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Abstract

Partial sequences of the RNase P RNA gene (rnpB) were obtained from a number of hemoplasmas and other Mycoplasma species. Phylogenetic analysis of these sequences showed that all hemoplasmas were present within a single clade and were most closely related to Mycoplasma fastidiosum, similar to the results found with 16S rRNA gene phylogeny.
**RNase P RNA Gene (rnpB) Phylogeny of Hemoplasmas and Other *Mycoplasma* Species**†

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Hemoplasmas are wall-less epicyclic erythrocytic bacterial parasites which have not yet been cultured in vitro. These organisms have recently been reclassified within the genus *Mycoplasma* based on phylogenetic analysis of 16S rRNA gene sequences (10, 11). Hemoplasmas have been identified in a number of domestic and wild animal species (6, 9–11, 14, 15, 21), and infection with these organisms causes anemia of various severities. For instance, acute infection of cats with either *Mycoplasma haemofelis* or *Candidatus Mycoplasma turicensis* is associated with mild to severe anemia, whereas infection with *Candidatus M. haemominutum* usually results in few clinical signs (5, 21).

Several studies have investigated the phylogeny of hemoplasma species on the basis of 16S rRNA gene sequences (7, 9, 10, 14, 17, 18, 22), and a single study has used the RNA subunit of the RNase P (rnpB) gene (18). The RNase P RNA study was limited by the inclusion of only three hemoplasma species and the available sequence data from other *Mycoplasma* species. The *rnpB* gene is universally present in bacterial species and encodes the RNA subunit of endoribonuclease P, which is approximately 400 bp in length. This gene has been shown to be more suitable for phylogenetic discrimination of closely related taxa when compared with 16S rRNA sequences due to its higher rate of nucleotide variation (16). The aim of this study was to expand upon the previous study of hemoplasma phylogeny based upon the *rnpB* gene by sequencing a larger number of hemoplasmas and other *Mycoplasma* species to compare this phylogeny with those based upon 16S rRNA sequences.

Hemoplasma-positive blood samples were obtained from experimentally and naturally infected animals and confirmed by PCR to have the organisms’ DNA present. Other *Mycoplasma* species were obtained as freeze-dried ampoules of type-specific organisms from the *Mycoplasma* collection at the Veterinary Laboratories Agency, (Weybridge, Surrey, United Kingdom). DNA was extracted from each sample type using the Macherey-Nagel Nucleospin kit (ABgene, Epsom, United Kingdom) as per the manufacturer’s protocol. Amplification of a portion of the *rnpB* gene was performed using ABsolute QPCR master mix (ABgene) and a primer set described previously (80F1, GAGGAAAGTCCRYGCTWGCAC; and 290R1, TCCCYTACCRRAATTTGTTTCTT) (1). Amplification was carried out with 2 μl of extracted DNA and 200 nM of each primer in a final volume of 25 μl using a PTC-200 DNA engine (Bio-Rad Laboratories, Ltd., Hemel Hempstead, United Kingdom) with an initial incubation of 95°C for 15 min and then 40 cycles of 95°C for 15 s, 45°C for 30 s, and 72°C for 30 s followed by a final incubation at 72°C for 5 min. Products were separated by 2% agarose gel electrophoresis, and products of the appropriate sizes were excised with a clean scalpel blade, purified using the Macherey-Nagel NucleoSpin extract II kit (ABgene), and submitted for fluorescent dideoxynucleotide sequencing (The Sequencing Service, School of Life Sciences, University of Dundee, Scotland; www.dnaseq.co.uk). Sequence data using both the forward and reverse primers was obtained from three separate PCR amplifications for each sample. The sequences were aligned using ClustalW run in MacVector version 9 (MacVector, Inc., Cambridge, United Kingdom) to obtain a final sequence for each of the available samples. These sequences, combined with the *rnpB* gene sequences available from GenBank, were aligned using MacVector, taking into account described secondary structures of the RNase P RNA subunit (2). Nearly full-length 16S rRNA gene sequences for the species from which *rnpB* data were available were also obtained from GenBank and aligned using MacVector with manual adjustment if visual examination revealed any areas of misalignment. Phylogenetic trees
were constructed with MacVector for both *rnpB* and 16S rRNA using the neighbor-joining program (13), from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model (8). Further phylogenetic trees for *rnpB* were constructed using the PHYLIP package (4) and the maximum likelihood and parsimony methods. The data set was resampled 1,000 times to generate bootstrap percentage values.

Partial *rnpB* gene sequences were obtained from all 11 hemoplasma and 17 other *Mycoplasma* species included in the study, which varied in length from 103 to 191 bp (approximately 30 to 48% of the complete gene). These sequences were aligned with the equivalent regions from 12 *Mycoplasma* species available from GenBank in order to construct a phylogenetic tree (see alignment in the supplemental material); an

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FIG. 1. Phylogenetic analysis of nearly complete 16S rRNA genes (A) and the RNase P RNA gene (*rnpB*) (B) for sequenced hemoplasma isolates and other *Mycoplasma* species. A phylogenetic tree was constructed by the neighbor-joining method. Evolutionary distances are to the scales shown. The data set was resampled 1,000 times to generate bootstrap percentage values, and values greater than 50% are given at the nodes of the tree. The 16S rRNA gene pneumoniae, hominis, and spiroplasma groups and the hominis clusters that were classified in previous reports are indicated (12, 20). The asterisks indicate *rnpB* sequences generated as part of the present study. GenBank accession numbers are shown for all sequences. Nodes present in the *rnpB* phylogenetic trees calculated using maximum likelihood and parsimony (data not shown) are highlighted with a plus sign.
equivalent phylogenetic tree was constructed using nearly complete 16S rRNA gene sequences obtained from GenBank (Fig. 1). All hemoplasma species were located within a single clade in both the \textit{rnpB} and 16S rRNA gene phylogenetic trees. The hemoplasma species were further subdivided into two distinct groups (Fig. 1), one containing \textit{M. haemofelis} and “\textit{Candidatus M. turicensis}” (haemofelis group) and the other containing “\textit{Candidatus M. haemominutum}” (haemominutum group). Both phylogenies showed that the hemoplasmas were most closely related to \textit{M. fastidiosum}, followed by other members of the \textit{M. pneumoniae} group (16S rRNA gene phylogeny) or \textit{M. iowae} and \textit{M. penetrans} (\textit{rnpB} phylogeny). However, the \textit{rnpB} phylogenetic tree did not separate the \textit{Mycoplasma} species into the same distinct groups as in the 16S rRNA gene phylogeny. Additional \textit{rnpB} phylogenetic trees were constructed using the maximum likelihood and parsimony methods, which supported the presence of the hemoplasma species in a separate clade with bootstrap values equivalent to or greater than (66 and 86%, respectively) those found by the neighbor-joining method (see Fig. S1 and S2 in the supplemental material). These additional trees also indicated a close relationship of the hemoplasma species not only to \textit{M. fastidiosum} but also to \textit{M. gallisepticum}.

\textbf{FIG. 1—Continued.}
This study represents an expansion of the phylogenetic data available for mycoplasmas, including the hemoplasmas, based upon \( rnpB \) gene data. Parallel attempts were made by our laboratory (unpublished data) to amplify other gene sequences (heat shock protein 70, elongation factor Tuf gene, and the 16S-to-23S rRNA intergenic spacer region) from the hemoplasmas and related Mycoplasma species for additional comparative phylogeny. This was performed using primers designed to amplify existing Mycoplasma species sequences, but PCR failed to yield products consistent with the gene being amplified. The results of this study support the findings of the previous limited report of phylogeny based upon \( rnpB \) (18), which also showed the division of the hemoplasma species into the “haemofelis” and “haemominutum” groups and their close relationship to some members of the \( M. \) pneumoniae group. The present study examined a region of the \( rnpB \) gene similar, but not identical, to that sequenced previously (18). Attempts were made to amplify a larger portion of the \( rnpB \) gene from the Mycoplasma and hemoplasma species using consensus primers for all available Mycoplasma species sequences, but this was unsuccessful. The bootstrap values for the separation of the hemoplasmas into a separate clade in the \( rnpB \) trees were significantly lower than those present in the 16S rRNA gene tree and were lower than the statistically significant value of >95% suggested by Felsenstein et al. (3). The failure of the \( rnpB \) phylogenetic trees to generate significant bootstrap values could have been due to the relatively short sequences amplified in our study (16), although the degree of variation present in the regions used for phylogeny will influence the outcome as well as sequence length, or repetitive sequence regions within the alignment. Some A/T-rich regions do occur within the sequences in the alignments, but there are no sections of perfect nucleotide repeats. Nevertheless, separation of the hemoplasma species into a separate clade was supported by the finding of this clade in all three trees constructed using the different phylogenetic methods.

The other Mycoplasma species in the \( rnpB \) phylogenetic tree were not separated into the five distinct groups previously described on the basis of 16S rDNA gene sequences (20), which is in agreement with the previous study using \( rnpB \) (18). The \( rnpB \) phylogenetic relationship of the hemoplasma species to \( M. \) fastidiosum agrees with the findings of previous studies using 16S rRNA sequence (7, 9, 10, 18). Johansson et al. (7) suggested that, based upon 16S rRNA gene phylogeny, \( M. \) fastidiosum, \( M. \) caviae, and the hemoplasmas are descended from a common ancestor and are all members of the \( M. \) pneumoniae group, whereas Messick et al. (9) suggested that specific residues and motifs within the 16S sequences showed that the hemoplasma species were distinct groups, separate from the \( M. \) pneumoniae group. The \( rnpB \) phylogenetic tree created in this study shows that some members of the \( M. \) pneumoniae group are most closely related to the hemoplasma species, but the latter do form a separate, distinct clade from those of the \( M. \) pneumoniae group, supporting the concept that the hemoplasmas are a distinct group. In line with this, it is interesting to note that the transfer of the hemoplasmas into the genus Mycoplasma has generated some controversy. Uilenberg et al. (19) have argued that insufficient evidence exists to reclassify the hemoplasmas within the genus Mycoplasma. Although supporting the inclusion of hemoplasmas within the class Mollicutes, they suggested that the hemoplasmas should form a separate genus from that of the mycoplasmas, due to their specific biological properties and sequence identities.

By using phylogeny on two gene sequences for 40 Mycoplasma species, this study strengthens the relationship of the hemoplasma organisms with those of the genus Mycoplasma and provides information about the relatedness of these organisms. All hemoplasmas were found to be present within a single clade using both 16S rRNA gene and \( rnpB \) phylogenetic trees and were further subdivided into two groups, one of which included the two feline hemoplasma species, \( M. \) haemofelis and “Candidatus \( M. \) turicensis” (haemofelis group), and the other of which included “Candidatus \( M. \) haemominutum” (haemominutum group).

**Nucleotide sequence accession numbers.** The GenBank accession numbers of the nucleotide sequences derived in this study are EF212002 and EU078593 to EU078619.

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**REFERENCES**


