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

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ABSTRACT

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 pneumoniae group 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.

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 rDNA-based phylogeny and shown that the haemoplasmas reside in a single clade, within the genus *Mycoplasma*, most closely related to the pneumoniae group of Mycoplasmas, with *Mycoplasma fastidiosum* and *Mycoplasma cavipharyngis* 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, uncultivatable 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

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conserved (Birkenheuer et al., 2002; Mignard and Flandrois, 2006; Stackebrandt and Goebel, 1994; Tasker et al., 2003). The *rnpB* 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 rRNA 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 kahaneii', '*Candidatus* Mycoplasma haemocervae', '*Candidatus* Mycoplasma haematoparvum', '*Candidatus* Mycoplasma haemohominis', '*Candidatus* Mycoplasma erythrocervae', *Mycoplasma ovis*, *Mycoplasma felis,* and *M. fastidiosum*. Additionally, EDTA blood samples of *M. wenyonii, Mycoplasma haemomuris*, '*Candidatus* Mycoplasma erythrocervae', '*Candidatus* Mycoplasma haemocervae' and '*Candidatus* Mycoplasma haemohominis' were obtained from clinical and experimentally infected cases, and a vial of *M. cavipharyngis* 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. cavipharyngis,* 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 Skaletsky, 2000) and alignments of selected available haemoplasma and *Mycoplasma* sequences downloaded from GenBank (National Centre for Biotechnology Information, USA).

Table 1
Primers for the amplification and sequencing of *gapA* and *dnaK* partial sequences.

Primer	Primer sequence 5′-3′
dnaK	
F34	GACCTAGGTACAACTAACTCYTGTG
F61	TWGGTGGTGATGATTGRGA
F146	GGDGGAGGWACWTTTGAYG
F350	GTTATTACTGTTCCAGCATACTTTAA
F603	DGGRGGWACWTTTGAYGTYT
R874	CKCCCTGWACTACRTGAATRTCT
R1052	ATTCKWGTWGAWCCHCCDAC
R1139	CCACCTAGTGTTTCAATACTTAGAGTT
R1367	CCGTTAGCGTCAATAGAGAAGG
R1802	TTAGTTTTATCTACCTCAGTCTTATCCT
gapA	
F22	GGATTCGGAAGAATCGGAAG
F27	TGGATTYGGAAGAATMGGWAG
F71	AATGGHTTYGGWMGDATYGG
F369	AGTTATCTCCGCTCCAGCAAA
R667	GGWGCATCHTGWADTYTTTG
R683	TWCCWATWGCNGCAGAWGCWCCKGT
R729	ACTCTRTGHGCAATHCCATC
R968	TGRYTNACATAAGAAGAYTCRTTATCRTA
R975	AACAAGCTGATTCACATAAGAAGA

2.4. Polymerase chain reaction

Polymererase 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 HotStar*Taq* Mastermix (Qiagen), $MgCl_2$ to a final concentration of 4.5 mM, primers (200 nM for *dnaK* primers F34, R1139, R1367, and R1802; 400 nM for *dnaK* primers F61, F146, F603, R874, and R1052; 200 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

Table 2
GapA and *dnaK* accession numbers for all sequences obtained in this study.

Sample	GapA	DnaK
'*Ca.* M. haemolamae'	KF151042	KF151053
'*Ca.* M. haemohominis'	No amplification	KF151052
'*Ca.* M. Kahaneii'	No amplification	KF151054
'*Ca.* M. erythrocervae'	KF151043	KF151050
'*Ca.* M. haemocervae'	KF151041	KF151051
M. coccoides	KF151044	No amplification
M. ovis	KF151048	KF151058
M. wenyonii	KF151049	KF151059
M. haemomuris	KF151047	KF151057
M. cavipharyngis	No amplification	KF151055
M. fastidiosum	KF151045	KF151056
M. felis	KF151046	No amplification

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*.