Phylogeny of the *Mycoplasma mycoides* cluster based on analysis of five conserved protein-coding sequences and possible implications for the taxonomy of the group

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A phylogenetic tree of the *Mycoplasma mycoides* cluster was inferred from a set of concatenated sequences from five housekeeping genes (*fusA*, *glpQ*, *gyrB*, *lepA* and *rpoB*). The relevance of this phylogeny was reinforced by detailed analysis of the congruence of the phylogenies derived from each of the five individual gene sequences. Two subclusters were distinguished. The *M. mycoides* subcluster comprised *M. mycoides* subsp. *mycoides* biotypes Small Colony (SC) and Large Colony (LC) and *M. mycoides* subsp. *capri*. The latter two groups could not be clearly separated, which supports previous proposals that they be united into a single taxonomic entity. The *Mycoplasma capricolum* subcluster included *M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae* and *Mycoplasma* sp. bovine group 7 of Leach, a group of strains that remains unassigned. This group constituted a distinct branch within this cluster, supporting its classification as a subspecies of *M. capricolum*. *Mycoplasma cottewii* and *Mycoplasma yeatsii* clustered in a group that was distinct from *Mycoplasma putrefaciens* and they were all clearly separated from the *M. mycoides* cluster. In conclusion, this approach has allowed us to assign phylogenetic positions to all members of the *M. mycoides* cluster and related species and has proved the need to adjust the existing taxonomy. Furthermore, this method may be used as a reference technique to assign an unequivocal position to any particular strain related to this cluster and may lead to the development of new techniques for rapid species identification.

INTRODUCTION

The *Mycoplasma mycoides* cluster, as described by Cottew *et al.* (1987), represents a group of six closely related mycoplasmas that are pathogenic for ruminants and which are currently referred to as *Mycoplasma mycoides* subsp. *mycoides* Small Colony biotype (MmmSC) and Large Colony biotype (MmmLC), *M. mycoides* subsp. *capri*

(Mmc), *Mycoplasma capricolum* subsp. *capricolum* (Mcc), *M. capricolum* subsp. *capripneumoniae* (Mccp) and *Mycoplasma* sp. bovine group 7 of Leach (MBG7). In addition, on the basis of 16S rRNA gene sequence similarity, other species such as the pathogen *Mycoplasma putrefaciens* (Weisburg *et al.*, 1989) and the related saprophytic species *Mycoplasma cottewii* and *Mycoplasma yeatsii* (Heldtander *et al.*, 1998) have been included in the phylogenetic *M. mycoides* cluster, within the *Spiroplasma* group.

Abbreviations: CBPP, contagious bovine pleuropneumonia; CCP, contagious caprine pleuropneumonia; MBG7, *Mycoplasma* sp. bovine group 7 of Leach; Mcc, *M. capricolum* subsp. *capricolum*; Mccp, *M. capricolum* subsp. *capripneumoniae*; Mmc, *M. mycoides* subsp. *capri*; MmmLC, *M. mycoides* subsp. *mycoides* Large Colony biotype; MmmSC, *M. mycoides* subsp. *mycoides* Small Colony biotype.

The GenBank/EMBL/DDJB accession numbers of the partial *fusA*, *glpQ*, *gyrB*, *lepA* and *rpoB* gene sequences of 26 strains analysed here are listed in Supplementary Table S1.

Accession numbers of the sequences used in this study are available with the online version of this paper.

MmmSC and Mccp are the causative agents of contagious bovine and contagious caprine pleuropneumonia (CBPP and CCP), respectively, which are diseases of major concern, classed by the World Organization for Animal Health (OIE) as notifiable animal diseases. Other members of the cluster (MmmLC, Mmc and Mcc) and *M. putrefaciens* are involved in the 'MAKePS' syndrome, characterized by mastitis, arthritis, keratoconjunctivitis, pneumonia and septicaemia in small ruminants (Thiaucourt & Bölske, 1996). Finally, MBG7 represents a

group of strains that are serologically distinct from other bovine mycoplasmas (Leach, 1967) and which cause mastitis and polyarthritis in cattle.

Because the different members of the *M. mycoides* cluster share many genotypic and phenotypic traits, their classification and evolutionary relationships have been difficult to establish. This may explain why the group of strains known as MBG7 remains unassigned, while Mccp was named only relatively recently (Leach *et al.*, 1993). Initial classification within this cluster was attempted based on DNA–DNA hybridization and biochemical and serological methods as well as 2D PAGE patterns (Cottew *et al.*, 1987), which resulted in differentiation of the *M. mycoides* subspecies on one side and the *M. capricolum* subspecies on the other. The position of MBG7 was controversial due to conflicting data obtained from the various methods applied and remains unresolved, though recent studies concur in favour of classification of this group of strains within a subspecies of *M. capricolum* (Harasawa *et al.*, 2000; Pettersson *et al.*, 1996; Thiaucourt *et al.*, 2000).

Another controversial issue is the distinction of MmmLC and Mmc as separate subspecies: numerous investigations, based on both genetic and antigenic analyses, suggest that they may be classed together as a single subspecies within the *M. mycoides* subcluster (Costas *et al.*, 1987; Leach *et al.*, 1989; Monnerat *et al.*, 1999; Olsson *et al.*, 1990; Rodwell, 1982; Salih *et al.*, 1983; Taylor *et al.*, 1992; Vilei *et al.*, 2006).

The phylogeny of the *M. mycoides* cluster inferred from 16S rRNA gene sequences (Pettersson *et al.*, 1996) relied on too few nucleotide variations to construct a reliable phylogenetic tree. It was then proposed that further studies be based on sequence analysis of alternative genes. To this aim, five housekeeping genes have been identified (*fusA*, *glpQ*, *gyrB*, *lepA* and *rpoB*), taking into consideration the requirements for phylogenetic studies described by Woese (1987). Phylogenetic analysis based on concatenated sequences from these five genes and reinforced by separate analysis of each of the five genes has enabled us to determine evolutionary relationships between all the members of the *M. mycoides* cluster.

METHODS

Determination of partial gene sequences. A sample of strains from various geographical origins was chosen carefully, including representatives of all members of the *M. mycoides* cluster and related species and adapting the number of strains to the known variability within each group. The 28 strains analysed are listed in Table 1, with reference to their origin. The strains were characterized according to results obtained using two different techniques: growth inhibition with hyperimmune sera (Clyde, 1983) and dot immunobinding (Poumarat *et al.*, 1992).

Mycoplasma strains were cultured in modified Hayflick's broth [PPLO broth without crystal violet (21 g l^{-1}), 20% horse serum de-complemented for 1 h at 56°C , 5% fresh yeast extract, 0.2% glucose, 0.4% sodium pyruvate] at 37°C in 5% CO_2 and were harvested in

the late exponential phase of growth. Preparation of PCR templates in lysis buffer was based on the method of Miserez *et al.* (1997).

The selection of primer pairs for amplification of *fusA*, *glpQ*, *gyrB*, *lepA* and *rpoB* sequences (Table 2) was based on initial alignments of sequences obtained from published mycoplasma genomes (<http://cbi.labri.fr/outils/molligen/home.php>) as well as from ongoing genome sequencing projects (MmmLC strain 95010-C1 and MmmSC strain 8740-Rita). Amplification of each of the target sequences was performed in 50 μl reactions containing $1 \times$ Taq buffer (Qiagen) (giving a final MgCl_2 concentration of 1.5 mM), 150 μM dCTP and dGTP, 300 μM dATP and TTP (dNTPs from Roche), 0.4 μM each of the corresponding forward and reverse primer, 1 U Taq polymerase (Qiagen) and 1 μl sample. The PCR thermal program consisted of an initial denaturation step of 2 min at 94°C followed by 35 cycles of 30 s at 94°C , 30 s at 52°C and 45 s at 72°C and a final extension step of 5 min at 72°C . An exception was made for the PCR with primers GlpQ-L-F/-R, for which the annealing temperature was 45°C , with the reaction conditions otherwise remaining the same.

The primer pairs listed in Table 2 were also used for sequencing of the corresponding PCR products by Genome Express (Meylan, France). Sequences were aligned using the AlignX tool of Vector NTI 9.0 (Invitrogen) and they were trimmed to the same size, providing fragments of 561 bp (*fusA*), 538 bp (*glpQ*), 533 bp (*gyrB*), 657 bp (*lepA*) and 625 bp (*rpoB*) for phylogenetic analyses. No deletions/insertions were observed except for the *glpQ* sequences of the two *M. putrefaciens* strains, which contained an extra codon. The partial nucleotide sequences determined in this study for each of the five protein-coding genes from 24 strains, as well as from MmmSC strain 8740-Rita and MmmLC strain 95010-C1, were deposited in GenBank under the accession numbers shown in Supplementary Table S1 (available in IJSEM Online). Sequences of these five genes were also retrieved from the published genomes of the type strains MmmSC PG1^T (GenBank accession no. BX293980) and Mcc California kid^T (CP000123). A total of 28 sequences were therefore available for each of the five genes studied.

Phylogenetic analyses. Sequence analyses were conducted using MEGA 3.1 (Kumar *et al.*, 2004; <http://www.megasoftware.net>), whereas Darwin 5.0 (Perrier *et al.*, 2003; <http://darwin.cirad.fr/darwin>) was used for diversity analyses. Phylogenetic trees were constructed using the neighbour-joining algorithm (Saitou & Nei, 1987) based on pairwise dissimilarities between strains. Since other methods such as minimum evolution and maximum parsimony by close-neighbour interchange (MEGA 3.1) generated nearly identical topologies, the neighbour-joining method was retained for its properties of convergence and consistency, as well as low computational complexity.

Pairwise distances were calculated as the proportion of non-matching sites between pairs of sequences. The three positions corresponding to an extra codon in the *glpQ* sequences of the *M. putrefaciens* strains were deleted to avoid gaps. Since the sequences were highly similar, the effect of multiple substitutions was considered negligible and no correction was applied to dissimilarities. The mean net distance between groups was also calculated as the arithmetic mean of the pairwise distances between two groups corrected by the corresponding mean distance within groups (MEGA 3.1).

A bootstrap analysis with 500 replicates was performed to test the stability on randomly chosen sets of positions. To analyse the stability through the sample of strains, the influence of each sequence on the tree structure was estimated using a tool available in Darwin 5.0. In a jackknife-like procedure, each unit is successively removed and a partial tree is inferred from all units except the removed one. This tree is compared to the initial tree using a topological criterion, the quartet tree distance (Qd), which measures among all quartets

Table 1. *Mycoplasma* strains analysed in this study

Suppliers of strains are abbreviated as follows: Arist. U., Professor K. Sarris, Aristoteles University, Thessaloniki, Greece; Bern U., Professor J. Nicolet, Institute for Veterinary Bacteriology, University of Bern, Switzerland; BgVV, Dr K. Sachse, Federal Institute for Health Protection of Consumers and Veterinary Medicine, Division 4, Jena, Germany; Cal. U., Dr A. J. DaMassa, Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, USA; CIRAD, Centre de Coopération Internationale en Recherche Agronomique pour le Développement, UPR-15, Montpellier, France; CIRG, Dr Rajneesh Rana, Central Institute for Research on Goats, Makhdoom, Mathura, India; FDVR, Dr E. P. Lindley, Federal Department of Veterinary Research, Vom, Nigeria; IVRI, Dr V. P. Singh, Indian Veterinary Research Institute, Izatnagar, India; LANAVET, Dr A. Martrenchar, Laboratoire National Vétérinaire, Garoua, Cameroon; LPA, Dr J. Domenech, Laboratoire Central de Pathologie Animale, Bingerville, Ivory Coast; NVI-E, Dr Y. Fikré, National Veterinary Institute, Debre Zeit, Ethiopia; NVI-S, Dr G. Bölske, National Veterinary Institute, Uppsala, Sweden; TiHo, Dr R. Schmidt, Tierärztliche Hochschule, Hannover, Germany. NK, Not known.

Strain	Abbreviation	Host	Lesion/organ	Year	Country	Supplier/reference(s)
<i>M. mycoides</i> subsp. <i>mycoides</i> SC						
PG1 ^{T*} (=NCTC 10114 ^T)	MmmSC-1	Bovine	Pleuropneumonia	NK	NK	Type strain
8740-Rita [*]	MmmSC-2	Bovine	Pleuropneumonia	1987	Cameroon	LANAVET
<i>M. mycoides</i> subsp. <i>mycoides</i> LC						
Y-goat ^{R†} (=NCTC 11706)	MmmLC-1	Caprine	Peritonitis	1956	Australia	Laws (1956)
95010-C1 [*]	MmmLC-2	Caprine	Arthritis	1995	France	CIRAD
55507-1	MmmLC-3	Caprine	Arthritis	1998	Germany	TiHo
Kombolcha	MmmLC-4	Caprine	Pleuropneumonia	1975	Ethiopia	NVI-E
WK354	MmmLC-5	Caprine	Arthritis	1980	Switzerland	Bern U.
<i>M. mycoides</i> subsp. <i>capri</i>						
PG3 ^T (=NCTC 10137 ^T =CIP 71.25 ^T)	Mmc-1	Caprine	Pleuropneumonia	1950	Turkey	Tully <i>et al.</i> (1974)
L	Mmc-2	Caprine	Mastitis	1975	France	CIRAD
N108	Mmc-3	NK	NK	1977	Nigeria	FDVR
2003-045-C2	Mmc-4	Caprine	Pneumonia	<2003	India	CIRG
2002-054 (VP9L)	Mmc-5	Caprine	NK	<2002	India	IVRI
<i>M. capricolum</i> subsp. <i>capricolum</i>						
California kid ^{T*} (=NCTC 10154 ^T =ATCC 27343 ^T =CIP 104620 ^T)	Mcc-1	Caprine	Arthritis	1955	USA	Cordy <i>et al.</i> (1955)
2002-053 (VP28L)	Mcc-2	Caprine	NK	<2002	India	IVRI
96038	Mcc-3	Caprine	Arthritis	<1996	Greece	Arist. U.
8086-1	Mcc-4	Caprine	Mastitis	1980	France	CIRAD
90122 (C1547)	Mcc-5	Caprine	Pneumonia	1990	Ivory Coast	LPA
<i>M. capricolum</i> subsp. <i>capripneumoniae</i>						
9231-Abomsa	Mccp-1	Caprine	Pleuropneumonia	1982	Ethiopia	NVI-E
95043	Mccp-2	Caprine	Pleuropneumonia	1995	Niger	CIRAD
<i>Mycoplasma</i> sp. bovine group 7 of Leach						
PG50 ^{R†} (=NCTC 10133)	MBG7-1	Bovine	Arthritis	1963	Australia	Simmons & Johnston (1963)
9733	MBG7-2	Caprine	Milk (no symptoms)	1993	India	BGVV
D424	MBG7-3	Bovine	Preputium (no symptoms)	<1990	Germany	NVI-S (Christiansen & Erno, 1990)
Calf 1	MBG7-4	Bovine	Septicaemia	<1990	Nigeria	NVI-S (Christiansen & Erno, 1990)
<i>Mycoplasma</i> sp.						
8756-C13‡	M sp.	Caprine (wild)	NK	<1987	USA	Cal. U.
<i>M. putrefaciens</i>						
KS1 ^T (=NCTC 10155 ^T =ATCC 15718 ^T)	Mput-1	Caprine?	Arthritis?	1954?	USA	Tully <i>et al.</i> (1974)
Tours 2	Mput-2	Caprine	Mastitis	1972	France	CIRAD
<i>M. cottewii</i>						
VIS ^T (=NCTC 11732 ^T =ATCC 51347 ^T =CIP 105678 ^T)	Mcot	Caprine	Ear (no symptoms)	1981	Australia	Cottew & Yeats (1981); DaMassa <i>et al.</i> (1994)
<i>M. yeatsii</i>						
GIH ^T (=NCTC 11730 ^T =ATCC 51346 ^T =CIP 105675 ^T)	Myea	Caprine	Ear (no symptoms)	1981	Australia	Cottew & Yeats (1981); DaMassa <i>et al.</i> (1994)

*Strains for which sequences were obtained from published genomes or from ongoing genome-sequencing projects.

†Proposed reference strain for this taxon.

‡The taxonomic position of strain 8756-C13 is uncertain.

(subsets of four sequences) the proportion of those that do not share the same topology. This distance is regarded as a measure of the influence that the removed sequence has on the tree structure. A high Qd value for a given unit means that this sequence conveys a strong and atypical signal, largely incompatible with the common signal of the other units, and is highly influential on the tree structure. On the other hand, a low Qd value means that the removed unit conveys a signal similar to that of other units and is not particularly influential on the structure. Similar and low Qd values are expected for a coherent set of units. The detection of particularly high Qd values warns of the presence of atypical units. Since tree construction methods are known to be very sensitive to atypical units, sequences with unusual strong influence were excluded in order to infer more reliable trees.

RESULTS AND DISCUSSION

Phylogenetic approach

Five conserved protein-coding genes, presenting greater variability than 16S rRNA gene sequences, were selected for phylogenetic analysis of the *M. mycoides* cluster. These genes were located at distant positions, distributed along the genome of sequenced mycoplasmas belonging to this cluster. Four of them are informational genes: *fusA* encodes the elongation factor G, *gyrB* encodes the DNA gyrase subunit B, *lepA* encodes the GTP-binding membrane protein or elongation factor LepA and *rpoB* encodes the DNA-dependent RNA polymerase β -subunit. These universally conserved genes have been selected as complementary to 16S rRNA gene analysis for bacterial identification and phylogenetic studies (Santos & Ochman, 2004) and *rpoB* has already been used for phylogenetic analysis of *Mycoplasma* species (Kim *et al.*, 2003; Vilei *et al.*, 2006). An operational gene, *glpQ*, was also included. It encodes glycerophosphoryl diester phosphodiesterase and has also been used to define prokaryote taxa (Schwan *et al.*, 2005).

Assuming that the evolutionary history of organismal lineages in the *M. mycoides* cluster can be properly estimated in the form of a phylogenetic tree, it was proposed that this 'species tree' may be inferred from the phylogenies based on the five selected genes, the 'gene trees'. However, it is now

well established that different genes sampled from the same set of taxa can produce markedly different phylogenies because of limited variability, inadequate sampling or random convergence, but also because of biological phenomena related to evolutionary events such as horizontal transfer or selection pressure. The problem is then to reconcile the trees derived from different genes to approximate the species tree. Two approaches are generally proposed: consensus methods and total evidence. The former (strict consensus, relaxed consensus or, more recently, consensus networks) considers discordance as uncertainty and produces trees that are often largely unresolved. In contrast, the total evidence approach infers a tree from a set of concatenated gene sequences assuming that each gene tree contains, at least in part, a common phylogenetic signal that will emerge with sufficient sampling. It has been shown that total evidence is often efficient in overcoming the incongruences present in single-gene analyses (Rokas *et al.*, 2003). Therefore, the approach chosen in this study was to infer the phylogeny of the *M. mycoides* cluster from a sequence generated by concatenation head-to-tail of the five selected gene sequences. However, since the expected statistical smoothing is not guaranteed with only five genes, the confidence attached to the inferred phylogeny would be strengthened if the common structure could be retrieved, at least at the level of the main subclusters, in each of the individual gene phylogenies or if the biological causes for strong incongruence on a particular gene could be recognized and explained. For this reason, each of the five gene trees was analysed separately and compared to the total evidence tree.

The *M. mycoides* cluster tree as inferred from a set of five concatenated protein-coding sequences

Primer pairs for amplification of *fusA*, *glpQ*, *gyrB*, *lepA* and *rpoB* sequences were selected on the basis of homologous regions detected by alignment of the available sequences of the type strains MmmSC PG1^T and Mcc California kid^T and sequences obtained through ongoing genome

Table 2. PCR primers

PCR product sizes and 5' positions of forward primers for each target were taken from the genome sequences of MmmSC PG1^T (GenBank accession no. BX293980) and Mcc California kid^T (CP000123).

Target	Forward primer		Reverse primer		Product (bp)	Position
	Name	Sequence (5'–3')	Name	Sequence (5'–3')		
<i>fusA</i>	FusA-F	TGAAATTTTATAGATGGTGGAGAA	FusA-R	GGTAATTTAATAGTTTCACGATATGAA	781	680
<i>glpQ</i>	GlpQ-L-F	GTGAATATAATGATCTTAGTAGC	GlpQ-L-R	TCAGGATAATCTGAAATATAACC	695	1
<i>gyrB</i>	GyrB-B	GTGTTGTTAATGCTTTATCTTTATATG	GyrB-R	TTGTGGATCTGTATGTCTAACTGATAAAA	635	359
<i>lepA</i>	LepA-L-F	AGAAATTTTAGTATTATTGCTCATA	LepA-L-R	CTTGGAGCAGTTGCAATTAGTTCAA	1097	19
<i>rpoB</i>	RpoB-F2	GCTCAATCTAATGTTAATCAAGATG	RpoB-R2	TCTTCTGGAGAAAGTTGAACTTGTC	824	1963

Fig. 2. Phylogenetic tree derived from distance analysis of five concatenated protein-coding sequences (*fusA*, *glpQ*, *gyrB*, *lepA*, *rpoB*). The tree was constructed using the neighbour-joining algorithm. Bootstrap percentage values were calculated from 500 resamplings and values over 70% are displayed. Bar, distance equivalent to 1 substitution per 100 nucleotide positions. The location of subspecies and groups of strains is indicated. See Table 1 for strain details and Supplementary Table S1 for sequence accession numbers.

placed in an intermediate position between the cluster and the other related species. *M. cottewii* and *M. yeatsii* clustered together, separate from *M. putrefaciens*, and they were all clearly separated from the *M. mycoides* cluster. All the main branches indicated here were supported by maximum bootstrap values (100%).

Assessment of the concordance of individual gene trees with the tree based on concatenated sequences

To assess the adequacy of the phylogeny inferred from concatenated sequences, separate analyses of each of the five genes were performed on 23 strains belonging to the *M. mycoides* cluster. To facilitate comparisons, the trees were rooted using the uncharacterized strain 8756-C13. However, since atypical sequences such as the outgroup can deeply disturb tree structures, this was not included in the set of analysed strains but was grafted onto the inferred trees afterwards. For the same reason, influential units were investigated systematically by calculating Qd values, as described in Methods. In order to verify the stability of the

phylogenetic signal along the gene sequences, separate analyses were performed on sequential subsequences. Finally, independent analyses were also performed on each of the three codon positions, which do not undergo the same evolutionary processes. The first two codon positions are strongly constrained, minimizing the risk of homoplasy, while the third codon position is often free. Modifications in the first two positions tend to result in a change in the amino acid residue. Substitutions in the second codon position were infrequent and were often associated with amino acid changes, accompanied by a change in the first position. The first codon position was therefore retained to avoid this possible redundancy.

The structure of each of the trees based on individual gene sequences was compared to the topology of the tree based on concatenated sequences, focusing comparisons on the distribution of the different subspecies according to the main branches supported by high bootstrap values.

Tree based on *fusA* sequences. The phylogenetic tree constructed from *fusA* sequences (Fig. 3a) was in general agreement with the tree based on concatenated sequences.

Fig. 3. Evolutionary distance trees based on *fusA* sequences of 561 bp (a) and *glpQ* sequences of 538 bp (b). The trees were constructed using the neighbour-joining algorithm on a set of 23 strains belonging to the *M. mycoides* cluster. They are rooted on the grafting position of the uncharacterized strain 8756-C13 (labelled 'M sp.'). used as an outgroup. Bootstrap percentage values were calculated from 500 replications and values over 70 % are displayed. Bars, distance equivalent to 1 substitution per 100 nucleotide positions. See Table 1 for strain details and Supplementary Table S1 for sequence accession numbers.

The two subclusters were clearly distinguished: *M. mycoides* subspecies on one side, *M. capricolum* subspecies and MBG7 on the other. Within the *M. mycoides* subcluster, the two MmmSC strains clustered together, though they were not neatly separated from the rest of the subcluster, where MmmLC and Mmc remained indistinguishable. As for the *M. capricolum* subgroup, Mccp strains could not be clearly separated from Mcc strains, whereas MBG7 constituted a distinct branch. All these main branches were supported by high bootstrap values (91–100 %).

Tree based on *glpQ* sequences. The general topology of the tree based on *glpQ* sequences (Fig. 3b) was similar to that based on *fusA*, with MmmSC strains more deeply embedded within the *M. mycoides* subcluster, being indistinguishable from the other strains.

Tree based on *gyrB* sequences. The tree inferred from *gyrB* sequences was incongruent with the phylogeny based on concatenated sequences (Fig. 4a). It differed from the *fusA* and *glpQ* trees in the position of the MmmSC strains,

which left the *M. mycoides* subcluster to join the *M. capricolum* subcluster (98 % bootstrap). The MBG7 strains were attached to the *M. capricolum* subcluster, where Mccp constituted a separate branch. Independent analyses on each codon position revealed the same incongruent topology, and no influential units were identified among the 23 sequences. However, closer examination of variable nucleotide positions showed that the MmmSC strains shared several variant nucleotides with MBG7 strains, which attracted them towards the *M. capricolum* subcluster. These nucleotides were concentrated (7 of 10) in a short subsequence between positions 140 and 240. Therefore, an analysis restricted to the second half of the sequence (Fig. 4b) agreed globally with the concatenated sequence phylogeny, with MmmSC placed in the *M. mycoides* subcluster.

Tree based on *lepA* sequences. The tree derived from *lepA* sequences (Fig. 5a) was largely incongruent with the common structure and did not allow a clear discrimination of the species *M. mycoides* and *M. capricolum*. As in the

Fig. 4. Evolutionary distance trees based on *gyrB* sequences of 533 bp (a) and based on the second half of these sequences from nucleotide position 267 (b). For further details refer to Fig. 3. See Table 1 for strain details and Supplementary Table S1 for sequence accession numbers.

case of *gyrB*, the attraction between MmmSC and MBG7 was apparent, in this case inducing a separate group (74 % bootstrap) linked to the *M. mycoides* subcluster. The strains referred to as MmmLC-5 and MmmLC-3 were linked to the *M. capricolum* subcluster. These, as well as strain MmmLC-2, were detected as highly influential units, with Qd values over 0.22, while the remaining values were below 0.12. MmmLC-2 and MmmLC-3 showed specific nucleotide variations, while MmmLC-5 shared variant nucleotides with the *M. capricolum* subcluster. In fact, the nine variable sites present in the first half of the *lepA* sequence of MmmLC-5 were characteristic of *M. capricolum*, whereas the second half contained seven variant nucleotides corresponding to a typical *M. mycoides* sequence. Analysis of the second half of this sequence (Fig. 5b) restored the three erratic strains to their subcluster but did not suppress the attraction between MmmSC and MBG7. When the analysis was restricted to the initial codon position (Fig. 5c), the common structure was recovered (except for strain MmmLC-5), with MmmSC linked to the *M. mycoides* subcluster and MBG7 to the *M. capricolum* subcluster, indicating that their shared nucleotide variations, as well as the specific variant nucleotides of the MmmLC-2 and MmmLC-3 sequences, mainly concerned the least-constrained codon positions.

Tree based on *rpoB* sequences. The tree derived from *rpoB* sequences (Fig. 6a) was highly incongruent with the common structure and neither of the two subclusters could be clearly discriminated. Analyses based on partial sequences and on each codon position did not allow this structure to be clarified. However, strain MmmLC-1, corresponding to strain Y-goat^R, was identified as a strongly influential unit (Qd value of 0.2, whereas the others did not exceed 0.02). In six variable positions, the

sequence of strain Y-goat^R shared variant nucleotides characteristic of *M. capricolum*, which were not shared by MBG7. This resulted in attraction of the MmmLC/Mmc group towards the *M. capricolum* subspecies, accompanied by repulsion of MBG7. The tree constructed by exclusion of strain Y-goat^R (Fig. 6b) restored an *M. capricolum* subcluster including MBG7 and an *M. mycoides* subcluster, though variant nucleotides specific to MmmSC, partly shared by strain MmmLC-4, kept them in a separate group.

Phylogeny of the *M. mycoides* cluster and taxonomic implications

The phylogeny of the *M. mycoides* cluster has been inferred from a set of five concatenated protein-coding gene sequences and strengthened by separate analyses performed on each individual gene. Although incongruences were observed for certain genes, which explain the ambiguities that have been found in previous studies, the 'mean structure' represented by the concatenated sequence tree could be recognized behind gene specificities when reinforced in this manner. This phylogeny does not contradict that based on 16S rRNA gene sequences (Pettersson *et al.*, 1996). However, the reduced variability of the 16S rRNA gene sequence, added to the specific evolutionary processes of this genetic marker, has not allowed the close relationships existing between these organisms to be completely resolved: only 36 variable positions were found in a comparison of 1467 nucleotides corresponding to the sequences of the two rRNA operons of 10 strains of the *M. mycoides* cluster, including all type strains, increasing to 66 (30 unique positions) when *M. putrefaciens* KS1^T was included. The use of five house-keeping gene sequences has not only reduced the bias generated by using a single gene sequence but has also

Fig. 5. Evolutionary distance trees based on *lepA* sequences of 657 bp (a) and based on the second half of these *lepA* sequences, from nucleotide position 328 (b), and a tree inferred from the first codon positions of the 657 bp *lepA* sequences (c). For further details refer to Fig. 3. See Table 1 for strain details and Supplementary Table S1 for sequence accession numbers.

Fig. 6. Evolutionary distance trees based on *rpoB* sequences of 625 bp constructed from the set of 23 strains belonging to the *M. mycoides* cluster (a) and constructed as above but excluding strain MmmLC-1 (b). For further details refer to Fig. 3. See Table 1 for strain details and Supplementary Table S1 for sequence accession numbers.

provided greater resolution by considerably increasing variability. The partial gene sequences used for phylogeny inference provided 106–185 variable positions in a comparison of the 28 strains analysed and 36–68 when limiting the comparison to the 23 strains of the *M. mycoides* cluster (Table 3). Concatenation of the five sequences increased the number of variable positions to 261, of which 203 were informative for phylogenetic analysis of the *M. mycoides* cluster.

This approach has allowed us to construct a reliable tree to assign phylogenetic positions to all members of the *M. mycoides* cluster, showing once more that the existing taxonomy needs to be reviewed.

Phylogenetic position of *Mycoplasma* sp. bovine group 7 of Leach. The greatest incongruences, found in two of the five gene sequences analysed in this study, resulted from the attraction between MmmSC and MBG7. For *gyrB*, this proximity was limited to a subsequence and, in the case of *lepA*, it essentially concerned the least-constrained codon positions. The common structure corresponding to *fusA* and *glpQ* (and *rpoB* when excluding the influential unit MmmLC-1), which places MmmSC in the *M. mycoides* subcluster and MBG7 in the *M. capricolum* subcluster, is not strongly contradicted by analyses of the other two genes and can therefore be retained. Furthermore, the partial attraction observed between MBG7 and MmmSC explains the ambiguity that persists in the present classification and why, unlike the other members of the *M. mycoides* cluster, the group of strains referred to as MBG7 has not yet been assigned a species name. Investigations based on DNA–DNA hybridization (Christiansen & Ernø, 1982), identification with a DNA probe (Taylor *et al.*, 1992) and electrophoretic analysis of isoenzymes and serology (Salih *et al.*, 1983) have suggested the classification of MBG7 as a distinct species. DNA–DNA hybridization studies by Askaa *et al.* (1978), lipoprotein sequence similarity and immunological cross-reactions (Frey *et al.*, 1998), as well as sequence similarity of the

glycerol transport locus *gtsABC* (Djordjevic *et al.*, 2003), have revealed a close relatedness to MmmSC. In contrast, serological cross-reactions instead suggested that MBG7 was closer to the *M. capricolum* subspecies (Cottew *et al.*, 1987; Ernø *et al.*, 1983), which was confirmed by 1D and 2D SDS-PAGE profile comparisons (Costas *et al.*, 1987; Olsson *et al.*, 1990). In addition, MSC4, a small stable RNA, was found to be specific to the species *M. capricolum* and MBG7 (Ushida *et al.*, 2003), and recent phylogenetic studies based on 16S rRNA gene sequences (Pettersson *et al.*, 1996), 16S–23S rRNA intergenic spacer sequences (Harasawa *et al.*, 2000) and a putative membrane protein gene (Thiaucourt *et al.*, 2000) all grouped MBG7 in the *M. capricolum* subcluster. The results presented here provide additional evidence regarding the genetic identification of this group of strains and support its classification as a subspecies of *M. capricolum*.

Interestingly, all strains of MBG7 provided identical sequences for each of the five genes analysed. This seems to indicate a very limited intraspecies variability for this group of strains and, presumably, a recent differentiation within the species *M. capricolum*.

All members of the *M. mycoides* cluster have been isolated from small ruminants (Atalaia *et al.*, 1987; Kusiluka *et al.*, 2001), where multiple infections are frequent (Cottew & Yeats, 1982). These conditions may well promote horizontal gene transfer between the different species. Genomic exchanges were suspected for several strains analysed in this study. This is the case for strain MmmLC-5, for which the first half of the *lepA* sequence was characteristic of *M. capricolum* while the second half corresponded to a typical *M. mycoides* sequence. A lateral transfer of genes or gene fragments may explain the local proximity that exists between MBG7 and MmmSC that has been observed previously and is again shown in two of the five genes analysed in this work.

Relatedness of *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*. The distinction of MmmLC and

Mmc as separate subspecies has been questioned for some time. Numerous investigations have provided evidence to suggest that they may be grouped as a single subspecies within the *M. mycoides* subcluster. Although early DNA–DNA hybridization studies by Askaa *et al.* (1978) suggested that Mmc was closer to MmmSC than to MmmLC, analyses of 1D and 2D PAGE protein patterns proved the opposite (Costas *et al.*, 1987; Leach *et al.*, 1989; Olsson *et al.*, 1990; Rodwell, 1982; Salih *et al.*, 1983). Identification using a DNA probe (Taylor *et al.*, 1992) and analysis of LppA sequence similarity and serological cross-reactions (Monnerat *et al.*, 1999) also suggested a very close relatedness between MmmLC and Mmc, and two phylogenetic studies of the *M. mycoides* cluster concluded that they should be grouped into a single entity (Pettersson *et al.*, 1996; Thiaucourt *et al.*, 2000). However, the International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Mollicutes* has indicated that additional comparative analysis is required in order to clarify this matter (Johansson & Bradbury, 2007).

Additional evidence has recently been presented by Vilei *et al.* (2006) based on *rpoB* sequence comparisons. In the present work, comparisons have been extended to five genes using a similar sample of strains, though strain WK354, included as an Mmc strain in previous studies (Monnerat *et al.*, 1999; Thiaucourt *et al.*, 2000), was characterized in this work as MmmLC by both growth inhibition and dot immunobinding techniques. As in previous studies, the data obtained in this work show that MmmLC is closer to Mmc than to the biotype MmmSC and that MmmLC and Mmc are not sufficiently distant to constitute separate entities. Furthermore, neither of these two organisms represents the aetiological agent of a characteristic disease, which may have been a justification for their separation. For example, this had been the rationale for the establishment of Mccp as a separate subspecies of *M. capricolum*.

In conclusion, MmmLC and Mmc appear to constitute a single entity, which could justify their combination into the same taxonomic subspecies.

Position of *M. putrefaciens* and other related species. *M. putrefaciens* is not included in the classical *M. mycoides* cluster and may be easily differentiated from the members of this cluster by biochemical and serological methods (Tully *et al.*, 1974) as well as by a specific PCR test (Peyraud *et al.*, 2003). However, on the basis of 16S rRNA gene sequence similarity, it was included in the phylogenetic *M. mycoides* cluster, within the *Spiroplasma* group (Weisburg *et al.*, 1989). Also based on 16S rRNA sequence data, the closely related species *M. cottewii* and *M. yeatsii* were included in this phylogenetic cluster, branching with *M. putrefaciens* (Heldtander *et al.*, 1998). Although the latter authors suggested that these three species should actually constitute a separate cluster, their precise taxonomic position remains uncertain. In this study, *M. putrefaciens*, *M. cottewii* and *M. yeatsii* were distant from the members of

the classical cluster, with an important number of strictly specific nucleotide variations, confirming that they should not be included in the *M. mycoides* cluster.

Application to identification and classification of strains

The great relevance of diseases such as CBPP and CCPP and the fact that several members of the *M. mycoides* cluster produce similar clinical signs in small ruminants with different virulence mean that precise identification is of paramount importance. However, some of these mycoplasmas are difficult to differentiate by conventional techniques (Cottew *et al.*, 1987). Growth inhibition, which is often used for species identification, is frequently hampered both by interspecies cross-reactions and by intraspecies variability. Cross-reactions are frequent between biotypes MmmSC and MmmLC (Cottew & Yeats, 1978; Thigpen *et al.*, 1983) and between MBG7 and Mccp (Cottew *et al.*, 1987; Ernø *et al.*, 1983; Kibe *et al.*, 1985), hindering confirmation of CBPP and CCPP, respectively. A high degree of intraspecies heterogeneity has also been shown using several techniques, particularly within MmmLC/Mmc and also Mcc to a certain extent, whereas MmmSC and Mccp appear rather homogeneous (Abu-Groun *et al.*, 1994; Christiansen & Ernø, 1990; Costas *et al.*, 1987; Kusiluka *et al.*, 2001). Our data correlate with these observations and emphasize once more the limitations encountered in the classification of certain strains and, furthermore, in the definition and delimitation of species and subspecies within the *M. mycoides* cluster.

Given the increased resolving power of the strategy presented in this work, it was hypothesized that it might allow strains that have proved difficult to affiliate to be classified. This is the case for strain 8756-C13, isolated from a Rocky Mountain goat, for which identification by classical methods is challenging. Cultural parameters indicated that it might be an MmmLC strain, though growth-inhibition tests were negative for both MmmLC and Mmc. It was finally classed as MmmLC on the basis of immunofluorescence results, although electrophoretic patterns were inconclusive. The data from this study show that this is not a typical strain of the *M. mycoides* cluster, but a nearby relative, located closer to this cluster than to other related species such as *M. putrefaciens*, *M. cottewii* and *M. yeatsii*. Presumably, study of new isolates originating from diverse geographical and host origins will result in the identification of further ‘atypical’ strains related to this cluster and, until whole-genome sequence comparisons become accessible to diagnostic laboratories, protein gene sequence data may prove to be a good addition to 16S rRNA genes for fine strain characterization. However, analyses based on multiple gene sequences are still difficult to use for routine diagnostic purposes. On the other hand, *fusA* may be an interesting alternative for rapid identification and phylogenetic positioning by PCR and sequencing, for which the set of sequences presented in this work may

serve as a reference: although variability was low among the *fusA* sequences from *M. mycoides* strains, the tree inferred from this gene was very similar to that based on the concatenated sequences.

Concluding remarks

Conserved protein-coding gene sequences may be used to complement and extend 16S rRNA gene sequence data to allow differentiation of closely related groups of organisms, such as the members of the *M. mycoides* cluster. However, a single gene tree can be largely incongruent with the species tree, which represents its true evolutionary history. Therefore, several genes must be analysed simultaneously. It is proposed here to consider the tree inferred from five concatenated gene sequences as a reliable estimation of the species phylogeny, since this 'mean structure' can be detected in each individual gene tree, even if it is partially masked by specific gene evolution.

This approach has resulted in the construction of a reliable phylogenetic tree, which will be a universal tool for the classification of strains of the *M. mycoides* cluster, including those difficult to identify by classical methods. Furthermore, it may lead to the development of new techniques for rapid species identification and molecular epidemiology.

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