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Non-ribosomal phylogenetic exploration of *Mollicute* species: New insights into haemoplasma taxonomy

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**A B S T R A C T**

Nine species of uncultivable haemoplasmas and several *Mycoplasma* species were examined by partial sequencing of two protein-encoding housekeeping genes. Partial glyceraldehyde-3-phosphate dehydrogenase (*gapA*) and heat shock protein 70 (*dnaK*) gene sequences were determined for these *Mollicute* species; in total nine *gapA* sequences and ten *dnaK* sequences were obtained. Phylogenetic analyses of these sequences, along with those of a broad selection of *Mollicute* species downloaded from GenBank, for the individual genes, and for the *gapA* and *dnaK* concatenated data set, revealed a clear separation of the haemoplasmas from other species within the *Mycoplasma* genus; indeed the haemoplasmas resided within a single clade which was phylogenetically detached from the pneumonias of Mycoplasmas. This is the first report to examine the use of *gapA* and *dnaK*, as well as a concatenated data set, for phylogenetic analysis of the haemoplasmas and other *Mollicute* species. These results demonstrate a distinct phylogenetic separation between the haemoplasmas and Mycoplasmas that corresponds with the biological differences observed in these species, indicating that further evaluation of the haemoplasmas’ relationship with the *Mycoplasma* genus is required to determine whether reclassification of the haemoplasmas is necessary.

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1. Introduction

The taxonomic position of the *Eperythrozoon* and *Haemobartonella* species has long been a subject of controversy. Originally classified within the order *Rickettsiales*, they were reclassified as members of the class *Mollicutes*, order *Mycoplasmatales* and family *Mycoplasmataceae*, genus *Mycoplasma* (Brown et al., 2010b) or family *Incertae Sedis*, genus *Eperythrozoon* or *Haemobartonella* (Brown et al., 2010a), on the basis of 16S rRNA gene sequence analysis, and given the trivial name haemoplasma (Messick et al., 2002; Neimark et al., 2001, 2002; Rikihisa et al., 1997). Phylogenetic characterisation using the RNaseP RNA (*rnpB*) gene has supported the 16S rRNA-based phylogeny and shown that the haemoplasmas reside in a single clade, within the genus *Mycoplasma*, most closely related to the pneumonias of *Mycoplasma*, with *Mycoplasma fastidiosum* and *Mycoplasma caviae* being their closest relatives (Johansson et al., 1999; Neimark et al., 2001; Peters et al., 2008; Rikihisa et al., 1997; Tasker et al., 2003). Haemoplasmas are, as yet, uncultivable bacteria, limiting their phenotypic characterisation. They adhere to red blood cells causing varying degrees of anaemia, and can infect a large range of mammalian species including, but not limited to, cats (Foley and Pedersen, 2001; Tasker et al., 2009; Willi et al., 2005), dogs, alpacas, opossums (Messick et al., 2002), sheep, goats (Neimark et al., 2004), and humans (Steer et al., 2011).

Dispute over the nomenclature and classification of the haemoplasmas as members of the genus *Mycoplasma* has left many of them within the order *Mycoplasmatales*, family *Incertae sedis* under the genus *Eperythrozoon* or *Haemobartonella*; *Incertae sedis* being a taxonomic description given to species whose position and relationship with other species is undefined (Brown et al., 2010a; Neimark et al., 2005; Uilenberg et al., 2006). Indeed, an insufficient level of similarity to justify the classification of the haemoplasmas within the genus *Mycoplasma* was reported by Uilenberg et al. (2004). Uilenberg et al. (2004) highlighted that only 77.3% 16S rRNA gene identity existed between *Mycoplasma wenyonii* (a haemoplasma species) and *M. fastidiosum* (a member of the genus *Mycoplasma*), and that significant differences in biological characteristics (e.g. biological niche, transmission methods, ability to culture in vitro) between the haemoplasmas and members of the genus *Mycoplasma* also existed.

Despite wide use of 16S rRNA gene and *rnpB* sequences to describe phylogenetic relationships between species of bacteria, both genes lack resolving power at the species level as they are highly
sequence used in a previous haemoplasma phylogeny study showed little variation and was too short to give high bootstrap values (Peters et al., 2008). The use of multilocus sequence analysis (MLSA) of protein encoding genes has been proven to be useful in the determination of the taxonomic position of many bacteria. This approach has been previously used to analyse members of the Mycoplasma genus, using genes such as tufA, fusA, gyrB, lepA, rpoB, efp, gmk and adk (Kamla et al., 1996; Manso-Silván et al., 2012; Manso-Silván et al., 2007; Thompson et al., 2011). It was reported that tufA was more able to demonstrate the phenotypic features of the bacteria than the 16S rRNA gene, and MLSA proved useful for discrimination at sub-species levels. gapA and dnaK are two protein-encoding housekeeping genes that have been previously used in phylogenetic analysis of other bacteria due to their identification as good taxonomic markers (Falah and Grupta, 1997; Fraga et al., 2010; Martens et al., 2008; Wertz et al., 2003). Both gapA and dnaK should provide more resolving power than the 16S rRNA gene and rnpB as they are highly conserved across species but offer higher variation within the sequences than those of RNA genes, and are well over twice the length of the rnpB gene; gapA and dnaK are approximately 1 Kbp and 1.8 Kbp respectively, in comparison to approximately 0.4 Kbp for rnpB.

The continued incorporation of the haemoplasmas within the order Mycoplasmatales family, Incertae sedis highlights the need to further explore the taxonomic position of these bacteria. This is the first report to examine the use of gapA and dnaK for phylogenetic analysis of a wide range of haemoplasmas and other Mollicute species, and furthermore the first to describe a concatenated data set for these genes in these species.

2. Materials and methods

2.1. Source of species

The samples used in the current study were DNA derived from species obtained for a previous study (Peters et al., 2008): Mycoplasma coccoides, Mycoplasma haemomuris, Candidatus Mycoplasma haemolamae, Candidatus Mycoplasma kahanei, Candidatus Mycoplasma haemocervae, Candidatus Mycoplasma haematoparvum, Candidatus Mycoplasma haemominimus, Candidatus Mycoplasma erythrophila, Mycoplasma ovis, Mycoplasma felis, and M. fastidiosum. Additionally, EDNA blood samples of M. wenyonii, Mycoplasma haemomuris, Candidatus Mycoplasma erythrophila, Candidatus Mycoplasma haemocervae and Candidatus Mycoplasma haemominimus were obtained from clinical and experimentally infected cases, and a vial of M. capreolyticus colonies on agar was kindly provided by Mycoplasma Experience (Reigate, UK).

2.2. DNA extraction

Genomic DNA was extracted from EDTA blood using the Nucleospin® Blood Kit (Macherey-Nagel) following the manufacturer’s protocol, eluting into 100 μl of buffer BE. For M. capreolyticus, the agar sample was spun at 600 g for 30 s and 100 μl of supernatant was then subjected to DNA extraction using the Nucleospin® Blood Kit as for the blood samples. DNA was stored at –20 °C until further use.

2.3. Primer design

Primers (Table 1) were designed for the amplification and sequencing of partial gapA and dnaK gene sequences using Primer3 v. 0.4.0 (Rozen and Skaltsky, 2000) and alignments of selected available haemoplasma and Mycoplasma sequences downloaded from GenBank (National Centre for Biotechnology Information, USA).

2.4. Polymerase chain reaction

Polymerase chain reaction (PCR) to amplify both gapA and dnaK was performed using DNA for all species and a combination of primers from Table 1. Each PCR reaction consisted of 12.5 μl of 2 X HotStarTaq Mastermix (Qiagen), MgCl2 to a final concentration of 4.5 mM, primers (200 nM for dnaK primers F27, F71, R139, R1367, and R1802; 400 nM for gapA primers F22, F369 and R975; 400 nM for gapA primers F27, F71, R667, R683, R729, and R968), 1 μl of template DNA and water to a final volume of 25 μl. A positive control (M. haemofelis"Ca. M. haemominutum") and a negative control (water) were run alongside the samples in all PCR runs. A MJ Research PTC-200 Peltier thermal cycler (Bio-Rad) was used for PCR, set to incubate at 95 °C for 15 min, then 45 cycles of 95 °C for 10 s, 50 °C for 15 s and 72 °C for 90 s, followed by 72 °C for 5 min.

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5’-3’</th>
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<tr>
<td>dnaK</td>
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<tr>
<td>F3A</td>
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<tr>
<td>F61</td>
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<tr>
<td>F146</td>
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<tr>
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<tr>
<td>R1139</td>
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<td>R1367</td>
<td>CGGATAGCTCAAMYAGACGG</td>
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<tr>
<td>R1802</td>
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<tr>
<td>gapA</td>
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</tr>
<tr>
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<td>F27</td>
<td>TGGATTYGGAAGAATCGGAAG</td>
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<tr>
<td>F71</td>
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<tr>
<td>F369</td>
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<tr>
<td>R667</td>
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<td>R729</td>
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Table 2

<table>
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<tr>
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<th>DnaK Accession</th>
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<td>No amplification</td>
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<tr>
<td>‘Ga. M. kahanei’</td>
<td>No amplification</td>
<td>No amplification</td>
</tr>
<tr>
<td>‘Ga. M. erythrophila’</td>
<td>KF151043</td>
<td>KF151050</td>
</tr>
<tr>
<td>‘Ga. M. haemocervae’</td>
<td>KF151041</td>
<td>KF151051</td>
</tr>
<tr>
<td>M. coccoides</td>
<td>KF151044</td>
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</tr>
<tr>
<td>M. ovis</td>
<td>KF151048</td>
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</tr>
<tr>
<td>M. wenyonii</td>
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<td>KF151059</td>
</tr>
<tr>
<td>M. haemomuris</td>
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<td>KF151057</td>
</tr>
<tr>
<td>M. capreolyticus</td>
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<td>No amplification</td>
</tr>
<tr>
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<td>KF151056</td>
</tr>
<tr>
<td>M. felis</td>
<td>KF151046</td>
<td>No amplification</td>
</tr>
</tbody>
</table>

For some samples only gapA or dnaK sequences could be amplified; these samples were thus not included in the concatenated data set. Attempts to amplify and sequence full length gapA and dnaK sequences from all species were unsuccessful; partial gene sequences were generated, and the length for which there was overlap in all species was subjected to phylogenetic analysis, corresponding to 466 bp for gapA and 509 bp for dnaK.
15 min. Products were separated on a 1% agarose gel, and products of the appropriate size were purified using NucleoSpin Extract II Kit (Macherey-Nagel). The amount of DNA present in each sample was quantified using the Quant-iT™ dsDNA Broad-Range or High-Sensitivity Assay Kits (Invitrogen) according to the manufacturer’s instructions. Re-amplification was carried out for reactions producing little product, using the procedure described above with 1 l of PCR product as template. Samples were submitted to the DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, www.dnaseq.co.uk) for sequencing using an Applied Biosystems model 3730 automated capillary DNA sequencer after being diluted to a specified concentration dependant on amplicon size. Primers were added at a concentration of 3.2 µM to the samples to be sequenced.

2.5. Sequence analysis and phylogenetic analysis

Forward and reverse sequences for each sample were assembled using ClustalW in MacVector (MacVector and Assembler 11.1.2) and primer sequence sites were removed. Each sample was sequenced twice in both forward and reverse directions, and all sequences derived from each sample aligned to resolve any discrepancies. The final sequences for all samples were aligned using MAFFT version 7 (Katoh and Standley, 2013), along with selected haemoplasma and other Molllicute dnaK and gapA sequences available online in GenBank. The section of each gene for which sequence data were available for all samples was then subjected to phylogenetic analysis.

To establish that the two data sets, gapA and dnaK, could be combined a partition homogeneity test was run using PAUP 4.0 (Swofford, 2003). Modeltest 3.7 (Posada and Crandall, 1998) was applied to the data to determine the model best suited for both the single gene data and the concatenated data set, for both aligned gene sequences and the concatenated aligned sequences the best fit evolutionary model determined by Modeltest 3.7 was the generalised time reversible model. The result of the modeltest was then used in Phyml 3.0 (Guindon et al., 2010) to generate maximum likelihood trees viewed using Treeview (Page, 1996). In addition,
neighbour-joining trees were constructed using the Kimura-2 parameter model in Mega5 (Tamura et al., 2011). Bootstrap analysis of the trees was performed to 1000 replicates and *Clostridium perfringens* (GenBank: BA000016) was chosen as the out-group.

3. Results and discussion

This study represents the first use of the housekeeping genes *gapA* and *dnaK*, as well as a concatenated data analysis, for a wide range of haemoplasma and other Mollicute species. Partial *gapA* and *dnaK* gene sequences were obtained for most species, as shown in Table 2. Only partial sequences could be obtained for the two genes: sequence lengths of 466 bp and 509 bp for *gapA* and *dnaK*, respectively, were analysed phylogenetically to produce maximum likelihood individual and concatenated trees. Nucleotide sequence data generated from this study are available from the GenBank database (Genbank: KF151041–151059).

Considering firstly the non-haemoplasma *Mycoplasma* species, the concatenated tree, as shown in Fig. 1, separates the non-haemoplasma *Mycoplasma* genus species into three separate groups: the hominis group, the pneumoniae group and the spiroplasma group, which is consistent with the 16S rRNA-based phylogenetic analysis of *Mycoplasma* species (Weisburg et al., 1989). Additionally, species of the order *Acholeplasmatales* (including the *Acholeplasma* and *Phytoplasma* species) were separated from those of the order *Mycoplasmatales*. It is apparent in all the trees produced in this study (Figs. 1–3) that the type species for the *Mycoplasma* genus, *Mycoplasma mycoides*, as well as *Mycoplasma capricolum*, cluster closely with *Mesoplasma florum* of the order *Entoplasma*.

![Fig. 2. Maximum likelihood tree for *gapA* sequences. A *gapA* sequence length of 466 bp was analysed for a number of haemoplasma and other Mollicute species, and a maximum likelihood tree was constructed. *Indicates sequences derived in the current study. Accession numbers are given. The data set was resampled 1000 times and the resulting bootstrap values are given as percentages at the nodes (values less than 50% are not shown).](image-url)
tales, family Entoplasmataceae, genus *Mesoplasma*. This further supports previous work suggesting that *M. mycoides* and *M. capricolum* do not belong in the order Mycoplasmatales and family Mycoplasmataceae, let alone the same genus as the other *Mycoplasma* species (Weisburg et al., 1989). However, despite the evidence these species remain within the genus *Mycoplasma* due to the confusion that reclassification of the type species outside of this genus would cause within the scientific community; re-naming of all other *Mycoplasma* species would be needed if this occurred, following the identification of a new type species for the *Mycoplasma* genus (Brown et al., 2010b; Gasparich et al., 2004; Tully et al., 1997).

It is evident from the concatenated tree (Fig. 1) that there is a distinct separation of the haemoplasmas from the other *Mycoplasma* species, supported by a bootstrap value of 100%, whilst the pneumoniae and hominis groups of the genus *Mycoplasma* reside in a separate clade. This observation has not been described before for haemoplasma phylogeny using 16S rDNA- and rnpB-based phylogenies (Johansson et al., 1999; Neimark and Kocan, 1997; Peters et al., 2008; Rikihisa et al., 1997). The construction of a neighbour-joining tree (data not shown) confirmed the separate clustering of the haemoplasma species from the other *Mycoplasma* species. The high level of support for this cluster is confirmed in both the individual gene trees (Figs. 2 and 3). This division of the haemoplasma species is most likely accounted for by the distinct biological differences between the haemoplasmas and other members of the *Mycoplasma* genus. The natural habitat of the Mycoplasmas is usually the mucosal surfaces of the respiratory and urogenital tracts of vertebrate species, as well as the eyes and joints (Razin et al., 1998), conversely the haemoplasmas reside attached to red blood cells. Other biological differences between
the haemoplasmas and members of the genus Mycoplasma include the haemoplasmas’ suspected arthropod transmission, with haemoplasma DNA being found in both fleas and ticks (Woods et al., 2005; Woods et al., 2006), and the haemoplasmas’ lack of ability to grow in an in vitro system. Despite multiple attempts, including the use of Mycoplasma-specific media, the haemoplasmas remain one of the few Mycoplasmas currently uncultivated in vitro, highlighting a specific growth requirement of the haemoplasmas not seen in the other species of the genus Mycoplasma. Additionally, as previously described, there is a lack of identity between the haemoplasmas and species of the genus Mycoplasma (Ullenberg et al., 2004, 2006).

Our study shows that there is also considerable distance between the haemoplasmas and their closest relatives, Mycoplasma penetrans, M. fastidiosum, M. capivaphyngis and the other members of the pneumococci group of Mycoplasmas. The concatenated tree does show the haemoplasmas sharing a node with M. penetrans, but there is no support given to this relationship due to its low bootstrap value (41.5%) (Fig. 1). This is in contrast to the closer relationship of the haemoplasmas to the pneumococci group reported previously (Johansson et al., 1999; Peters et al., 2008; Tasker et al., 2003). Although the concatenated tree still shows the pneumococci group to be the haemoplasmas’ closest relatives, the phylogenetic separation between these species is great enough to suggest that the haemoplasmas should comprise a separate distinct genus. Messick et al. (2002) have also reported that the haemoplasmas are only peripherally linked to the pneumoniae group. Our work has shown that the resulting phylogeny separates haemoplasmas from those of the genus Mycoplasma. Additionally, as previously described, there is a lack of identity between the haemoplasmas and species of the genus Mycoplasma (Ullenberg et al., 2004, 2006).

The derived concatenated maximum likelihood tree (Fig. 1) confirmed that the haemoplasma species reside within a single clade consisting of two subgroups: the so-called haemofelis cluster (consisting of M. haemofelis, M. haemominutum, M. haemolamae, M. erythrocervae, M. wenyoni, and M. ovis), as has been described by Peters et al for both 16S rDNA- and rnbP-based phylogeny (Peters et al., 2008). Both individual trees (Figs 2 and 3) also support that the haemoplasma clade consists of two subgroups (Peters et al., 2008; Tasker et al., 2003). Like rnbP-based phylogeny, both gapA and dnaK were able to discriminate between the closely related M. haemofelis and M. haemominutum (Birkenheuer et al., 2002), which 16S rRNA gene-based phylogeny has failed to do (Birkenheuer et al., 2002).

Here we report the use of gapA and dnaK sequences for the analysis of the phylogenetic relationships of the haemoplasmas within the Mollicutes. Our work has shown that the resulting phylogeny using these non-ribosomal genes clearly differentiates the haemoplasmas and other species of the Mycoplasma genus into separate clades, divides the haemoplasmas into the previously reported haemofelis and haemominutum subgroups, and distinguishes between the haemoplasma species, especially the closely related species M. haemocanis and M. haemominutum. The separation of the haemoplasmas from those of the Mycoplasma genus strongly indicates that these results correspond with the differences in biological characteristics of these bacteria, suggesting that the haemoplasmas may not be closely related to the Mycoplasma species as has been previously reported. These results suggest the possibility that the haemoplasmas could reside within their own genus, but further analysis, using more genes, would be required to determine whether this is true. We suggest that the taxonomic position of these bacteria may be better evaluated by MLSA, and that further evaluation may provide support for a new genus for the haemoplasmas.

**Acknowledgments**

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**Appendix A. Supplementary material**

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.meegid.2014.02.001](http://dx.doi.org/10.1016/j.meegid.2014.02.001).

**References**


