Re-evaluation of the classical *Mycoplasma lipophilum* cluster (Weisburg *et al.* 1989) and description of two new clusters in the hominis group based on 16S rDNA sequences

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The *Mycoplasma lipophilum* cluster (Weisburg *et al.* 1989) in the hominis group of the mollicutes is re-evaluated in this work to update the phylogenetic framework for classification of species within the genus *Mycoplasma*. Therefore, sequences of the 16S rRNA gene were determined from previously described species, and 11 were found to be closely related to the *M. lipophilum* cluster. A selection of members of the other hitherto defined clusters of the hominis group was included for phylogenetic analysis, revealing that the classical *M. lipophilum* cluster could be re-organized into two clusters, namely the *M. lipophilum* cluster and the *Mycoplasma bovis* cluster. The former was found to contain two species, while the latter contained 20 species. The two clusters were closely related, sharing an ancestral branch with the *Mycoplasma synoviae* cluster. Furthermore, the *M. bovis* cluster could be divided into subclusters. Interestingly, two species, *Mycoplasma equigenitalium* and *Mycoplasma elephantis*, formed a distinct and early branch of the *M. lipophilum*, *M. bovis* and *M. synoviae* clusters. This entity was termed the *M. equigenitalium* cluster. The clusters and subclusters could be verified by using neighbour-joining and maximum-likelihood analyses on a variety of data sets, bootstrap calculations, secondary structure analysis and signature nucleotides. Therefore, the new 16S rDNA data presented in this work were used to re-evaluate the *M. lipophilum* cluster, leading to the definition of two additional clusters. At present, the mollicutes belonging to the hominis group can be classified into ten evolutionary lineages.

Keywords: 16S rRNA, hominis group, *Mollicutes*, *Mycoplasma lipophilum* cluster, phylogeny

INTRODUCTION

The class *Mollicutes* (trivial name mycoplasmas) evolved from the *Clostridium–Streptococcus–Lactobacillus* branch of the phylum harbouring Gram-

positive organisms with a low G+C content in their genomes (Maniloff, 1992). A sparse array of biochemical features characterizes the mollicutes and the use of molecular data is important for taxonomic work on these micro-organisms (ICSB Subcommittee on the Taxonomy of Mollicutes, 1995; Heldtander *et al.*, 1998; Lee *et al.*, 1998; Pettersson *et al.*, 2000; Weisburg *et al.*, 1989). At present, the mollicutes contain five distinct phylogenetic groups, as revealed by analysing the primary structures of the 16S rRNA molecule (Weisburg *et al.*, 1989). The major group is the so-called hominis group, which was named after the human mollicute *Mycoplasma hominis*. The hominis

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Abbreviations: FCB, *Flavobacterium–Cytophaga–Bacteroides* division; ML, maximum-likelihood; NJ, neighbour-joining.

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group has been subdivided into eight sublineages (clusters and branches containing single species) by Weisburg *et al.* (1989) and Pettersson *et al.* (1996, 2000).

Many species of the genus *Mycoplasma* have been described, primarily by serological methods, but without determining the actual evolutionary relationship to other mollicutes. The aim of the present study was to investigate the current status of the *Mycoplasma lipophilum* phylogenetic cluster based on 16S rRNA gene sequences. The following species of the genus *Mycoplasma* were sequenced and found to belong to the *M. lipophilum* cluster or to form a new cluster within the phylogenetic hominis group. *Mycoplasma caviae* (G122^T) was first isolated from different tissues of guinea pigs by Hill (1971). Although one strain of this species was associated with uterine infection, it is not known whether the species is pathogenic. *Mycoplasma columbinasale* (694^T) was first isolated by Yoder & Hofstad (1964) from pigeon nasal turbinates and its taxonomic status was later established by Jordan *et al.* (1982). *Mycoplasma columbinum* (MMP-1^T) was first isolated from the oropharynx and trachea of pigeons and described by Shimizu *et al.* (1978). Whether this organism is pathogenic has not been established. A collection of mycoplasmas isolated from the respiratory tract of chickens, all apparently non-pathogenic, was examined by Edward & Kanarek (1960) and designated as *Mycoplasma iners*. An almost complete 16S rDNA sequence has been deposited under the accession number AF064063. However, the strain designation of the isolate associated with this sequence is uncertain and the established type strain, PG30^T (Edward & Freundt, 1973), was therefore selected for sequencing in this work. *Mycoplasma lipofaciens* (R171^T) was first isolated from the infra-orbital sinus of a chicken and described by Bradbury *et al.* (1983). Again, pathogenicity has not been established. *Mycoplasma maculosum* was first isolated by Edward & Fitzgerald (1951) from the throat and genital tracts of dogs and designated as strain γ . Later, Edward & Freundt (1956) established its taxonomic status and designated PG15^T as the type strain. *Mycoplasma* strains isolated from the canine throat and bladder by Armstrong *et al.* (1970) were characterized as members of a putative new species (canine serogroup D). Rosendal (1975) introduced a new species, *Mycoplasma opalescens* (MH5408^T), collectively classifying these strains. The human *Mycoplasma* sp. strain Navel and related simian mycoplasmas were named *Mycoplasma primatum* by DelGiudice *et al.* (1971). The simian strain HRC292^T was selected as the type strain. A partial 16S rDNA sequence (about 900 bp) of *M. primatum* strain Navel (not the type strain) has been deposited in GenBank by Rawadi *et al.* (1998). *Mycoplasma spermatophilum* was isolated from sperm and cervical specimens of patients with infertility problems (Hill, 1991). A partial sequence of *M. spermatophilum* strain AH159^T (about 900 bp) has been deposited in GenBank by Rawadi *et al.* (1998).

Mycoplasma strains isolated from the cervix of mares by Krabisch *et al.* (1973) were later classified as *Mycoplasma equigenitalium* (T37^T) by Kirchhoff (1978). *Mycoplasma elephantis* (E42^T) was first isolated from the genital tract of female elephants by Clark *et al.* (1978) and later characterized by Kirchhoff *et al.* (1996). Pathogenicity has not been established for either *M. equigenitalium* or *M. elephantis*.

The phylogenetic analysis presented in this work reveals that species showing close relationships to *M. lipophilum* can be grouped into new and revised clusters.

METHODS

Bacterial strains, growth conditions and preparation of genomic DNA. All strains sequenced in this study were obtained from the mycoplasma culture collection at the National Veterinary Institute in Uppsala, Sweden, and from the former mycoplasma culture collection at the Mycoplasma Section of the National Institute of Allergy & Infectious Diseases in Frederick, MD, USA (Table 1). The mycoplasmas were grown in appropriate media as described previously (Bölske, 1988). The cells were washed three times in PBS and DNA was prepared by phenol-chloroform extraction according to standard procedures (Johansson *et al.*, 1998b).

Determination of 16S rDNA sequences. Genomic DNA (10 ng) was used for amplification of virtually complete 16S rRNA genes with primers RIT593 and RIT620B. In order to increase yield and specificity, the PCR products obtained were subjected to two different semi-nested amplifications by using 1 μ l of the product as template in the subsequent reactions. Primers RIT593 and RIT390B were used in one of the amplifications and RIT388 and RIT620B were used in the other semi-nested reaction. All PCR primers have been published previously (Pettersson *et al.*, 1996). The first set covered the U1 to U5 regions (Gray *et al.*, 1984) of the 16S rRNA gene and the second set defined the segment between the U2 and U8 regions. Biotinylated PCR products, suitable for solid-phase DNA sequencing, were generated with 10 pmol of each primer. The following thermo-cycling profile was used in all amplifications. Denaturation at 96 °C for 15 s and a combined primer annealing/extension step at 70 °C for 90 s was repeated 30 times. A final extension at 72 °C for 10 min was also used. One primer was biotinylated in each of the two sets in order to generate products suitable for solid-phase 16S rDNA sequencing. Immobilization of the biotinylated PCR products followed by strand separation and template preparation were performed with superparamagnetic beads (Dynabeads M-280 Streptavidin) according to the instructions of the manufacturer (DynaLabs). The nucleotide sequences from both strands were determined by direct solid-phase 16S rDNA sequencing using protocols and primers detailed previously (Johansson *et al.*, 1998a; Pettersson *et al.*, 1996).

Phylogenetic analysis. The sequences were aligned manually by using the Genetic Data Environment (GDE) software (Smith, 1992). A set of differently modified alignments was obtained by the removal of gaps and ambiguously aligned positions and by excluding positions that were less than 50% conserved. The non-corrected and corrected alignments were used to establish evolutionary relationships

Table 1 Species and strains belonging to the *M. bovis*, *M. lipophilum* and *M. equigenitalium* clusters

Sequences with the following accession numbers were also used in the phylogenetic analysis: 'Candidatus Mycoplasma ravidipulmonis' (AF001173), 'Mycoplasma agassizii' PS6 (U09786), *Mycoplasma alkalescens* D12^T (U44764), *Mycoplasma bovirhinis* PG43^T (U44766), *Mycoplasma conjunctivae* HRC581^T (U44770), *Mycoplasma felis* CO^T (U09787), *Mycoplasma gallinaceum* DD^T (L24104), *Mycoplasma gypis* B1/T1^T (AF125589), *M. hominis* PG21^T (AJ002265), *Mycoplasma hyorhinis* BTS7^T (M24658), *M. iowae* 695^T (M24293), *Mycoplasma mobile* 163K^T (M24480), *M. mycoides* subsp. *mycoides* SC, PG1^T (U26038), *Mycoplasma neurolyticum* Type A^T (M23944), *Mycoplasma ovipneumoniae* Y98^T (U44771), *M. pneumoniae* FH^T (M29061), *Mycoplasma pulmonis* PG34^T (AF125582), *Mycoplasma salivarium* PG20^T (AF125583), *Mycoplasma svalvi* Mayfield B^T (M23936) and *M. synoviae* WVU 1853^T (L07757).

Species and strain	Main host	Glu/Arg*	Accession no.†	Reference
<i>M. adleri</i> G-145 ^T	Goat	–/+	U67943	Heldtander <i>et al.</i> (1998)
<i>M. agalactiae</i> PG2 ^T	Goat, sheep	–/–	U44763	Pettersson <i>et al.</i> (1996)
<i>M. bovirhinis</i> PG11 ^T	Cattle	–/–	M24291	Weisburg <i>et al.</i> (1989)
<i>M. bovis</i> Donetta ^T	Cattle	–/–	U44767	Pettersson <i>et al.</i> (1996)
<i>M. californicum</i> ST-6 ^T	Cattle	–/–	M24582	Weisburg <i>et al.</i> (1989)
<i>M. caviae</i> G122 ^T	Guinea pig	+/-	AF221111	This study
<i>M. columbinasale</i> 694 ^T	Bird (pigeon)	–/+	AF221112	This study
<i>M. columbinum</i> MMP-1 ^T	Bird (pigeon)	–/+	AF221113	This study
<i>M. felifaucium</i> PU ^T	Puma	–/+	U15795	Brown <i>et al.</i> (1995)
<i>M. fermentans</i> PG18 ^T	Human, primate	+/+	M24289	Weisburg <i>et al.</i> (1989)
<i>M. gallinarum</i> PG16 ^T	Bird (chicken, goose)	–/+	L24105	Boyle & Morrow (1994)
<i>M. iners</i> PG30 ^{T‡}	Bird (chicken)	–/+	AF221114	This study
<i>M. leopharyngis</i> LL2 ^T	Lion	–/–	U16760	Brown <i>et al.</i> (1995)
<i>M. lipofaciens</i> R171 ^T	Bird (chicken)	+/+	AF221115	This study
<i>M. maculosum</i> PG15 ^T	Dog	–/+	AF221116	This study
<i>M. meleagridis</i> 17529 ^T	Bird (turkey)	–/+	L24106	Boyle & Morrow (1994)
<i>M. opalescens</i> MH5408 ^T	Dog	–/+	AF221117	This study
<i>M. primum</i> HRC292 ^T	Human, primate	–/+	AF221118	This study
<i>M. simbae</i> LX ^T	Lion	–/+	U16323	Brown <i>et al.</i> (1995)
<i>M. spermatophilum</i> AH159 ^T	Human	–/+	AF221119	This study
<i>M. hyopharyngis</i> H3-6BF ^T	Pig	–/+	U58997	Blank <i>et al.</i> (1996)
<i>M. lipophilum</i> MaBy ^T	Human, primate	–/+	M24581	Weisburg <i>et al.</i> (1989)
<i>M. equigenitalium</i> T37 ^T	Horse	+/-	AF221120	This study
<i>M. elephantis</i> E42 ^T	Elephant	+/-	AF221121	This study

* Glu indicates glucose fermentation and Arg denotes arginine hydrolysis. +, Positive; –, negative.

† Accession numbers in bold indicate almost-complete 16S rDNA sequences determined in this study.

‡ A 16S rDNA sequence for *M. iners* was deposited in GenBank under the accession number AF064063. However, the strain designation was not available from this entry and the type strain was therefore resequenced in this work.

among the mollicutes investigated in this study. Phylogenetic calculations were performed by using algorithms implemented in the phylogenetic program package PHYLIP (Felsenstein, 1993). Infiles in the format compatible with that of PHYLIP were generated by using an option implemented in GDE. Both neighbour-joining (NJ) and maximum-likelihood (ML) programs were used to compute phylogenetic trees. Distances were corrected for multiple substitutions at single locations by the one-parameter model of Jukes & Cantor (1969) and statistical evaluation was performed by resampling the data 1000 times. The F84 evolutionary model with empirical nucleotide frequencies and a transition/transversion ratio set to 2.0 (Felsenstein, 1993) were used to calculate evolutionary trees by ML. The option for global rearrangement was invoked to find the best tree.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences determined in this work have been deposited

in GenBank. The accession numbers of these sequences and those retrieved for phylogenetic analysis are listed in Table 1.

RESULTS AND DISCUSSION

16S rDNA sequences

Primary structures of the 16S rRNA molecule were determined for 11 previously described species (Table 1). Almost complete sequences were generated, as determined for both strands. Polymorphisms in the two 16S rRNA genes were found in the following strains: *M. columbinum* MMP-1^T, A/G₍₁₉₃₎; *M. caviae* G122^T, A/G₂₀₆ and A/G₂₂₄; and *M. columbinasale* 694^T, C/T₇₄₈ and C/T₁₁₈₉ (according to *Escherichia coli* numbering; Brosius *et al.*, 1978). The position in parentheses indicates that the corresponding position is absent from the 16S rRNA gene of *E. coli*. All

species studied in this work were found to have a uridine residue at position 912 in the 16S rRNA molecule. This is distinctive for members of the hominis group, occurring in a highly conserved locale of the 16S rRNA molecule, and it is observed rarely in the domain *Bacteria* (Pettersson *et al.*, 1996, 2000; Weisburg *et al.*, 1989). Therefore, U₉₁₂ still proves to be synapomorphic for mollicutes of the hominis group.

Phylogenetic analysis

The sequences determined in this work were aligned with more than 300 other 16S rDNA sequences from mollicutes. Phylogenetic trees were constructed with all operational taxonomic units (OTUs) contained in this alignment and the trees served as a guide for the selection of appropriate OTUs for a more detailed phylogenetic analysis of the *M. lipophilum* cluster. Representative mycoplasmas from all other hitherto defined clusters and lineages within the hominis group were included for proper analysis of the evolutionary relationships of the species studied in this work. *Mycoplasma pneumoniae* strain FH^T and *Mycoplasma iowae* strain 695^T belong to the pneumoniae group (Weisburg *et al.* 1989) and were included for comparison. The type species of the mollicutes, *Mycoplasma mycoides* subsp. *mycoides* SC strain PG1^T, which is positioned phylogenetically in the spiroplasma group, served as an outgroup in the tree. Different criteria were applied in order to filter the data set for nucleotide positions to be used for phylogenetic analysis. By this procedure, subsets for final phylogenetic calculations were generated by removing gaps and ambiguously aligned positions and by applying a nucleotide consistency filter of 50%, i.e. positions for which a certain nucleotide composition could not be observed in more than half of the sequences. The NJ and ML algorithms were used to analyse the resulting data sets and all trees showed overall agreement concerning topology; a representative evolutionary distance tree is displayed in Fig. 1. The tree was computed from a data set from which only gaps were removed, finally containing 1370 nucleotide positions. Alternative reference and outgroup organisms did not alter the topology of the tree significantly. The stability of the tree was tested statistically by bootstrap analysis and the percentage values as calculated from 1000 replicates are given at the individual nodes of the tree. The cladistic procedure revealed that the species studied in this work formed three distinct lines of descent, including a splitting of the classical *M. lipophilum* cluster (Weisburg *et al.*, 1989) within the hominis group (Fig. 1).

Phylogenetic considerations and nomenclature

The inclusion of several mycoplasmas that have not previously been classified phylogenetically indicated clearly that the classical *M. lipophilum* cluster should be divided into two separate clusters. Consequently, the revised *M. lipophilum* cluster contained only two species, *M. lipophilum* and *Mycoplasma hyopharyngis*,

and this reduced cluster bisects the *Mycoplasma synoviae* cluster and a clade that harbours 20 species, including the bovine pathogen *Mycoplasma bovis* (Fig. 1). A third entity to be considered in this work was found at the tip of an early branch of the *M. synoviae*, *M. lipophilum* and *M. bovis* clades. This entity was formed by *M. equigenitalium* strain T37^T and *M. elephantis* strain E42^T; they showed a tight and distinct clustering in all trees.

Convincingly, certain nucleotide positions in the 16S rRNA gene (Table 2) supported the lines of the new clusters. Moreover, a few of the positions were regarded as signatures and are shown in bold in Table 2. A signature nucleotide in this context is a nucleotide residue found explicitly in a certain position within the sequences of the particular cluster, where the base that is present differs from those found in the majority of the other prokaryote taxa. The clusters could also be defined by unique nucleotide positions in the 16S rRNA molecule (Table 2). The characterization of a unique nucleotide feature was restricted to the hominis group and/or the class *Mollicutes*. Thus, a nucleotide residue at a certain position was said to be unique when present in the molecules of all strains within the particular cluster and absent, with no or only a few exceptions, in the strains of any other cluster or group of the mollicutes. These features are useful as an aid in molecular phylogeny of the mollicutes for the evaluation of the validity of the groups, clusters and subclusters (Weisburg *et al.*, 1989; Pettersson *et al.*, 2000). Therefore, discriminative nucleotide information (detailed below) at defined positions further justifies the introduction of two new clusters within the hominis group. The two new phylogenetic clusters are named according to the binomial nomenclature, acquiring their epithets from a representative species in the respective cluster, as suggested for classificatory work based on molecular phylogeny of the mollicutes (Weisburg *et al.*, 1989; Pettersson *et al.*, 2000). Accordingly, the two new clusters will hereafter be referred to as the *M. equigenitalium* cluster and the *M. bovis* cluster, while the third lineage retains the name the *M. lipophilum* cluster, as introduced previously by Weisburg *et al.* (1989).

The statistical support and branch lengths suggested a shared common ancestry of the *M. lipophilum*, *M. bovis* and *M. synoviae* clusters (Pettersson *et al.*, 1996), as indicated in Fig. 1. Moreover, these clusters possessed an apomorphic homologous structural feature between positions 722 and 723 (*E. coli*), with an extension of two extra bases. This attribute is found in the 16S rRNA molecule of neither the early branching *M. equigenitalium* cluster nor other mollicute groups or other taxa of the prokaryotes (Weisburg *et al.*, 1989; Pettersson *et al.*, 1996). It is likely that this idiosyncrasy was acquired somewhere on the ancestral branch of the *M. lipophilum*, *M. bovis* and *M. synoviae* clusters. The ancestral relationship was also reflected by the presence of derived nucleotide characters that were common to the three clusters, to the exclusion of

Fig. 1. Evolutionary distance tree based on 16S rRNA gene sequences, showing the representative phylogeny of the hominis group, but with emphasis on the *M. equigenitalium*, *M. lipophilum* and *M. bovis* clusters. The letters alongside vertical lines indicating subclusters of the *M. bovis* cluster correspond to the nomenclature used in the text and in Table 1. *M. mycoides* subsp. *mycoides* SC PG1^T of the spiroplasma group served as the outgroup and *M. pneumoniae* FH^T and *M. iowae* 695^T, both belonging to the pneumoniae group of the mollicutes, were included for comparison. Bootstrap percentage values obtained from 1000 resamplings of the data set are given at the nodes. Clusters denoted in bold were introduced in this work and species in bold were sequenced in this work. The accession numbers of these sequences are given in Table 1. The scale bar shows a distance equivalent to 0.1 substitutions per nucleotide position.

most other mollicutes. Among these positions were for example the canonical base pairs U·A and A·U in positions 612-628 and 833-853 (to be detailed elsewhere). Thus, the tree is likely to reflect a true evolutionary linking of the *M. lipophilum*, *M. bovis* and *M. synoviae* clusters.

The *M. lipophilum* cluster

Previously, the classical *M. lipophilum* cluster contained 11 characterized species (Weisburg *et al.*, 1989; Pettersson *et al.*, 1996; Heldtander *et al.*, 1998). In this study, the *M. lipophilum* cluster has been revised, now

Table 2 Unique and signature nucleotide positions in the 16S rRNA of the *M. equigenitalium*, *M. lipophilum* and *M. bovis* clusters

The numbers of particular positions correspond to those in the 16S rRNA molecule of *E. coli* (Brosius *et al.*, 1978). Signatures are shown in bold; lower-case letters denote residues found occasionally.

Position of base or base pair	<i>M. equigenitalium</i> cluster	<i>M. lipophilum</i> cluster	<i>M. bovis</i> cluster	Hominis group	<i>Mollicutes</i>	Exceptions
129·232	U·G	U·G	C·G	U·G	C·G, U·G	
147·175	G·C	A·U	G·C ^{a*}	G·C	G·C	A·U: <i>M. adleri</i> , <i>M. columbinasale</i> . G·U: <i>M. fermentans</i>
148·174	G·A	C·U	G·A ^a	A·G, G·A	A·G, G·A	C·U: <i>M. fermentans</i> , <i>M. bovisgenitalium</i> subcluster
152	A	A	G	A, C	A	G: haemotrophic mycoplasmas
169	C	C	A ^a	C, U	a, C, u	U: <i>M. gallinarum</i>
242·284	U·A	C·G	C·G	C·G ^b	C·G, U·A, U·G	U·A: <i>M. sualvi</i> , <i>M. mobile</i>
264	U	U	C ^a	a, U	A, c, U	A: <i>M. lipofaciens</i> . U: <i>M. iners</i> , <i>M. gallinarum</i>
293·304	G·C	G·U	G·U	G·U ^b	A·U, G·C, G·U, U·A	A·U: ' <i>M. agassizii</i> ', <i>M. pulmonis</i> , <i>M. sturni</i>
307	C	A, U	A, U	A, U ^b	A, c, U	C: <i>M. gypis</i>
407·435	A·U	C·G	C·G ^a	C·G	A·U	A·U: <i>M. fermentans</i> subcluster
408·434	A·U	C·G	A·U	A·U	A·U, G·C ^c	C·G: <i>Spiroplasma</i> spp. (few)
409·433	C·G	U·G	G·C	C·G, G·C ^b	C·G, G·C, U·A, U·G	U·G: <i>M. auris</i> , <i>M. buccale</i>
417·426	C·G	A·U, G·U	G·U	G·U, U·A	G·U, U·A ^c	C·G: <i>Acholeplasma modicum</i> , <i>Acholeplasma axanthum</i>
446·488	A·U	G·C	G·C ^a	G·C	A·U, G·C	A·U: <i>M. gallinarum</i> . U·G: <i>M. opalescens</i>
501·544	U·A	C·G	C·G	C·G ^b	C·G, u·a, u·g	U·A: ' <i>M. agassizii</i> ', <i>M. sualvi</i> . U·G: ' <i>Candidatus Mycoplasma ravigulmonis</i> ', <i>M. ovipneumoniae</i>
546	G	A	A	A	A ^c	G: haemotrophic mycoplasmas, mycoplasma-like organisms (some)
680·710	A·U	C·G	C·G	C·G, u·g	C·G, u·g ^c	A·U: <i>M. fastidiosum</i> cluster, haemotrophic mollicutes
771·808	G·C	A·U	G·C	G·C	G·C ^c	A·U: mycoplasma-like organisms (few)
812	G	G	C	G ^b	c, G	C: ' <i>Candidatus M. ravigulmonis</i> '
893	U	U	C	U ^b	C, U	U: ' <i>Candidatus M. ravigulmonis</i> '
895·904	C·G	C·G	U·A	C·G	G·C, U·A	
906	G	G	A	G ^b	G	A: <i>M. hominis</i> , <i>M. hyosynoviae</i>
930·1387	U·A	C·G	C·G, U·G ^a	C·G, U·G	C·G, U·G	A·G: <i>M. californicum</i> . C·U: <i>M. simbae</i>
1048·1209	G·U	A·U	A·U	A·U	G·C	
1120·1153	A·U	C·G	C·G	C·G, U·A	a·u, c·g, G·C, U·A	
1124·1149	U·A	G·C	G·C	a·u, G·C ^b	a·u, G·C, G·U	U·A: ' <i>M. agassizii</i> '
1129·1143	C·G	U·A	U·A ^a	U·A ^b	C·G, U·A	C·G: <i>M. spermatophilum</i> . C·A: ' <i>M. agassizii</i> '
1131	U	—	A, C	—, A	A, G, U	
1424·1476	A·U	U·A	U·A	U·A, u·g	C·G, g·u, U·A, U·G ^c	A·U: haemotrophic mycoplasmas (few)
1436·1465	C·U	U·C	U·C	U·C, A·U, U·U ^b	A·U, C·A, C·U, U·A	C·U: <i>M. gypis</i> , <i>M. mobile</i>

*Exceptions listed are those found among the members of the *M. bovis* cluster (a), the hominis group (b) or mollicute taxa not belonging to other mollicute groups (c).

harbouring only two species. The current members, *M. lipophilum* MaBy^T and *M. hyopharyngis* H3-6BF^T, were 96.5% similar in 16S rDNA sequence to each other, but only ≤ 93.3 , ≤ 91.7 and ≤ 88.8 % similar to the neighbouring *M. bovis*, *M. synoviae* and *M. equigenitalium* clusters. It is notable that the value of ≤ 93.3 % similarity to the *M. bovis* cluster approached the lower limit of the intraspecies 16S rDNA percentage similarity found among the individual members of the *M. bovis* cluster (see below). Despite this, the *M. lipophilum* cluster can be regarded as a separate phylogenetic unit as judged by the evolutionary distance tree and the presence of sequence signatures (Fig. 1; Table 2). This observation underlines the importance of performing phylogenetic computations instead of relying entirely on results obtained by simple similarity-based methods such as BLAST, which might be misleading.

Besides a marked and stable clustering in all derived trees, there are also 16S rRNA gene sequence positions that are signatory for the *M. lipophilum* cluster (Table 2). Some compositions are found rarely in other bacterial phyla, such as the adjacently positioned base pairs A·U and C·U in positions 147·175 and 148·174. Exceptions to the former pair are found in the 16S rRNA molecules of six members of the *Flavobacterium*–*Cytophaga*–*Bacteroides* (FCB) phylum, including *Blattabacterium*. This composition is also present in *Petrogala miotherma*, *Fibrobacter succinogenes* and *Acetohalobium arabaticum*. In the *Archaea*, the residues A·U at 147·175 are found only in a few halophiles and in *Sulfolobus solfataricus* and *Sulfolobus shibatae*. The feature C·U at position 148·174 is found in *Mycoplasma fermentans* and the *Mycoplasma bovigenitalium* subcluster (this study), as well as in *Prevotella oralis* and *Isosphaera pallida* in the domain *Bacteria*. It can also be found in some members of the domain *Archaea*. These positions are therefore likely to constitute suitable targets for probe-based diagnostic assays of the *M. lipophilum* cluster. Another very rare feature is the base pair C·G at 408·434, which is shared only by a few bacterial taxa and by four spiroplasmas among the mollicutes and occurs sparsely among the archaea. Furthermore, the *M. lipophilum* cluster has A·U at positions 771·808, shared with the order *Chlamydiales* and the genera *Acinetobacter*, *Nocardiosis*, *Arthrobacter* and their relatives (most), *Corynebacterium*, *Lactobacillus* (most), the *Actinomyces* group (most), *Actinopolyspora* and a few other members of the *Bacteria*.

A higher-order structural feature was identified for the *M. lipophilum* cluster, namely a truncation of position 1131. However, this one-base deletion is also present in some members of the β -*Proteobacteria*, some spiroplasmas and the *Clostridium leptum* subgroup. It nevertheless constitutes a rarely observed attribute in the 16S rRNA molecule. The members of the pneumoniae group and the *M. synoviae* cluster have deletions in this region of several base pairs. Moreover, *M. lipophilum* MaBy^T and *M. hyopharyngis* H3-6BF^T

also share biochemical properties, in that both hydrolyse arginine but lack the ability to ferment glucose (Table 1).

The *M. bovis* cluster

The *M. bovis* cluster was found to contain 20 species, isolated from a variety of hosts (Table 1). Thus, this cluster is one of the largest entities of the hominis group, being about the size of the *M. hominis* cluster (Pettersson *et al.*, 2000). The interspecies 16S rDNA similarities ranged between 91.9 and 99.5%. Most of these values fell in the range 93–97%, and only 9.5% of all the calculated percentage values in the distance matrix (190 in total) of this cluster fell outside this range. Typically, the *M. bovis* cluster was ≤ 92.2 % similar to the *M. synoviae* cluster and ≤ 89.1 % similar to the *M. equigenitalium* cluster. Moreover, the *M. bovis* cluster could be divided further into a set of subclusters (detailed below), indicated in Fig. 1.

A sequence attribute, an adenosine residue, at position 906 can be found in other bacterial divisions, but occurs rarely among the Gram-positives with low G + C content in their genomes. Therefore, this residue is shown in bold face, despite not having the status of a true signature.

All members of the *M. bovis* cluster, except *Mycoplasma californicum*, were found to have an extra nucleotide between positions 419 and 420. This extra base will lead to the formation of a C·G base pair, thereby engaging the fourth nucleotide of the loop, starting at position 420. The stem of this helix in the *M. bovis* cluster is therefore one base pair longer, due to a reduction of the tetra-loop found normally in this helix to a tri-loop. *Mycoplasma meleagridis* is characterized by having a non-canonical pair of the U·G type in the particular region of the 16S rRNA molecule. Members of the FCB phylum, the *Mycoplasma fastidiosum* cluster and ureaplasmas of the pneumoniae group of the mollicutes also have a tri-loop in this part of the molecule.

The *M. equigenitalium* cluster

The two members of this cluster, *M. equigenitalium* strain T37^T and *M. elephantis* strain E42^T, were found to be closely related, with a 16S rDNA similarity of 99.1%. *M. elephantis* and *M. equigenitalium* showed a distinct line of descent, sharing a common ancestral node with members of the *M. lipophilum*, *M. bovis* and *M. synoviae* clusters. Generally, the *M. equigenitalium* cluster was < 90% similar to any other cluster or lineage within the hominis group, and its distinct position was justified from both the tree (Fig. 1) and nucleotide features (Table 2). A few of these were characterized as signatory for this cluster. One was observed to be the base pair C·G at 417·426, with only a few exceptions within the bacterial divisions such as the early bacterial lines *Aquificales* and

Thermotogales, the genera *Thermus* and *Fibrobacter* and some environmental clones, *Campylobacter* and relatives and the subgroup of *Thermoanaerobacter*. Members of the *Archaea* have deletions in this region. The composition U·A at 930·1387 can be observed in subgroups of the FCB division, the spirochaete genus *Leptospira*, the *Rickettsia* and *Orientia* subgroups of the α -*Proteobacteria* and the *Calyptogenia* symbionts belonging to the *Thiothrix nivea* subgroup of the γ -*Proteobacteria*. Despite these exceptions, this feature is observed rarely in the domain *Bacteria*. A third attribute, the base pair A·U at positions 1424·1476, was observed in the *Treponema pallidum* subgroup, the genus *Colwellia*, *Helicobacter* and relatives, the *Campylobacter fetus* subgroup (*Arco-bacter* and *Sulfurospirillum*) and the *Haloanaerobium* group. Two additional clusters also shared this composition, namely the *Acetohalobium arabaticum* and *Alloiococcus otitis* subgroups (three and two species, respectively).

Subclusters within the *M. bovis* cluster

In general, the members of the *M. bovis* cluster showed a repeatedly stable topology in the different calculated phylogenies, which allows for the definition of subclusters (Fig. 1). Also, the subclusters were supported by high bootstrap percentage values of over 80%. Nucleotide features that justify this fine-tuned classification, at least to a certain extent, have been compiled in Table 3. However, we believe that these subclusters should be regarded as tentative, because some of the subclusters predicted here might be destabilized as more taxa are added to the *M. bovis* cluster in future, with the need to re-evaluate them. Nevertheless, they will facilitate work significantly on the classification of organisms that are phylogenetically related to members of the *M. bovis* cluster. A short presentation, with the actual names of the subclusters and lines within the *M. bovis* cluster, follows below and the letter codes given in parentheses are the same as those used in Fig. 1 and Table 3. The names of the subclusters have been introduced by following the previously outlined scheme for phylogenetic grouping of the mycoplasmas by using binomial epithets (Pettersson *et al.*, 2000).

Species *M. opalescens* (a), *M. spermatophilum* (f) and *M. lipofaciens* (h). The species *M. opalescens* MH5408^T, *M. spermatophilum* AH159^T and *M. lipofaciens* R171^T did not cluster specifically with any other species in the *M. bovis* cluster and are defined as constituting single-species lines of descent. These species showed 16S rDNA similarity of $\leq 96.2\%$ to each other or to any species of the other subclusters of the *M. bovis* cluster.

The *Mycoplasma leopharyngis* subcluster (b). The *M. leopharyngis* subcluster was supported by a bootstrap value of 100%, containing *M. leopharyngis* LL2^T and *M. maculosum* PG15^T. They were 99.5% similar at the 16S rDNA sequence level and shared biochemical

properties to some extent, in that both mycoplasmas do not ferment glucose (Table 1).

The *Mycoplasma felifaucium* subcluster (c). This entity harboured *M. felifaucium* PU^T and *Mycoplasma adleri* G-145^T, with a statistical bootstrap support of 100%. They were 97.7% similar at the 16S rDNA sequence level and shared biochemical properties (Table 1).

The *M. bovis* subcluster (d). Three species formed the *M. bovis* subcluster, namely, *M. bovis* Donetta^T, *Mycoplasma agalactiae* PG2^T and *M. primatum* HRC 292^T. The 16S rRNA similarity ranged between 97.7 and 99.0% and all species are incapable of fermenting glucose (Table 1). They grouped together in 100% of the consensus and majority trees. Strikingly, this entity also showed a higher-order structural attribute in the 16S rRNA molecule, the insertion of a purine between positions 197 and 198 (*E. coli*). This feature is not present in other mollicutes (except *M. hyopharyngis*) or prokaryotes, thus being idiosyncratic for the *M. bovis* subcluster.

The *M. fermentans* subcluster (e). *M. fermentans* PG18^T and *M. caviae* G122^T were constituents of the *M. fermentans* subcluster, grouping together with a bootstrap value of 100%. Both species ferment glucose and their 16S rRNA gene sequences were 98.8% similar.

The *M. iners* subcluster (g). With its five members, the *M. iners* subcluster was the largest phylogenetic subcluster in the *M. bovis* cluster. The members were *M. iners* PG30^T, *Mycoplasma gallinarum* PG16^T, *M. meleagridis* 17529^T, *M. columbinasale* 694^T and *M. columbinum* MMP-1^T. This clade was formed in as many as 80% of all trees that were obtained by 1000 resamplings of the data set. They only shared 16S rDNA similarities ranging between 94.0 and 96.4%. These relatively low values suggest that they are separate species, without the need for the final justification of this status by performing DNA–DNA reassociation experiments (Stackebrandt & Goebel, 1994). Phenotypically, all species are united by their capacity to hydrolyse arginine and their inability to ferment glucose. Interestingly, this was the only subcluster that shared the same group of hosts, i.e. all are of avian origin (Table 1).

The *M. bovigenitalium* subcluster (i). *M. bovigenitalium* PG11^T, *M. californicum* ST-6^T and *Mycoplasma simbae* LX^T belonged to an entity that was termed the *M. bovigenitalium* subcluster. The primary structures of their 16S rRNA genes were 96.0–97.4% similar to each other. The branch of this subcluster was the most distinct, with a bootstrap support of 100%. The three species are all unable to ferment glucose.

Considerations on the clusters and subclusters for taxonomic work

The updates and details of the part of the hominis group studied and discussed in this work will have a profound impact on taxonomic work on mollicutes belonging to the *M. equigenitalium*, *M. lipophilum* and

Table 3 Unique and signature nucleotide positions in the 16S rRNA molecules of the different subclusters and lines within the *M. bovis* cluster

Letters a–i correspond to the subclusters and lines of descent indicated in Fig. 1. Unique nucleotide positions in the 16S rRNA genes of members of a particular subcluster are shown in bold. The letter codes R and Y follow the IUPAC code of the International Union of Biochemistry. Abbreviations for single-species lines and subclusters correspond to the line of *M. opalescens* (*Mopa*), the *M. leopharyngis* (*Mleo* subcl.), *M. felifaucium* (*Mfel* subcl.), *M. bovis* (*Mbovis* subcl.) and *M. fermentans* (*Mfer* subcl.) subclusters, the line of *M. spermatophilum* (*Mspe*), the *M. iners* subcluster (*Mine* subcl.), the line of *M. lipofaciens* (*Mlipof*) and the *M. bovisgenitalium* subcluster (*Mbovig* subcl.), as defined in the text and in Fig. 1. The numbers of particular positions correspond to those in the 16S rRNA molecule of *E. coli* (Brosius *et al.*, 1978).

Position of base or base pair	(a) <i>Mopa</i>	(b) <i>Mleo</i> subcl.	(c) <i>Mfel</i> subcl.	(d) <i>Mbovis</i> subcl.	(e) <i>Mfer</i> subcl.	(f) <i>Mspe</i>	(g) <i>Mine</i> subcl.	(h) <i>Mlipof</i>	(i) <i>Mbovig</i> subcl.	Comments*
82·87	A·U	A·U	A·U	A·U	A·U	A·U	C·G	C·G	A·U	
85	A	A	A	A	A	A	C	C	U	
128·233	U·G	U·G	U·G	U·A	U·G	U·G	U·G	U·G	U·G	U·A: hominis group (few), mollicutes (few)
148·174	G·A	G·A	G·A	G·A	C·U	G·A	G·A	G·A	C·U†	C·A: <i>M. simbae</i>
154·167	G·C	U·G	G·C	G·C	G·C	A·U	A·U†	A·U	G·C	G·C: <i>M. columbinum</i>
183	U	U	U	U	U	U	G	C	U	
200·217	A·U	R·U	A·U	A·U	R·U	A·U	A·U	U·A	C·G	C·G: <i>M. hominis</i>
305	G	G	A	G	G	G	G	G	G	A: bacteria (few)
407·435	C·G	C·G	C·G	C·G	A·U	C·G	C·G	C·G	C·G	A·U: hominis group (few)
443·491	A·U	A·U	A·U	U·G	R·U	A·U	A·U	A·U	A·U	U·G: hominis group (few)
450·483	G·C	G·C	G·C	G·C	G·C	G·C	G·C	G·C	U·A	U·A: mollicutes (few), bacteria (few)
484	G	G	G	G	G	G	G	G	U	U: pneumoniae group, bacteria (few)
590·649	U·A	U·G	U·A	U·R	U·G	U·G	U·U	U·A	U·R	U·U: hominis group (none)
659·746	A·U	A·U	A·U	A·U	G·U	A·U	A·U	A·U	A·U	
722	A	U	A	A	A	A	A	A	A	U: hominis group (none)
819	A	A	A	A	A	A	A	A	U	U: hominis group (few)
830·856	C·G	C·G	C·G	U·A	C·G	C·G	C·G	C·G	C·G	
1163·1173	C·G	G·C	C·G	C·G	C·G	C·G	C·G	C·G	C·G	
1243·1294	C·G	A·U	C·G	A·U	C·G	A·U	U·A	A·U	C·G	
1254·1283	A·U	A·U	A·U	A·U	A·U	A·U	A·U	A·U	Y·A	U·A: hominis group (few)
1264·1271	U·A	U·A	A·U	U·A	U·A	U·A	U·A	U·A	U·A	A·U: hominis group (none)
1273	G	C	G	G	G	G	G	G	U, A	A, U: hominis group (few)
1274	G	A	G	G	G	G	G	G	G, C	
1310·1327	A·U	A·U	A·U	A·U	U·A	U·A	U·A	U·A	G·C	

*Comments are given as to what extent the actual nucleotide composition can be regarded as discriminatory.

†Taxa listed under comments are exceptions in nucleotide composition found among members of that particular subcluster.

M. bovis clusters. In accordance with previous discussions on facilitating the classification of mycoplasmas (Heldtander *et al.*, 1998; Johansson *et al.*, 1998a; Pettersson *et al.*, 1996, 2000), for a new mycoplasma strain that belongs phylogenetically to any of these clusters or subclusters, it should be sufficient to perform serological tests against the members of that cluster or subcluster. However, if the organism in question shows a 16S rDNA similarity of > 97% to another member(s) of the phylogenetic cluster or subcluster, but is serologically negative with antisera to this member, it is recommended that DNA–DNA hybridization experiments should be performed in order to reveal whether or not the organism should be given novel species status (Stackebrandt & Goebel, 1994).

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