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Phylogenetic Analysis of Hemoplasma Species: an International Study

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Nearly complete 16S rRNA gene sequences for feline and canine hemoplasma isolates from Europe, Australia, Africa, and Asia showed almost 100% identity to those previously reported for United States isolates. Partial sequences of the RNA subunit of the RNase P gene were also determined, and RNase P-based phylogenetic analysis showed that the hemoplasmas are most closely related to the members of the *Mycoplasma pneumoniae* group.

The hemoplasmas are a group of hemotropic bacterial species (6, 11–13). Hemoplasma organisms have recently been reclassified within the genus *Mycoplasma* based on phylogenetic analysis of 16S rRNA gene sequences from a number of United States isolates. *Mycoplasma haemocanis* infects dogs, while two distinct feline hemoplasma species, *Mycoplasma haemofelis* and "*Candidatus* Mycoplasma haemominutum" (6, 8, 11, 12, 16), are known to infect cats. *M. haemofelis* induces anemia in immunocompetent hosts (12), while "*Candidatus* M. haemominutum" appears to be less pathogenic (6). *M. haemocanis* causes anemia primarily in splenectomized or immunocompromised dogs (11). Very few papers describing hemoplasma isolates from outside the United States have been published (2, 19), and the worldwide distribution of these pathogens is unknown. The aim of the present study was to determine selected gene sequences of several hemoplasma isolates collected from different parts of the world and to perform phylogenetic studies to further define the nature of these hemotropic pathogens.

Blood samples were obtained from cats and dogs in the United Kingdom, Israel, South Africa, Australia, France, and Germany that were believed to be hemoplasma infected. Following DNA extraction (DNeasy Tissue Kit; Qiagen, Crawley, United Kingdom), amplification of nearly complete 16S rRNA gene sequences was carried out using universal 16S rRNA gene primers (10, 22). Successfully amplified products of the appropriate size (approximately 1,500 bp) were purified (QIAquick gel extraction kit; Qiagen) and then cloned by using a TOPO pCR 2.1 kit (Invitrogen, Groningen, The Netherlands). Plasmid DNA was purified (Qiaprep Plasmid Spin Miniprep Kit; Qiagen) and submitted for fluorescent dideoxynucleotide sequencing (University of Dundee Sequencing Service, Dundee, Scotland). Sequences of the RNA subunit of the RNase P gene were generated using two primer pairs (RNasePFor1 [5′-CTGC GATGGTCGTAATGTTG-3′] plus RNasePRev1 [5′-GAGGA GTTTACCGCGTTTCA-3′] and RNasePFor2 [5′-TATTTAAA GTAGAGGAAAGTC-3′] plus RNasePRev1). Following PCR amplification, PCR products (approximately 160 to 210 bp) were purified as described above and submitted to the same sequencing facility. The sequence data derived from all PCR products amplified from each hemoplasma isolate were aligned (AssemblyLIGN software; Oxford Molecular, Oxford, United Kingdom) and combined to generate a final sequence. Sequences were aligned using CLUSTAL X (version 1.8 EMBL) (20), and further manual adjustment was performed if visual observation showed regions of misalignment. Phylogenetic trees were constructed with the PHYLIP package (3), using the neighbor-joining program (18), from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model (9), and parsimony analysis by the method of Fitch (4) was used to count the number of base changes required on a given tree. The data set was resampled 100 times to generate bootstrap percentage values.

In total, 12 nearly complete 16S rRNA gene sequences were generated for different feline and canine hemoplasma isolates. These sequences showed 97 to 99% identity to United States "*Candidatus* M. haemominutum," *M. haemofelis*, or *M. haemocanis* sequences. Phylogeny studies using both distance and discrete methods yielded similar results (data not shown), with no obvious geographical or host specificity grouping of isolates evident (Fig. 1). Partial sequences of the RNA subunit of the RNase P gene were generated for six hemoplasma isolates. Phylogenetic analyses of the RNase P gene sequences available for representatives of the class *Mollicutes* and those sequenced in this study gave similar results with both distance (Fig. 2) and discrete (data not shown) methods in that all hemoplasmas fell within one clade, with the closest relatives being species in the *Mycoplasma pneumoniae* group. No obvious geographical grouping of isolates was observed, and although host specificity grouping was seen when the distance method was employed, this was not as conclusive with the discrete method.

These studies represent the first report of sequencing of nearly complete 16S rRNA genes from non-United States *M. haemofelis* and *M. haemocanis* hemoplasma isolates, and they confirm the presence of species nearly identical to those reported in the United States based on 16S rRNA gene data (6,

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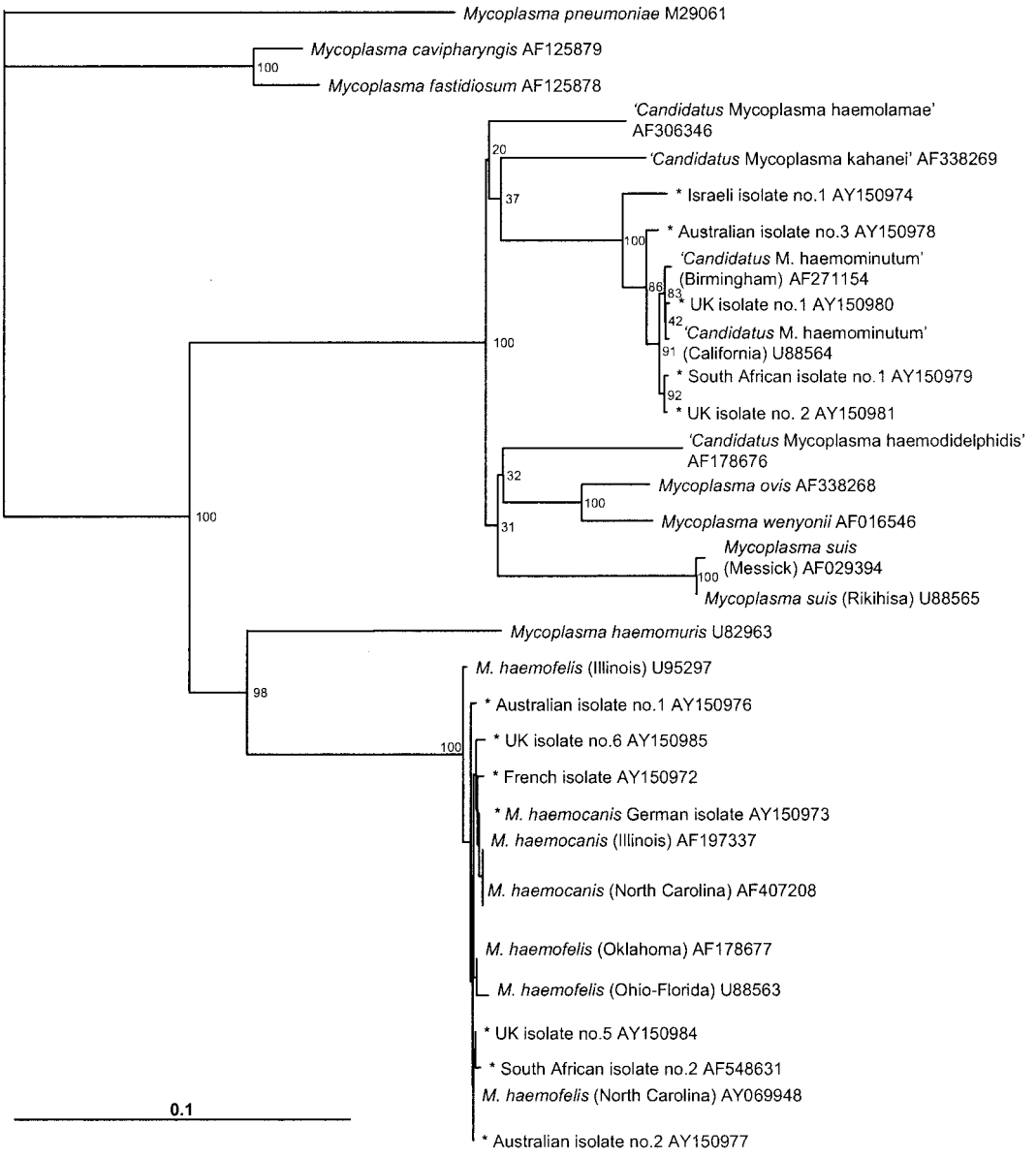

FIG. 1. Phylogenetic analysis of nearly complete 16S rRNA genes for sequenced hemoplasma isolates and related organisms. A phylogenetic tree was constructed by the neighbor-joining method. Evolutionary distances are to the scale shown. Bootstrap percentage values are given at the nodes of the tree. The asterisks indicate isolates sequenced in the present study. GenBank accession numbers are shown.

11, 12). Additionally, hemoplasma sequences for the RNA subunit of the RNase P gene were determined, allowing for the first time phylogenetic analysis of these organisms to be performed using non-16S rRNA gene data.

Analysis of 16S rRNA gene phylogeny of hemoplasmas from different countries revealed grouping of these organisms into one of two distinct clades, one clade comprising the "*Candidatus* M. haemominutum" isolates and related species and the other consisting of *M. haemocanis* and *M. haemofelis* isolates along with *M. haemomuris*, as reported previously with sequences derived in the United States (8, 11, 12). Although *M. haemocanis* and *M. haemofelis* isolates grouped together consistently, no reliable division of *M. haemocanis* and *M. haemofelis* was seen using either distance or discrete phylogenetic methods. This posed the question of whether the *M. haemocanis* and *M. haemofelis* isolates truly represent different species. Host specificity of canine and feline hemoplasmas had been suggested in an early study in which attempts to transmit feline hemoplasma infection to dogs failed (5). Although 16S rRNA sequences have proved to be a very effective tool in determining the phylogeny and taxonomy of the mollicutes, additional phylogenetic markers would be helpful to support the conclusions based on 16S rRNA gene data (15). It was, therefore, decided to determine the sequences of non-16S rRNA genes for further evaluation of the relationship between *M. haemocanis* and *M. haemofelis* as well as other hemoplasmas.

In this study, partial sequences of the RNA subunit of the

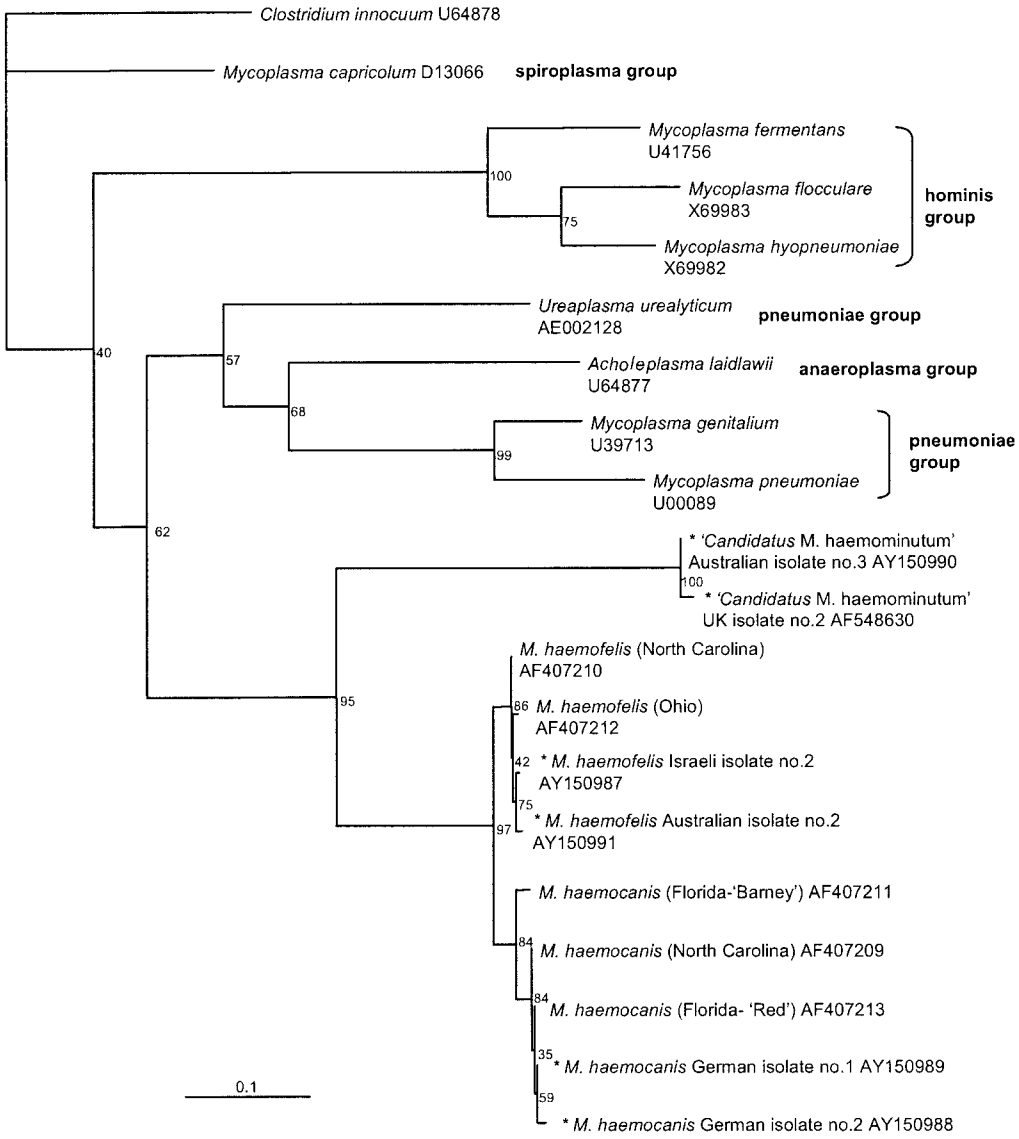

FIG. 2. Partial RNase P phylogenetic analysis of hemoplasma species and available members of the class *Mollicutes*. The phylogenetic tree was constructed by the neighbor-joining method. *Clostridium innocuum*, a walled relative of the mollicutes, was included as an outgroup. The previously described major phylogenetic groups of the mollicutes, based on 16S rRNA gene data (23), are represented in this tree and are shown in bold. Evolutionary distances are to the scale shown. Bootstrap percentage values are given at the nodes of the tree. Asterisks indicate isolates sequenced in the present study, and GenBank accession numbers are shown.

RNase P gene from a number of hemoplasma isolates were determined. The RNase P gene sequences available for mycoplasmas were limited in number, but preliminary phylogenetic analysis of those available did not generate the distinct five groupings of mycoplasmas described in studies of 16S rRNA gene phylogeny (23). Reports of studies of phylogeny of the genus *Mycoplasma* based on RNase P sequences have not been published to date, and further analysis of a larger number of *Mycoplasma* species would be helpful in defining these relationships. Based on 16S rRNA phylogeny studies, the hemoplasmas are most closely related to the *M. pneumoniae* group (14); this is supported by the findings of our studies using RNase P sequence data. Distance method phylogeny analysis of the derived RNase P sequences showed division of the *M. haemofelis* and *M. haemocanis* isolates into two distinct clusters, accompanied by high bootstrap percentages. However, the discrete method yielded no such division, although the bootstrap percentages associated with this method were lower. It appears that the RNase P gene may more reliably differentiate between *M. haemofelis* and *M. haemocanis* than 16S rRNA gene sequencing and supports the concept that these sequences represent distinct species infecting cats and dogs, respectively. A previous study, which looked at sequence identity only, also suggested that RNase P gene data might be more reliable for distinguishing *M. haemofelis* and *M. haemocanis* (1). Future research should focus on determining the RNase P gene sequences of other hemoplasma isolates for use in phylogenetic analyses.

There is no specific definition of exactly what constitutes a species (17). A polyphasic approach to taxonomy and species description, using phenotypic, genotypic, and phylogenetic information to describe an organism and deduce its status, has been suggested (21). The inability to culture hemoplasmas means that phenotypic characteristics cannot be described adequately to differentiate among species, resulting in a reliance on molecular methods (6, 11–13). DNA-DNA hybridization has been used to distinguish species (isolates from a single species having at least 70% DNA relatedness) (7, 17) and would be a useful technique to apply to hemoplasmas, although culturing of organisms is usually required to generate the large quantities of DNA needed for such studies. Phylogenetic analysis provides valuable information on the taxonomy of unculturable organisms such as the hemoplasmas. The studies reported here provide additional information on the relationship of the hemoplasmas and support the existence of distinct hemoplasma species in dogs and cats. Additionally, these studies report the existence in Germany, the United Kingdom, France, Australia, South Africa, and Israel of canine and feline hemotropic pathogens with 16S rRNA gene sequences nearly identical to those described in the United States. These organisms should be considered possible etiological agents in cases of anemia in recognized host species.

Nucleotide sequence accession numbers. The GenBank accession numbers of the nucleotide sequences derived in this study are AY150972 to AY150974, AY150976 to AY150981, AY150984, AY150985, AY150987 to AY150991, AF548630, and AF548631.

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