected to direct sequencing in both directions using the same primers used for PCR, in a 3500 Genetic Analyzer (Applied Biosystems).

The nucleotide sequences of rnpB from the eight strains of *M. haemomuris* along with the 16 established species of the genus *Mycoplasma* were aligned using CLUSTAL W (Thompson et al., 1994). A phylogenetic tree based on nucleotide sequences of rnpB was generated using the neighbour-joining method (Saitou & Nei, 1987) from a distance matrix corrected for nucleotide substitutions according to the Kimura two-parameter model (Kimura, 1980). The eight strains of *M. haemomuris* were divided into two subgroups with a high bootstrap value; subgroup A consisted of S151-2, S152-2-4, S152-5-7, S159-11-13 and Shizuoka strains detected in small field mice, and subgroup B consisted of S154, Ikemajima5-1 and Ikemajima14-1 strains detected in black rats (Fig. 1). In addition, we examined the secondary structure predicted for the P12 portion of RNase P RNA, which is a product of rnpB and a ribozyme responsible for processing the 5' end of tRNA molecules. This could provide a key piece of information with which to distinguish between closely related species of the genus *Mycoplasma*, such as *M. haemocanis* and *M. haemofelis* (Sasaoka et al., 2011), which have 99 % similarity in their 16S rRNA sequences. The secondary structures of the P12 helix were predicted according to the algorithm of Zuker & Stiegler (1981). The GAAA tetra-nucleotide at the top of the P12 helix on the RNase P RNA of *M. haemomuris* was conserved in both subgroups, though there was a palindromic nucleotide substitution on the stem region due to a transversion between adenine and uracil (Fig. S1).

Amino acid sequences predicted from the dnaK of the eight strains from the two subgroups of *M. haemomuris* were also aligned using CLUSTAL W (Thompson et al., 1994). Although the dnaK nucleotide sequences showed 84 % similarity between the subgroups (data not shown), the amino acid sequences showed 96 % similarity (Fig. S2). Thus, dnaK analysis, including both nucleotide and amino acid sequences, supported separation of the two subgroups of *M. haemomuris*.

The strains of the species *M. haemomuris* examined in the present study have previously been reported to be divisible into two subgroups based on their ITS sequences (Sashida et al. 2013). Our study further confirmed the existence of two distinct subgroups among the strains of the species *M. haemomuris* that may not be attributable to the differences in the geographical locations of strain collection, because subgroup A included strains from Aomori, Fukushima and Shizuoka Prefectures, and subgroup B included strains from Fukushima and Okinawa Prefectures. This variation, thus, most likely depends on species differences between the natural hosts of these strains of the species *M. haemomuris*. Specifically, strains of subgroup A were detected in small field mice, while strains of subgroup B were detected in black rats. Our analyses confirmed the existence of two genetically distinct subgroups among strains of the species *M. haemomuris*.

*M. haemomuris* was first observed in the blood of rats. At that time, it was named *Bartonella muris ratti* (Mayer, 1921), and was confirmed to be the causative agent of infectious anaemia in splenectomized albino rats (Ford & Eliot, 1928). Subsequently, another type of *Bartonella*, called at that time *Bartonella muris musculi*, was found in the blood of albino mice (Schilling, 1929). It was discovered later that the previous scientific name *Bartonella muris* (currently *M. haemomuris*) was more accurately separated into *Bartonella muris* subsp. *ratti* in rats and *Bartonella muris* subsp. *musculi* in mice (Noguchi, 1928; Regendanz & Kikuth, 1928), despite the possibility of cross-transmission between these rodent species by experimental infection. This raises the question of whether *M. haemomuris* Shizuoka, a strain isolated from a
small field mouse, would formerly have been classified as *B. muris* subsp. *musculi*. This allows us to propose that the species *M. haemomuris* be separated into ‘*Candidatus Mycoplasma haemomuris musculi*’, detected in small field mice, and ‘*Candidatus Mycoplasma haemomuris ratti*’, detected in black rats, based on the accumulated findings.

**Description of ‘*Candidatus Mycoplasma haemomuris subsp. musculi*’ (basonym *Mycoplasma haemomuris* (Mayer 1921) Neimark et al. 2002)**


In Giemsa-stained peripheral blood smears, tiny round bodies appear as deep purple projections from the erythrocyte surface. Susceptible to chlortetracycline and oxytetracycline. Most infections are latent, but may become apparent following splenectomy. Causes infectious anaemia or splenomegaly in mice. Nucleotide sequence of the 16S rRNA gene are deposited in DNA databases under accession numbers U82963 and AB918692, and PCR primers to amplify the 16S rRNA gene are described by Rikihisa et al. (1997).

**Description of ‘*Candidatus Mycoplasma haemomuris subsp. ratti*’ (basonym *Mycoplasma haemomuris* (Mayer 1921) Neimark et al. 2002)**


Tiny coccoid bodies sometimes in chains are found on the erythrocytes in Giemsa-stained peripheral blood smears. Susceptible to chlortetracycline and oxytetracycline. Latent infection is common among rats including laboratory rats. Causes infectious anaemia or splenomegaly in rats. The rat louse (*Polypax spinulosa*) is a putative vector. Nucleotide sequences of the 16S rRNA gene, ITS and partial 23S rRNA gene are deposited in DNA databases under accession numbers AB758434, AB758435 and AB758439, and PCR

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**Fig. 1.** A neighbour-joining phylogenetic tree based on a nucleotide sequence comparison of *rnpB* among haemotropic species of the genus *Mycoplasma* including eight strains of the species *M. haemomuris*. Nucleotide sequences obtained from the DNA databases are shown with accession numbers in parentheses. A strain name was not available for the accession numbers EU078610 to EU078619. *Mycoplasma fermentans* PG18 (U41756) was included as an out-group. Numbers at the relevant branch points refer to values of bootstrap probability of 1000 replications. Bar, the estimated evolutionary distance (0.1 nt substitutions per site), computed in CLUSTAL W (Thompson et al., 1994) using the neighbour-joining method (Saitou & Nei, 1987).
primers to amplify the sequence are described by Sashida et al. (2013).

References


