The genus *Spiroplasma* and its non-helical descendants: phylogenetic classification, correlation with phenotype and roots of the *Mycoplasma mycoides* clade

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The genus *Spiroplasma* (helical mollicutes: *Bacteria*: *Firmicutes*: *Mollicutes*: *Entomoplasmatales*: *Spiroplasmataceae*) is associated primarily with insects. The *Mycoplasma mycoides* cluster (*sensu* Weisburg *et al*. 1989 and Johansson and Pettersson 2002) is a group of mollicutes that includes the type species – *Mycoplasma mycoides* – of *Mycoplasmatales*, *Mycoplasmataceae* and *Mycoplasma*. This cluster, associated solely with ruminants, contains five other species and subspecies. Earlier phylogenetic reconstructions based on partial 16S rDNA sequences and a limited sample of *Spiroplasma* and *Mycoplasma* sequences suggested that the genus *Mycoplasma* was polyphyletic, as the *M. mycoides* cluster and the grouping that consisted of the hominis and pneumoniae groups of *Mycoplasma* species were widely separated phylogenetically and the *M. mycoides* cluster was allied with *Spiroplasma*. It is shown here that the *M. mycoides* cluster arose from *Spiroplasma* through an intermediate group of non-helical spiroplasmal descendants – the *Entomoplasmataceae*. As this conclusion has profound implications in the taxonomy of *Mollicutes*, a detailed phylogenetic study of *Spiroplasma* and its non-helical descendants was undertaken. These analyses, done with maximum-parsimony, provide cladistic status; a new nomenclature is introduced here, based on 'bottom-up' rather than 'top-down' clade classification. The order *Entomoplasmatales* consists of four major clades: (i) the Mycoides–Entomoplasmataceae clade, which contains *M. mycoides* and its allies and *Entomoplasma* and *Mesoplasma* species and is a sister lineage to (ii) the Apis clade of *Spiroplasma*. *Spiroplasma* and the *Entomoplasmataceae* are paraphyletic, but this status does not diminish their phylogenetic usefulness. Five species that were previously unclassified phylogenetically are basal to the Apis clade *sensu strictu* and to

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Abbreviations: DF, deformation; MI, metabolism inhibition; PES, polyoxyethelene sorbitan; PHUH clade, Pneumoniae–Hominis–Ureaplasma–Haemoplasma clade; SEM clade, Spiroplasma–Entomoplasmataceae–Mycoides clade.

The GenBank/EMBL/DDBJ accession numbers for new *Spiroplasma* 16S rDNA sequences are: *Spiroplasma* sp. strain 277F, AY189312; *Spiroplasma* sp. strain LB-12, AY189313; *S. insolitum*, AY189133; *S. floricola*, AY189131; *S. syrphidicola*, AY189309; *S. chrysopicola*, AY189127; *Spiroplasma* sp. strain TAAS-1, AY189314; *S. culicicola*, AY189129; *S. velocicrescens*, AY189311; *S. sabaudiense*, AY189308; *S. corruscae*, AY189128; *Spiroplasma* sp. strain CB-1, AY189315; *Spiroplasma* sp. strain Ar 1357, AY189316; *S. turonicum*, AY189310; *S. litorale*, AY189306; *S. lampyridicola*, AY189134; *S. leptinotarsae*, AY189305; *Spiroplasma* sp. strain W115, AY189317; *S. chinense*, AY189126; *S. diminutum*, AY189130; *S. alleghenense*, AY189125; *Spiroplasma* sp. strain TIUS-1, AY189318; *Spiroplasma* sp. strain BIUS-1, AY189319; *S. montanense*, AY189307; *S. helicoides*, AY189132; *Spiroplasma* sp. BARC 1901, AY189320.

Tables summarizing the phylogenetic trees constructed for the genus *Spiroplasma* and their support are available as supplementary material in IJSEM Online.

the Mycoides clade. One of these species, *Spiroplasma* sp. TIUS-1, has very poor helicity and a very small genome (840 kbp); this putative species can be envisioned as a 'missing link' in the evolution of the Mycoides–Entomoplasmataceae clade. The other two *Spiroplasma* clades are: (iii) the Citri–Chrysopicola–Mirum clade (serogroups I, II, V and VIII) and (iv) the ixodetis clade (serogroup VI). As *Mesoplasma lactucae* represents a basal divergence within the Mycoides–Entomoplasmataceae clade, and as *Entomoplasma freundtii* is basal to the Mycoides clade, *M. mycoides* and its allies must have arisen from an ancestor in the *Entomoplasmataceae*. The paraphyletic grouping that consists of the Hominis and Pneumoniae groups (*sensu* Johansson & Pettersson 2002) of *Mycoplasma* species contains the ancestral roots of *Ureaplasma* spp. and haemoplasmas. This clade is a sister lineage to the Entomoplasmatales clade. Serological classifications of spiroplasma are very highly supported by the trees presented. Genome size and G+C content of micro-organismal DNA were moderately conserved, but there have been frequent and polyphyletically distributed genome reductions. Sterol requirements were polyphyletic, as was the ability to grow in the presence of polyoxyethylene sorbitan-supplemented, but not serum-supplemented, media. As this character is not phylogenetically distributed, *Mesoplasma* and *Entomoplasma* should be combined into a single genus. The phylogenetic trees presented here confirm previous reports of polyphyly of the genus *Mycoplasma*. As both clades of *Mycoplasma* contain several species of great practical importance, a change of the genus name for species in either clade would have immense practical implications. In addition, a change of the genus name for *M. mycoides* would have to be approved by the Judicial Commission. For these reasons, the Linnaean and phylogenetic classifications of *Mycoplasma* must for now be discrepant.

INTRODUCTION

The genus *Spiroplasma* contains a group of motile, helical, wall-less prokaryotes that are associated primarily with insects, but much less frequently with ticks and plants (Williamson *et al.*, 1989, 1998; Tully & Whitcomb, 1990). In addition to their unique cellular morphology, species of this genus have attracted recent attention as the root of a small clade that contains the type species of the genus *Mycoplasma*. The genus *Spiroplasma* was discovered relatively recently. The first species described in this genus, *Spiroplasma citri* (Saglio *et al.*, 1973), is the aetiological agent of citrus stubborn disease (Calavan & Bové, 1989) and resides in an obligate cycle in the plant phloem and the insect vector. Another spiroplasma – thought at the time to be a spirochaete – was shown to cause a sex-ratio abnormality in *Drosophila* (Poulson & Sakaguchi, 1961; Williamson & Poulson, 1979). A third spiroplasma, eventually named *Spiroplasma kunkelii*, discovered shortly before *S. citri* was described (Davis *et al.*, 1972; Whitcomb *et al.*, 1986), is also an inhabitant of the plant phloem/insect habitat (Hackett & Clark, 1989). Spiroplasmas were then found to inhabit ticks (Burgdorfer *et al.*, 1975; Tully *et al.*, 1976, 1982, 1995). One of these, *Spiroplasma mirum*, can cause experimental disease in suckling rodents (Tully *et al.*, 1977). It was not realized until 1977 (Clark, 1977, 1982) that the major reservoir of spiroplasmas was insects and that the surfaces, rather than the phloem, of flowers and other plant parts were the major site for spiroplasma acquisition and transmission (Davis, 1978; McCoy *et al.*, 1979; Clark *et al.*, 1987).

The general phylogenetic position of the genus *Spiroplasma* was determined as a result of the pioneering work of Carl Woese and his associates [summarized by Woese (1987)], which utilized 16S rRNA for the study of prokaryote phylogeny. As the class *Mollicutes* was of special interest (Woese *et al.*, 1985), these studies led to an in-depth study of its evolution [reviewed by Johansson & Pettersson (2002)]. The class *Mollicutes* was shown to be a terminus in the evolution of Gram-positive bacteria. Early studies (Woese *et al.*, 1980) indicated that the class *Mollicutes* was divided into four major phylogenetic groups – a clade that contained *Anaeroplasmatales* and *Acholeplasmatales*, a clade that consisted of the hominis and pneumoniae groups of *Mycoplasma*, a clade that contained *Spiroplasma* and *Mycoplasma mycoides* and a monospecific clade that contained *Asteroleplasma anaerobium*. Although early studies were non-committal on the monophyly of the Acholeplasmatales–Anaeroplasma and Spiroplasma–Entomoplasmataceae–Mycoides clades (Woese *et al.*, 1980), subsequent phylogenetic analyses have led workers to hypothesize that they are monophyletic. However, the studies of Weisburg *et al.* (1989) indicated that one of the three major groups of *Mollicutes* – the asteroleplasma group – may be allied more closely with certain genera of low-G+C content, Gram-positive bacteria than with other mollicutes. Thus, wall-lessness probably evolved at least twice in the evolution of Gram-positive bacteria. In addition to the asteroleplasma group, Weisburg and colleagues defined the pneumoniae, hominis, anaeroplasma and spiroplasma groups.

The *M. mycoides* cluster of Weisburg *et al.* (1989) is a group that, with all recent additions, contains 23 species (J. G. Tully, personal communication). Five of these form a monophyletic grouping that is restricted to ruminant

animals. One of these, *M. mycoides*, is the type species of *Mollicutes*, *Mycoplasmataceae* and *Mycoplasma*. *M. mycoides* is divided into several subtaxa (some of which have been recognized at the subspecies level) that differ in their host range, pathogenicity and serological and genomic characters (Heldtander *et al.*, 1998; Persson, 2002). *M. mycoides* subsp. *mycoides* SC is the causative agent of contagious bovine pleuropneumonia [reviewed by Persson (2002)]. This disease, which was recognized as long ago as the middle ages (reviewed by Provost *et al.*, 1987), is the only bacterial disease on the A-list of communicable animal diseases (FAO EMPRES, 2000). This classification places it as one of the 15 most serious animal diseases in the world. The agent was cultivated in 1898 by Nocard and Roux. Dujardin-Beaumetz, a co-worker of Nocard and Roux, introduced filters to separate the organism from contaminants and was the first to cultivate the organisms on agar, where they formed fried-egg colonies [summarized by Bové *et al.* (1994)]. The organism was described as *Asterococcus mycoides* in 1910 and later reclassified in a novel genus, *Mycoplasma* (Nowak, 1929). In 1956, the current classification was established (Edward & Freundt, 1956). A second subspecies of the *M. mycoides* cluster, *Mycoplasma capricolum* subsp. *capripneumoniae*, is a serious pathogen of caprine animals (Johansson & Pettersson, 2002). A genome-sequencing project for *M. mycoides* has shown that the genome is 1140 kbp in size, with a very low G + C content of 24 mol%. The *M. mycoides* genome has 1067 fully annotated ORFs and many have been correlated with metabolic functions (Persson, 2002).

Earlier phylogenetic studies (Woese *et al.*, 1980; Weisburg *et al.*, 1989; Maniloff, 1992) produced a major surprise – that some members of the genus *Mycoplasma*, including the type species, *M. mycoides*, appeared to belong to the spiroplasma group and that the genus *Mycoplasma*, like *Mollicutes*, might therefore be polyphyletic. These studies suggested that a small group of species, including *M. mycoides* and its cohorts – all from bovine and caprine sources – were placed properly in the spiroplasma group of mollicutes.

The discovery of non-helical mollicutes in the insect gut/plant surface habitat (Clark, 1977; Davis, 1978; Clark *et al.*, 1986; Tully *et al.*, 1994) revealed a large assemblage of previously unrecognized mollicutes. These organisms appeared at first to be *Acholeplasma* or *Mycoplasma* species, but some were later found to be phylogenetically distinct from those genera (Weisburg *et al.*, 1989). This newly discovered group of organisms was later referred to a novel family, *Entomoplasmataceae* (Tully *et al.*, 1993), which had two genera. *Entomoplasma* was proposed for organisms that did not require sterol and *Mesoplasma* was proposed for certain species that could grow in the absence of sterol if, and only if, cultures of the organism were grown in media that were supplemented with 0·04 % polyoxyethelene sorbitan (PES).

The apparent polyphyly of *Mycoplasma* has profound implications for mollicute taxonomy. *M. mycoides* is the type species of the order *Mycoplasmatales*, the family *Mycoplasmataceae* and the genus *Mycoplasma*. If this species was shown conclusively to be phylogenetically remote from other *Mycoplasma* species, in an era that features strong attempts to reconcile binomial and phylogenetic classification, a major taxonomic quandary would be presented. Earlier phylogenies (Weisburg *et al.*, 1989; Maniloff, 1992) were derived from a database of largely incomplete sequences and were analysed by phenetic analytical methods (distance and the largely phenetic neighbour-joining method). However, now that a much more complete dataset exists and cladistics is becoming one of the cornerstones of microbial systematics (Ludwig & Schleifer, 1999), it is important that this profound conclusion be revisited and confirmed.

An appendix of the taxonomic terms used in the text is given in Table 1.

METHODS

Strains and growth conditions. The strains used in this study are listed in Table 2. Most strains of *Spiroplasma*, *Entomoplasma* and *Mesoplasma* were isolated, cloned and preserved in laboratories at USDA, Beltsville, MD, USA; SUNY, Stony Brook, NY, USA; or at the NIAID laboratory at Frederick, MD, USA. Type or representative strains were submitted to, and are available from, the American Type Culture Collection (ATCC). Representative cultures of these strains and of other non-type strains have been deposited at Purdue University (West Layfayette, IN,USA). Most *Spiroplasma*, *Mesoplasma* and *Entomoplasma* strains were grown in M1D broth medium (Whitcomb, 1983), but some were cultured in SP-4 broth medium (Whitcomb, 1983). Cultures of *Spiroplasma leptinotarsae* were grown in DCCM broth medium (Hackett *et al.*, 1996a). All cultures were maintained at their optimum temperatures (Konai *et al.*, 1996).

DNA isolation. DNA was extracted by using either the chelex resin/boil protocol (Walsh *et al.*, 1991) or the SDS lysis protocol, as described previously (Gasparich *et al.*, 1993).

***In vitro* amplification and DNA sequencing of the 16S rRNA gene.** 16S rRNA genes of the species investigated were PCR-amplified from genomic DNA. PCR sequence mixtures contained 2 µl extracted DNA, 50 µl MicroSeq 16S rDNA PCR kit (PE/Applied Biosystems) PCR mastermix and 48 µl water. PCR conditions were those recommended by the manufacturer (PE/Applied Biosystems). The PCR product generated was 1540 bp in length. PCR products were purified by using a Microcon-100 column (Amicon), following the protocol designated by the manufacturer. Reaction products were cycle-sequenced by using a MicroSeq 16S rRNA Gene Sequencing kit (PE/Applied Biosystems). Excess dye terminators were removed by using Centri-Sep spin columns (Princeton Separations) as recommended by the manufacturer. Reaction products were then dried in a Speed Vac (Savant) and resuspended in 4 µl sequencing gel loading buffer (25 mM EDTA, 50 mg Blue Dextran ml^{-1} and 1 : 5 deionized formamide). A 2 µl aliquot was then loaded onto an ABI 377 DNA sequencer (PE/Applied Biosystems) and electrophoresed at 1650 V and 52 °C for 7 h.

Assembly of sequences. Sequences were analysed by using the software programs Sequencing Analysis (PE/Applied Biosystems) and Factura (PE/Applied Biosystems). Analysed sequences were then assembled and edited by using Auto Assembler (PE/Applied Biosystems) and a consensus sequence was generated.

Nucleotide sequence accession numbers. The new 16S rDNA sequences that were used in this study have been deposited in GenBank under the accession numbers listed in Table 2.

Table 1. Taxonomic terms used in the text

More detailed definitions of cladistic terms are provided at http://www.bioinf.org/molsys/glossary.html.

Term	Definition
Autapomorphy	An apomorphy that diagnoses a terminus, but that is uninformative about relationships to other terminals and is therefore of no use for cladistic tree-building
Apomorphy (adj. apomorphic)	A relatively derived, advanced or unique character state
Attribute	Possession of a particular feature by an organism
Character	A heritable attribute that varies among termini and that is therefore useful for phylogenetic reconstruction
Clade	A monophyletic group (a branch on a cladogram)
Cladogram	A tree that comprises nested clades
Consensus	A class of methods that is used to estimate the amount of agreement among incongruent or partially congruent trees. A majority-rule consensus tree identifies all clades that are found in $>50\,\%$ of input trees
Long-branch attraction	A process in cladistic analysis that results in tree positions that are artificially close; may occur when a tree branch represents a large evolutionary distance
Monophyly	A group that has a unique origin in a single ancestral species; it includes the ancestor and all of its descendants. It is recognized by a homologous character state (synapomorphy) in all of its members
Outgroup	A terminal taxon (or group of taxa), preferably the sister group of the ingroup, that is used to root a cladogram (cf. ingroup). The root is placed between the outgroup(s) and the ingroup. Multiple outgroups may be used
Paraphyly (adj. paraphyletic)	A paraphyletic group originates from a single common ancestor (which is included in the group), but does not include all of the descendants of that ancestor. Its members share only ancestral character states; they do not uniquely share any synapomorphies. In micro-organisms, paraphyly may be confounded with polyphyly, as a result of presumed high extinction rates
Phenetic	Similarity of characters without regard to the distinction between synapomorphy, homoplasy and symplesiomorphy. Phenetic methods are poor at reconstructing phylogeny
Phylogenetic signal	A property of data that indicates the presence of informative characters, whose presence affects the topology of cladistic trees (cf. uninformative characters)
Plesiomorphy (adj. plesiomorphic)	A relatively primitive or ancestral character state
Polarity	Evolutionary ordering of character states, determined either independently of tree construction (direct method) or, more usually, from a rooted phylogenetic tree (indirect method)
Polyphyly (adj. polyphyletic)	A group that does not include a unique common ancestor, i.e. it has multiple evolutionary origins. Designation based upon convergent characters
Polytomy	A branch-point in a tree with more than two descendent branches. A polytomy referred to as 'hard' results from an absence of data to resolve branching dichotomously. Frequently found in groups whose members are too closely related to be resolved with the method used for analysis
sensu latu	An appendix to a taxic name used to define a taxon whose composition has been redefined, but whose name has been retained. See *sensu strictu*
sensu strictu	Used to define a taxon as originally circumscribed, prior to a recent change in the definition of the group's boundaries or composition
Synapomorphy	An apomorphy that is shared by two or more termini and therefore diagnoses a clade or monophyletic group
Terminus	One of the units whose collective phylogeny is reconstructed; in other words, the undivided tips of a tree (usually contemporary taxa). Termini may be higher taxa, species, populations, individuals, fossils or even genes
Topology	Structural details of trees
Total evidence	Reconstruction of phylogeny by analysing combined data of different kinds
Uninformative character	A character that provides no phylogenetic signal. In maximum-parsimony methods, only characters whose number of steps can vary on trees are informative; autapomorphic and invariant characters are uninformative

Phylogenetic analyses. Sequences were aligned by using CLUSTALX (Thompson *et al.*, 1994) and then aligned manually in MacClade (Maddison & Maddison, 1992). Maximum-parsimony, maximum-likelihood, distance and neighbour-joining analyses were performed by using PAUP (version 4.0b10; Swofford, 1998). Parameters for analyses are presented in Supplementary Table A (available in IJSEM Online).

RESULTS AND DISCUSSION

Choice of phylogenetic method

As indicated, trees generated by using four different phylogenetic methods were used in the analysis of the 16S

Table 2. Bacterial strains included in this study, their group designations and GenBank accession numbers for their 16S rRNA gene sequences

Genus, species and strain	Group designation	GenBank accession no.
Spiroplasma citri Maroc R8A2^T	I-1	M23942
Spiroplasma melliferum BC-3^T	I-2	AY325304
Spiroplasma sp. 277F	I-4	AY189312
Spiroplasma sp. LB-12	I-5	AY189313
Spiroplasma insolitum M55^T	I-6	AY189133
Spiroplasma floricola 23-6^T	III	AY189131
Spiroplasma apis B31^T	IV	M23937
Spiroplasma mirum SMCA^T	V	M24662
Spiroplasma ixodetis Y32^T	VI	M24477
Spiroplasma monobiae MQ-1^T	VII	M24481
Spiroplasma syrphidicola EA-1^T	VIII-1	AY189309
Spiroplasma chrysopicola DF-1^T	VIII-2	AY189127
Spiroplasma sp. TAAS-1	VIII-3	AY189314
Spiroplasma clarkii CN-5^T	IX	M24474
Spiroplasma culicicola AES-1^T	X	AY189129
Spiroplasma velocicrescens MQ-4^T	XI	AY189311
Spiroplasma diabroticae DU-1^T	XII	M24482
Spiroplasma sabaudiense Ar-1343^T	XIII	AY189308
Spiroplasma corruscae EC-1^T	XIV	AY189128
Spiroplasma sp. CB-1	XVI-2	AY189315
Spiroplasma sp. Ar 1357	XVI-3	AY189316
Spiroplasma turonicum Tab-4c^T	XVII	AY189310
Spiroplasma litorale TN-1^T	XVIII	AY189306
Spiroplasma lampyridicola PUP-1^T	XIX	AY189134
Spiroplasma leptinotarsae LD-1B	XX	AY189305
Spiroplasma sp. W115	XXI	AY189317
Spiroplasma taiwanense CT-1^T	XXII	M24476
Spiroplasma gladiatoris TG-1^T	XXIII	M24475
Spiroplasma chinense CCH^T	XXIV	AY189126
Spiroplasma diminutum CUAS-1^T	XXV	AY189130
Spiroplasma alleghenense PLHS-1^T	XXVI	AY189125
Spiroplasma sp. TIUS-1	XXIX	AY189318
Spiroplasma sp. BIUS-1	XXX	AY189319
Spiroplasma montanense HYOS-1^T	XXXI	AY189307
Spiroplasma helicoides TABS-2^T	XXXII	AY189132
Spiroplasma sp. BARC 1901	XXXIV	AY189320
Mesoplasma entomophilum TAC^T	NA	M23931
Mesoplasma lactucae 831-C4^T	NA	AF303132
Entomoplasma ellychniae ELCN-1^T	NA	M24292
Entomoplasma freundtii BARC 318^T	NA	AF036954
Entomoplasma melaleucae M1^T	NA	M24478
Mycoplasma mycoides subsp. *mycoides* PG1^T	NA	U26039
Mycoplasma capricolum subsp. *capripneumoniae* Yatta B	NA	AF202928
Mycoplasma putrefaciens KS1^T	NA	M23938
Mycoplasma cottewii VIS^T	NA	U67945
Mycoplasma yeatsii GIH^T	NA	U67946
Mycoplasma pneumoniae FH^T	NA	M29061
Mycoplasma hominis PG21^T	NA	M24473
Mycoplasma synoviae WVU 1853^T	NA	X52083
Mycoplasma pulmonis PG34^T	NA	M23941
Mycoplasma neurolyticum Type A^T	NA	M23944
Mycoplasma sualvi Mayfield B^T	NA	M23936

Table 2. cont.

Genus, species and strain	Group designation	GenBank accession no.
Mycoplasma equigenitalium T37^T	NA	AF221120
Mycoplasma bovis 422/88	NA	AF332757
Mycoplasma lipophilum MaBy^T	NA	M24581
Mycoplasma cavipharyngis 117C^T	NA	AF125879
Ureaplasma urealyticum 960^T	NA	M23935
Acholeplasma laidlawii JA1	NA	M23932
Phytoplasma sp. Vigna LL	NA	AJ289195
Mycoplasma haemomuris Shizuoka	NA	U82963
Anaeroplasma bactoclasticum JR^T	NA	M25049
Asteroleplasma anaerobium 161^T	NA	M22351
Erysipelothrix rhusiopathiae α-P15	NA	M23728
Clostridium innocuum B-3	NA	M23732
Bacillus subtilis TB11	NA	AF058766
Escherichia coli (strain not specified)	NA	J01859

rDNA sequence dataset (maximum-parsimony, distance, neighbour-joining and maximum-likelihood). All four methods have been used previously to analyse mollicute phylogeny (e.g. Woese *et al.*, 1980; Rogers *et al.*, 1985; Weisburg *et al.*, 1989; Maniloff, 1992; Johansson & Pettersson, 2002). The extensive work published by K. E. Johansson's group [reviewed in part by Johansson & Pettersson (2002)] has been done by using neighbour-joining, maximum-parsimony and maximum-likelihood. The large and globally inclusive trees generated by Gundersen *et al.* (1994) were constructed by using maximum-parsimony. Distance analyses (using *p*-distance) were performed in the initial work on mollicute phylogeny by Woese *et al.* (1980) and Weisburg *et al.* (1989), and neighbour-joining analyses were performed by Maniloff (1992). We are aware that each method has its detractors, especially distance (e.g. Maniloff, 1992; Ludwig & Schleifer, 1994; Farris *et al.*, 1999). Despite intense debate (Ludwig & Schleifer, 1994), a single method for phylogenetic reconstruction that can be used with complete confidence has not yet been identified. Therefore, in the current study, we used all four major algorithms to analyse the dataset (see Supplementary Table B in IJSEM Online). Consensus for spiroplasma groupings was obtained by using maximum-parsimony analyses (See Figs 1–4).

Our trees varied somewhat in topology, not only among analytical methods, but also with choice of positions for analysis. Trees generated with <1200 positions tended to show suspiciously unusual groupings. Although we determined bootstrap values routinely, we are aware of some of the limitations of this analytical method. For example, the presence of uninformative characters may affect its utility (Carpenter, 1996). Some authors have discussed the influence of position choice on the topology of phylogenetic trees (Hansmann & Martin, 2000). These authors point out that there is no *a priori* rationale for inclusion or exclusion of variable regions of a molecule, which, while difficult to align, may contain valuable phylogenetic signal. It is not clear why variable regions, if they can be aligned with reasonable certainty, should be excluded from analyses. In fact, when workers turn to analysis of the 16S–23S spacer region, variability is considered valuable for the resolution of intraspecific variability or variability among candidate members of very closely related species. Given the small evolutionary distances among some members of our strain complexes (e.g. serogroups I and VIII), an inclusionary strategy was followed in selecting the positions for most of our trees.

Under all these circumstances, results produced by trees generated under a wide range of assumptions offer important evidence. Support generated by the totality of the matrix of generated trees is important and where unanimous or nearly unanimous conclusions are reached, the phylogenetic inferences are surely robust.

Problems in nomenclature of groupings

Weisburg *et al.* (1989) named 'groups' by choosing a well-known species that was represented in the clusters they obtained with their distance analyses. We use the term 'clade' rather than 'group' in this paper, as all of the groupings that we designate have been obtained or confirmed by maximum-parsimony analysis. Johansson & Pettersson (2002) have followed Weisburg's precedent with considerable success; however, in our study, we found it difficult to adapt the existing nomenclature to certain clade structures.

One problem is that the concept of 'group' was first introduced into spiroplasma taxonomy (Junca *et al.*, 1980; Bové *et al.*, 1983; Whitcomb *et al.*, 1987; Williamson *et al.*, 1989) to denote putative species that were awaiting full characterization, according to proposed minimal standards

(International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1995). In this paper, to avoid confusion between this conflicting usage of 'group', we use the term 'serogroup' to denote spiroplasma strain assemblages that were classified under this system. However, in addition, problems arose with attempts to adapt the historical group–cluster–subcluster terminology (Weisburg *et al.*, 1989; Johansson & Pettersson, 2002) to a rapidly growing array of mollicute taxa – the Apis clade.

All except one of the clades in the trees that we present here could be classified by Weisburg's group concept. *Spiroplasma ixodetis* could be easily designated as either a monospecific clade or group. Spiroplasmas in serogroups I and II or serogroup VIII, which each, respectively, form distinct clades, could be viewed as subclusters under the Weisburg nomenclature. *Spiroplasma mirum*, which we treat here as a monospecific clade, could be treated as a third subcluster of the spiroplasma group (*sensu* Weisburg *et al.*, 1989). Also, the 18 species of *Entomoplasmataceae* and the five species of the *M. mycoides* cluster (which we show here to have clade status) could be classified as subclusters of Weisburg's '*M. mycoides* cluster'. However, the remaining 26 *Spiroplasma* species, which we define herein to comprise the 'Apis clade *sensu latu*', are difficult to classify without creating either a large number of subclusters or none at all. Weisburg *et al.* (1989), who studied only six of these species, termed this grouping the '*Spiroplasma apis* cluster'. However, as a result of discoveries of spiroplasmas in tabanids (Diptera: Tabanidae), mosquitoes (Diptera: Culicidae) and flower surfaces, the '*S. apis* cluster' (which we show herein to have clade status) has now expanded to include 21 species that clearly fall within the topologic boundaries that were established by the Weisburg study. Five species sequenced in this study occupy topological positions and are moderately outside the original boundaries. Should the newly designated 'Apis clade' accrete these five species? In this paper, we have added these five species to the 21 species that can be assigned on the basis of their topological relationship to the Weisburg species set, to form an 'Apis clade *sensu latu*'. The clade, which consists of the 21 species that fit within the boundaries of Weisburg's concept, we term the 'Apis clade *sensu strictu*'. The designations '*latu*' and '*strictu*' are used commonly in taxonomy when group boundaries are redefined.

It was in the classification of the Apis clade that our ability to use the established 'group classification' vanished. We were unable to separate the 26 species of the Apis clade *sensu latu* into subclusters without considerable fragmentation. If this large clade had not been subdivided, however, a substantial number of clades with clear biological significance would have escaped naming. Subdivision into 'subclusters' fails in the Apis clade, as it must in many cases of rapid taxic expansion, in that by starting at the top of the hierarchical level with 'group', defining only 'cluster' and 'subcluster' beneath, one is likely – in large groups – to exhaust the available nomina before all useful subdividing has been accomplished. To impose this kind of nomenclature onto a phylogenetic classification duplicates one of the most serious shortcomings of Linnaean nomenclature: the imposition of a template on clade structures that are unsuited to it (see discussion on Linnaean classification versus phylogenetic classification). These comments should not be construed in any way as criticism of the classification of Johansson & Pettersson (2002). Their useful classification represents the best possible attempt – in the interest of stability – to maintain the initial nomenclature of Weisburg *et al.* (1989) and has served well, even for the large and heterogeneous genus *Mycoplasma*. In summary, structural problems with clade topologies and the previous use of 'group' in *Spiroplasma* taxonomy in an entirely different sense make it difficult or impossible to use the 'group' concept for *Spiroplasma* clade nomenclature.

As noted in this paper, we approach nomenclature by defining clades. We use this term because all groupings to which we assign this term are derived from cladistic analyses (Swofford, 1998) – in most cases, maximum-parsimony. We also use the term 'clade' because the groups so defined will not be confused with any of the groups, clusters or subclusters of Johansson & Pettersson (2002). However, the difference in nomenclature does not imply that the underlying tree structures are substantially different; in fact, in all cases where the trees of Johansson & Pettersson (2002) and our trees describe the same species, the underlying topologies are quite similar and are sometimes identical.

Clade nomenclature

Clades mentioned in the text can be defined by referring to Tables 3 and 4 and Figs 1 and 2. Our nomenclatural approach differs from previous ones in that it starts not at a higher level, but at the level of the smallest clades, beginning with monospecific clades and clades that are composed of sister species. We present the data in this way as it is often clades of two to six species that are the most biologically and/or phenotypically significant. Our terminology uses: (i) lower-case species names (without italics) for clades with three or fewer species. For example, the sister species *Spiroplasma lampyridicola* and *S. leptinotarsae* form the lampyridicola–leptinotarsae clade. (ii) Clades with four or more species were named by using one or more of the component species, whose names served as nomina. These clades were named by using the single species name serving as the nomen, but in capital letters without italics. In Figs 1 and 2, we present phylogenetic trees that, with Tables 3 and 4, show the clade labelling. As the discussions in this paper involve not only *Spiroplasma* clades, but also clades of *Mollicutes* at higher hierarchical levels, we have adopted the standard clade terminology for higher-level taxa shown in Fig. 1. (iii) Where higher-level clades are identified, we retain the initial capital letter of the Linnaean name and the Linnaean spelling of the taxon, not italicized.

Phylogenetic trees. In all, 23 trees were generated by maximum-parsimony, maximum-likelihood, neighbour-joining

Table 3. Nomenclature for clades that represent higher hierarchical levels

Clade	No. (nodes in Fig. 1)	Synonymy with Weisburg *et al.* (1989) and Johansson & Pettersson (2002)
Acholeplasmatales clade	4	*A. laidlawii* cluster
Anaeroplasma clade	3	anaeroplasma group
Asteroleplasma clade	2	asteroleplasma group
Apis clade	14	apis cluster
Citri–Chrysopicola–Mirum clade	13	citri cluster
Hominis clade	8	hominis cluster
ixodetis clade*	12	ixodetis cluster
Mollicutes 'clade'†	1	*Mollicutes*
Mycoides–Capricolum–Putrefaciens clade	16	mycoides cluster
Mycoides–Entomoplasmataceae clade	15	mycoides cluster
Mycoplasmatales–Entomoplasmatales clade	6	None
Phytoplasma clade	5	*Candidatus* Phytoplasma cluster
Pneumoniae clade	9	pneumoniae cluster
Pneumoniae–Hominis–Ureaplasma–Haemoplasma (PHUH) clade	7	None
Spiroplasma–Entomoplasmataceae–Mycoides (SEM) clade	11	None
Ureaplasma clade	10	ureaplasma cluster

*This clade is in lower case because it is monospecific.
†Mollicutes, according to most phylogenetic reconstructions.

and distance (Supplementary Table B, available in IJSEM Online). Two of these trees are presented herein: Fig. 3 is a parsimony majority-rule consensus tree and Fig. 4 shows the same dataset with bootstrap values included (500 replicates).

Highly supported features of spiroplasma trees

The fundamental premise of cladistic taxonomy is that appropriate analyses will yield nested clusters (clades) of taxa that, having arisen from a single ancestor, share a common heritage. Twenty-four such clades are supported strongly by our studies, in that they occur in all, or almost all, trees constructed, regardless of the algorithm used in the analysis, and are usually afforded high support in bootstrap analyses. Supplementary Table B (available in IJSEM Online) lists the fraction of the 23 trees analysed that support each clade. In the following section, we list these highly supported clades and features and briefly discuss the biological characteristics of the clades that they define.

Monophyly of the Mycoplasmatales–Entomoplasmatales clade of *Mollicutes*. The orders *Mycoplasmatales* and *Entomoplasmatales* form a clade that is a sister to the Acholeplasma–Anaeroplasma–Phytoplasma clade of *Mollicutes*. The order *Mycoplasmatales* is polyphyletic, in that *Mycoplasma* species are split into two phylogenetically separate sections that do not share a common ancestor. Nevertheless, the two orders, taken together, are shown herein to have derived from a single common ancestor. Unlike most other prokaryotes, members of the Mycoplasmatales–Entomoplasmatales clade – so far as is known – use UGA as a codon for tryptophan and not as a stop codon. This character, although it has not been determined for most mollicutes, has been hypothesized to be synapomorphic for the Mycoplasmatales–Entomoplasmatales clade. Although this clade has no formal recognition in Linnaean taxonomy, it represents a profound split in the mollicute lineage and may have diverged from an ancestral lineage hundreds of millions of years ago (Hackett, 1990; Hackett *et al.*, 1990; Maniloff, 2002).

Position of the Spiroplasma–Entomoplasmataceae–Mycoides grouping (SEM) clade as a sister lineage to the Pneumoniae–Hominis–Ureaplasma–Haemoplasma (PHUH) clade. As in other trees that represent mollicute phylogeny [reviewed by Johansson & Pettersson (2002)], our trees showed consistently that the paraphyletic SEM clade is a sister lineage to a large, also paraphyletic, clade (PHUH) that contains over 100 species of *Mycoplasma*, including the (paraphyletic) Pneumoniae clade, the Hominis clade, ureaplasmas and haemoplasmas. *Mycoplasma* species of this clade form a paraphyletic unit that has no Linnaean designation, but given its origin from a common ancestor, should be treated as monophyletic for purposes of binomial nomenclature. The Pneumoniae clade (e.g. *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma iowae* and *Mycoplasma pirum*) has many species in which cytoskeletal modifications, including terminal blebs, are present. This technically paraphyletic clade contains the ancestral root of the genus *Ureaplasma*, an apomorphic taxon that presumably arose by transfer of a lineage of the Pneumoniae clade into the urogenital tract of vertebrates. The Pneumoniae clade also is the ancestral root of 'haemoplasmas' (Neimark & Kocan, 1997), a

Table 4. Clade nomenclature

Clade	No. (nodes in Fig. 2)	Features
Apis clade *sensu latu*	14	Two new clades (five spp.) added to Apis clade *sensu strictu* to form Apis clade *sensu latu*
Apis clade *sensu strictu*	32	Tabanid, mosquito and flower spiroplasmas
Apis–Litorale–Helicoides–Chinense	34	Tabanid or mosquito spiroplasmas
BIUS-1–W115	31	Flower spiroplasmas
BIUS-1–Diminutum–Floricola–Monobiae	25	Flower and mosquito spiroplasmas
CB-1–Ar 1357	29	Serogroup XVI Cantharid beetles, mosquitoes
chinense–velocicrescens*	35	Flower spiroplasmas
chrysopicola–syrphidae–TAAS-1	19	Serogroup VIII Tabanid spiroplasmas
Citri–Chrysopicola	18	Surprising sister clades; apomorphic morphology
Citri–Chrysopicola–Mirum	13	*S. mirum* has modal spiroplasma morphology
Citri–Melliferum–Insolitum–277F	21	Insect/plant phloem, leaf surfaces
citri–melliferum	22	Leafhopper/plant phloem, honeybee
clarkii (*S. clarkii*)	36	Green june beetle spiroplasma
culicicola (*S. culicicola*)	33	Mosquito spiroplasma
diminutum (*S. diminutum*)	30	Mosquito spiroplasma
diminutum–CB-1–Ar 1357	28	Mosquito, cantharid beetle spiroplasmas
Mycoides–Entomoplasmataceae	15	Includes clade that radiated to ruminants
Floricola–Diabroticae	27	Flower spiroplasmas
helicoides–gladiatoris–bARC1901	37	Tabanid spiroplasmas
ixodetis (*S. ixodetis*)	12	*Ixodetis* tick spiroplasma
leptinotarsae–lampyridicola	24	Beetle spiroplasma
litorale–turonicum–corruscae	39	Tabanid spiroplasmas
mirum (*S. mirum*)	17	Rabbit tick spiroplasma
monobiae (*S. monobiae*)	26	Flower spiroplasma
montanense–apis	40	Tabanids, other insects, flowers
Mycoides–Capricolum–Putrefaciens	16	Important ruminant pathogens
Mycoides	42	*Mycoplasma* spp.
putrefaciens–yeatsii–cottewii	41	Ruminant mycoplasmas
sabaudiense–alleghenense–TIUS-1	23	Spiroplasmas of Mosquitoes, tiphiid wasp
Spiroplasma–Entomoplasmataceae–Mycoides (SEM)	11	Order *Entomoplasmatales* plus Mycoides clade
taiwanense (*S. taiwanense*)	38	Mosquito spiroplasma

*Clade names with three or fewer species are not capitalized.

trivial name that was proposed by Neimark *et al.* (2001) for a cluster of haemophilic mycoplasmas, which had been named before the general concept of *Candidatus* status had been proposed (Murray & Stackebrandt, 1995). *Haemoplasma* species have recently been transferred to the genus *Mycoplasma* (Neimark *et al.*, 2001).

Position of the Mycoides clade within *Entomoplasmatales*. Here, we confirm and extend, by addition of 26 new complete *Spiroplasma* sequences (designated by GenBank accession numbers starting with AY in Table 2), the reports of others (Weisburg *et al.*, 1989; Maniloff, 1992; Gundersen *et al.*, 1994; Johansson & Pettersson, 2002) that *M. mycoides* and four allied *Mycoplasma* species, together with the Entomoplasmataceae clade, with which it has a sister relationship, comprise an evolutionary terminus that is derived from a spiroplasmal ancestor. Together, these groupings comprise the Mycoides–Entomoplasmataceae clade. Our trees demonstrate that the Mycoides clade is apomorphic in this clade of 23 species. Derivation of the Mycoides clade from the *Entomoplasmataceae* renders this family technically paraphyletic. We use the word 'technically' because we regard an emphasis on paraphyly as detrimental to the quest for groupings of species with common ancestors. The fact that a lineage has split off from an otherwise cohesive clade, in our view, does nothing to alter the evolutionary history of the clade itself or to change its status as having arisen from a common ancestor. Hence, realizing that not all systematists agree with us, we have chosen to note paraphyly without proposing it to be a nomenclatural determinant in Linnaean systems. It is polyphyly, not paraphyly, that threatens the utility of Linnaean classifications. The Mycoides–Entomoplasmataceae clade is a sister lineage to the Apis clade *sensu latu*, which is shown here

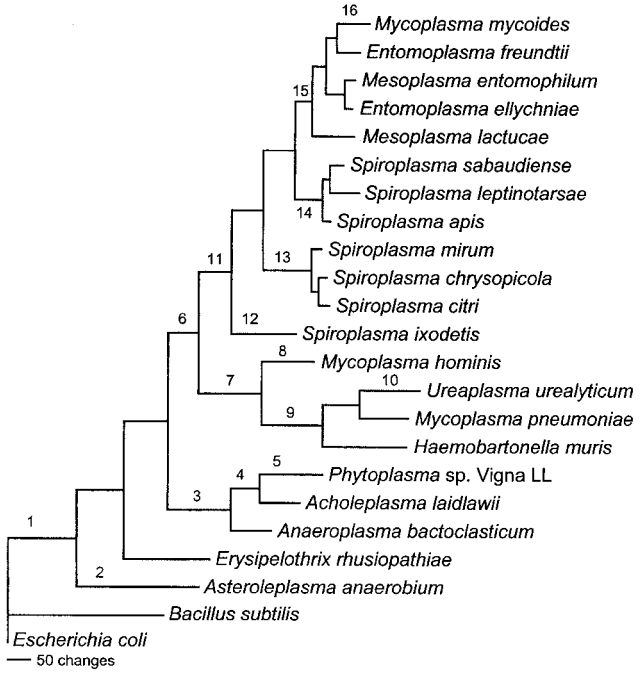

Fig. 1. Phylogram showing positions of clades at higher-level hierarchies of *Mollicutes*. See Table 3 for synonymy of clades described herein with the groups, clusters and subclusters of Weisburg *et al.* (1989) and Johansson & Pettersson (2002).

to contain the 21 species (or putative species) of the Apis clade *sensu strictu* [the six species analysed by Weisburg *et al.* (1989) plus 15 spiroplasmas sequenced herein, whose phylogenetic position falls within the boundaries established by that study] plus two novel clades (the lampyridicola–leptinotarsae and sabaudiense–alleghenense–TIUS-1 clades), which together have a total of five species, all topologically placed outside the original group boundaries; thus, there are 26 spiroplasmas in the Apis clade *sensu latu*. Both novel clades are basal to the Apis clade *sensu strictu* and are thus related more closely to the *Entomoplasmataceae* and *M. mycoides* than members of the Apis clade *sensu strictu*. The discovery of other spiroplasmas that are related more closely to *M. mycoides* than those previously known enables us to better evaluate the relationship of the Mycoides clade (and its sister, the Entomoplasmataceae clade) to spiroplasmas.

Essential monophyly of *Spiroplasma* and subdivision of the SEM clade. All currently classified *Spiroplasma* species form a single evolutionary unit that is derived from a common ancestor. As explained above, we do not believe that the status of *Spiroplasma* as being technically paraphyletic should be considered in the construction of Linnaean classifications. All trees and bootstrap values for the major nodes strongly support the subdivision of this clade into the ixodetis clade, the Citri–Chrysopicola–Mirum

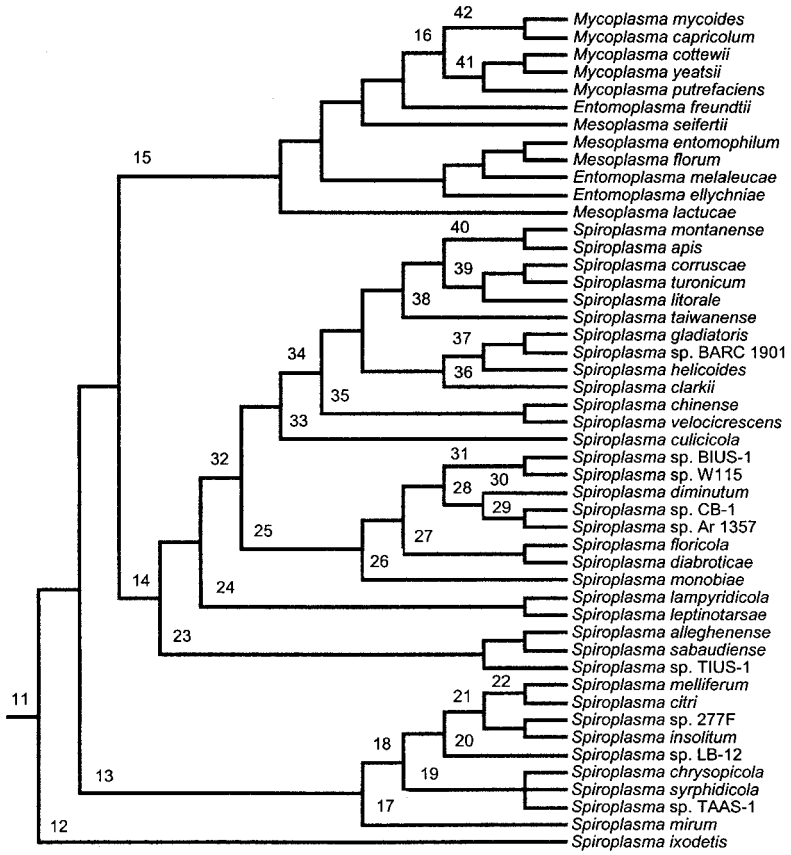

Fig. 2. Majority-rule maximum-parsimony tree showing position of major clades of the Spiroplasma–Entomoplamataceae–Mycoides (SEM) clade. Note that *Spiroplasma* is, technically, paraphyletic. See Table 4 for clade information.

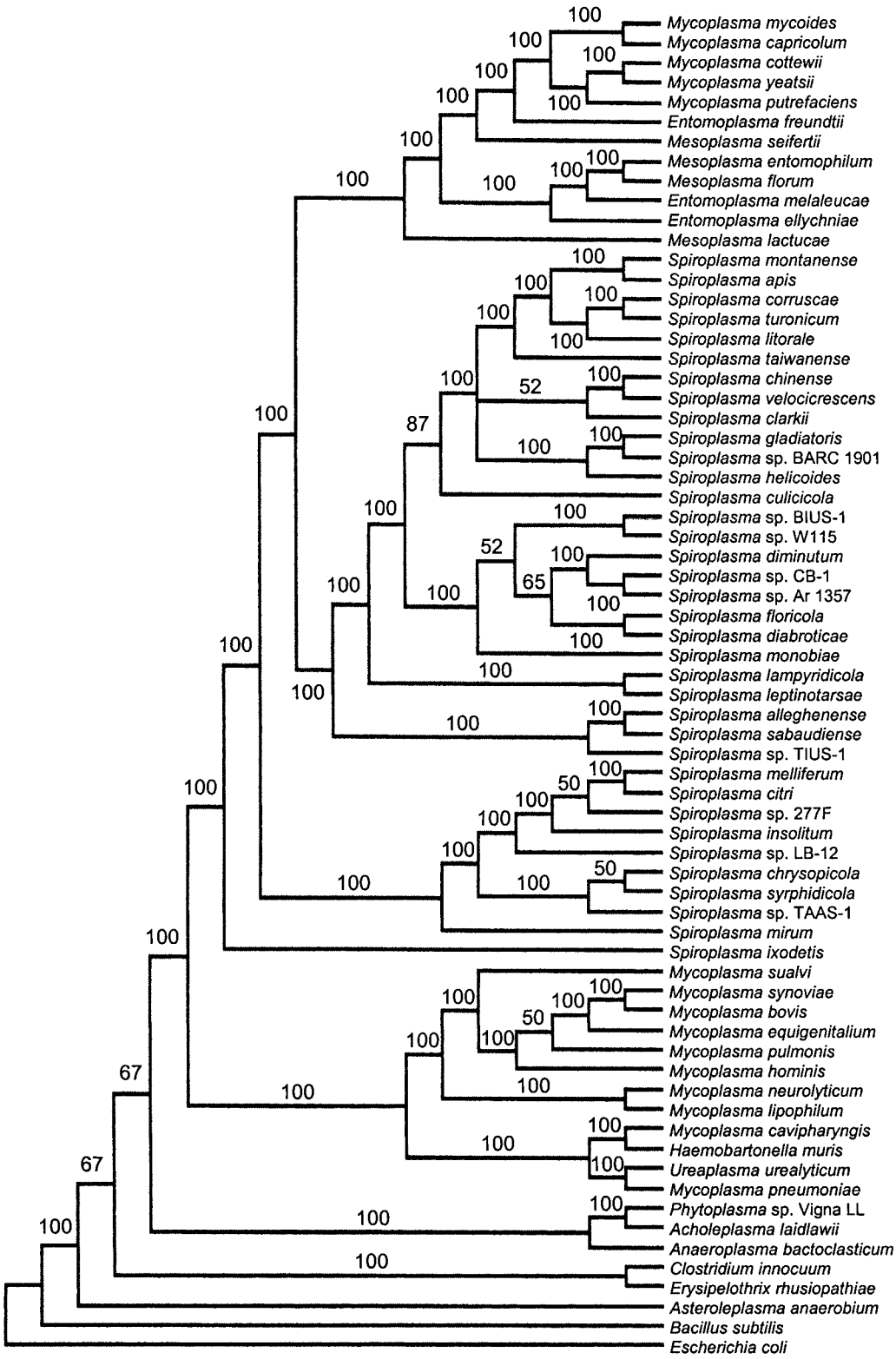

Fig. 3. Phylogenetic reconstruction using maximum-parsimony. Four Gram-positive bacterial species and *Escherichia coli* served as outgroups. Analysis was confined to those positions in the alignment that satisfied the condition that one base accounts for at least 50 % of the total composition of that position (a total of 1428 characters). The analysis used a heuristic search and the tree bisection–reconnection maximum-parsimony algorithm for branch-swapping. Note: *Phytoplasma* is a *Candidatus* genus designation.

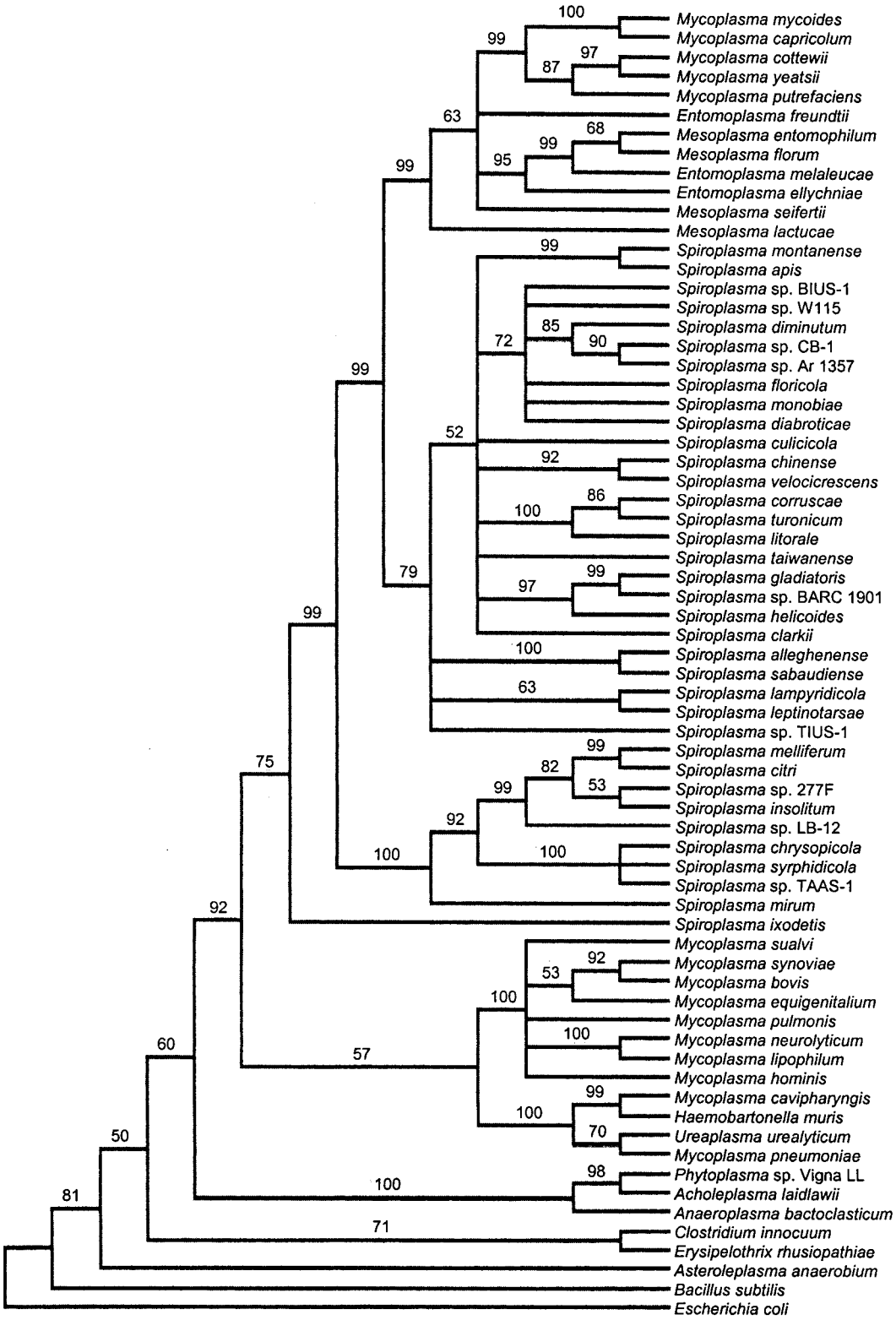

Fig. 4. Phylogenetic tree showing the position of the class *Mollicutes* and representative members of the phylum *Firmicutes*. *Escherichia coli* and three Gram-positive bacteria served as outgroups. In total, 1428 positions were used in the analysis, with the tree bisection–reconnection maximum-parsimony algorithm for branch-swapping. The dataset was resampled 500 times; bootstrap percentage values are given at nodes. Note: *Phytoplasma* is a *Candidatus* genus designation.

clade, the Apis clade *sensu latu* and the Mycoides–Entomoplasmataceae clade.

The ixodetis clade is basal in the spiroplasma lineage. The ixodetis clade diverges at the base of the *Spiroplasma* evolutionary tree. Originally, this clade was thought to consist of a single species, *S. ixodetis* (serogroup VI) and, technically, it still does. However, there have been several recent claims that certain micro-organisms that are associated with insects cluster with *S. ixodetis*. In each case, evidence consists of the 16S rDNA sequence similarity of the candidate organisms to spiroplasmal DNA of *S. ixodetis*, as revealed by BLAST searches. It should be noted that BLAST searches may not reveal the nearest neighbour and are by no means final arbiters of phylogenetic position (Koski & Golding, 2001). Also, in no case were micro-organisms envisioned or cultured. In some cases (Hurst *et al.*, 1999; Hurst & Jiggins, 2000; Jiggins *et al.*, 2000), organisms were reported to be associated with sex-ratio abnormalities in coccinellid beetles (*Adalia bipunctata*) or a butterfly (*Danaus chrysippus*) (Jiggins *et al.*, 2000). A publication from Russia (Sokolova *et al.*, 2002) reported electron microscopic visualization of spiroplasmas in ovarioles of *A. bipunctata*. The structures depicted appear to be filamentous, but do not display clear helicity. There is no way of knowing whether the envisioned organisms are actually spiroplasmas and, if so, whether they represent the *Adalia* sex-ratio agent. In any event, phylogenetic placement of the *Adalia* agent is problematical, especially when one considers that the taxic sampling used in phylogenetic analyses concerning them was extremely limited. Other isolates with an apparent topological relationship were from the bamboo pseudococcid (Fukatsu & Nikoh, 2000) and green pea aphid (Fukatsu *et al.*, 2001). The affinity of these hypothetical 'group members' *to S. ixodetis* must be examined in the context of long-branch attraction (Carmean & Crespi, 1995; Siddall & Whiting, 1999; Stiller & Hall, 1999). *S. ixodetis* exhibits an unusual, tightly coiled helicity, whereas other spiroplasmas display a less tightly coiled helicity. Whether this morphology is apomorphic or plesiomorphic for spiroplasmas is at present unknown. Detailed study of the cytoskeletal structure of spiroplasmas suggests that helicity itself is a fundamental condition that can be modified without substantial genomic change (Trachtenberg & Gilad, 2001; S. Trachtenberg, personal communication). The *S. ixodetis* genome is 2220 kbp in size, which is the largest in the genus *Spiroplasma* and just 500 kbp smaller than the genome of *Acholeplasma laidlawii*. However, the complete genome sequence of *S. ixodetis* has not been determined, so we do not know how much of this genome represents ORFs or, alternatively, repeat sequences or integrated viral or plasmid DNA, which represent a substantial portion of some spiroplasmal genomes (U. Melcher, personal communication).

Monophyly of the Citri–Chrysopicola–Mirum clade. High support is accorded to the monophyly of the clade Citri–Chrysopicola–Mirum, which contains serogroup I and II spiroplasmas (Citri–Poulsonii clade), serogroup VIII spiroplasmas (chrysopicola–syrphidicola–TAAS-1 clade) and the mirum clade [*S. mirum* (serogroup V)], despite the substantial phenotypic differences that characterize each of these three component clades. Given the conspicuous phenotypic and genotypic differences between the Chrysopicola–Syrphidicola–TAAS-1 and Citri–Poulsonii clades, their sister status is surprising.

Monophyly of the Citri–Poulsonii clade. High support is obtained for the clade (Citri–Poulsonii) that contains the seven sequenced serogroup I spiroplasmas and the serogroup II species *Spiroplasma poulsonii*. Serogroup I spiroplasmas form an ecologically diverse cluster that has been divided into eight subgroups on the basis of serology and DNA–DNA reassociation (Junca *et al.*, 1980, Bové *et al.*, 1983). In this paper, we include in our trees only five of the eight sequenced members of the Citri–Poulsonii clade; *S. poulsonii*, *Spiroplasma phoeniceum* and *S. kunkelii*, which have been sequenced, are not represented in Figs 3 or 4, and strain N525, the representative strain for subgroup I-7, has not been sequenced. Trees for the Citri–Poulsonii clade that contained all eight available sequences showed some minor inconsistencies in their topologies. We have chosen to omit the three sequenced species from the trees we present here, as topologies for this group have not yet been resolved completely. Of course, the small inconsistencies observed in internal topology do not negate the integrity of the clade itself. It is to be expected that the nodes of the serogroup I cluster should be difficult to resolve (Stackebrandt & Goebel, 1994), as the similarity coefficients of their 16S rDNA sequences ranged from 0·986 to 0·991.The Citri–Poulsonii clade is of great interest, in that it has a wide diversity of spiroplasma–host associations. Members of this cluster occur in ticks, honeybees, leafhoppers (plant phloem-sucking insects), plant/flower surfaces and *Drosophila*, in which they cause sex-ratio abnormalities (Williamson & Poulson, 1979; Williamson *et al.*, 1989). Given these host relationships, each node in the Citri–Poulsonii clade is of critical importance.

Basal position of subgroup I-5 spiroplasmas in the Citri–Poulsonii clade. The basal position of subgroup I-5 strain LB-12 and its allies is supported by all topologies we obtained for the Citri–Poulsonii clade. This spiroplasma was isolated from the green leaf bug in Taiwan (Lei *et al.*, 1979). As noted in the section on Evolution, this position is critical, as it implies that the plesiomorphic habitat in the Citri–Poulsonii clade may have been the plant phloem/sucking insect habitat.

Position of subgroups I-4 and I-6 as sister species in the Citri–Poulsonii clade. Strain 277F, the sole member of subgroup I-4, and subgroup I-6, represented by *Spiroplasma insolitum*, were classified as sister species in all topologies. Although strain 277F was isolated from a

tick, only a single strain is available, so the true habitat relationship of this spiroplasma is uncertain. *S. insolitum* has been isolated frequently from flowers and insects, including butterflies, in Maryland, USA (Hackett *et al.*, 1984).

Monophyly of the chrysopicola–syrphidicola–TAAS-1 clade. *Spiroplasma chrysopicola*, *Spiroplasma syrphidicola* and *Spiroplasma* sp. TAAS-1 (serogroup VIII) always formed a monophyletic clade. In addition, strains BARC 1357 and BARC 2649, candidate subgroups of serogroup VIII, also belong to this clade. However, the evolutionary distances among these five serogroup VIII strains are very small (16S rDNA similarity coefficients of 0·992–0·999) so these sequences failed to resolve the relationships among them (G. E. Gasparich, unpublished data), as would be expected (Stackebrandt & Goebel, 1994). Members of the chrysopicola–syrphidicola–TAAS-1 clade have been isolated almost exclusively from tabanid flies. Members of this group are, physically, the smallest spiroplasmas – passing quantitatively through 220 nm filters. This group also has a DNA G+C content (28–31 mol%) at the high end of the spiroplasma range and all members utilize arginine. More than 100 serogroup VIII isolates have been obtained from eastern Canada, south to Georgia, west to Idaho and Texas. The group is also known from Europe (Le Goff *et al.*, 1991). Recently, more than a dozen isolates have been obtained, ten from Costa Rica and three from Australia (F. E. French, unpublished data). Three formal subgroups have been proposed for serogroup VIII strains on the basis of DNA–DNA hybridization, DNA G+C content and serological analyses (Gasparich *et al.*, 1993). Type or representative strains are: EA-1 (*S. syrphidicola*, subgroup VIII-1); DF-1 (*S. chrysopicola*, subgroup VIII-2) and TAAS-1 (subgroup VIII-3). In addition, strain BARC 2649 was classified by Williamson *et al.* (1998) as an undesignated subgroup of serogroup VIII. However, as new isolates continue to accumulate, it has become more and more difficult to assign them to a subgroup (Stewart, 2001; F. E. French and R. F. Whitcomb, unpublished data).

Position of *S. mirum* as basal in the Citri–Chrysopicola–Mirum clade. The phylogenetic position of the tick spiroplasma, *S. mirum*, as basal in this clade of insect specialists was highly supported. *S. mirum* is the most basal species of *Spiroplasma* that shows the modal spiroplasma helicity and motility.

Position of the Mycoides and Entomoplasmataceae clades as sister lineages. High support is obtained for the apomorphic status of the Mycoides clade as a derivative of the Entomoplasmataceae clade. Hence, the taxon *Entomoplasmataceae* is technically paraphyletic. *Mesoplasma lactucae* was basal to the Entomoplasmataceae clade and *Entomoplasma freundtii* was basal to the Mycoides clade. As discussed below, divergence of the Mycoides and Entomoplasmataceae clades is in complete accord with the host affinities of the organisms. All species of the Mycoides clade are from ruminant animals and all entomoplasmas and mesoplasmas derive from the insect/plant surface habitat.

Polyphyly of *Entomoplasma* and *Mesoplasma* species. All trees constructed to date, starting with the original distance trees of Weisburg *et al.* (1989) and including later trees of Johansson [summarized by Johansson & Pettersson (2002)] and Maniloff (2002) have indicated that species of the genera *Mesoplasma* and *Entomoplasma* do not form clades, but are intermixed, with small interspecies evolutionary distances. Our trees also fail to show distinct clusters for these genera. Furthermore, the similarity coefficients for 16S rDNA sequences are extremely high in some intergeneric comparisons [*Entomoplasma melaleucae* versus *Mesoplasma entomophilum* (0·992)].

The two serogroup XVI species studied are sisters. Strains CB-1 (subgroup XVI-2) and Ar 1357 (subgroup XVI-3) were shown invariably to be sisters in the cladograms. Members of this serogroup are either isolates from flower surfaces or from cantharid beetles, which are frequent visitors of flowers. Several isolates in this clade have also been isolated from mosquitoes. The third subgroup, *Spiroplasma cantharicola* (XVI-1), was not sequenced in this study, but DNA–DNA hybridization data (Abalain-Colloc *et al.*, 1993) indicate that this species is related closely to subgroups XVI-2 and XVI-3.

Sister taxa status of serogroup XVI spiroplasmas and *Spiroplasma diminutum*. All trees showed *S. diminutum* and the CB-1–Ar 1357 (serogroup XVI) clade as sister lineages. However, *S. diminutum* is not related serologically to serogroup XVI spiroplasmas. *S. diminutum* and certain serogroup XVI strains have been isolated from mosquitoes.

Monophyly of the helicoides–gladiatoris–BARC 1901 clade. This clade of three species (or putative species), all isolated from tabanid flies, was highly supported in trees generated by all four phylogenetic methods.

Monophyly of the corruscae–turonicum–litorale clade. The monophyly of these three tabanid *Spiroplasma* species was also supported strongly. *Spiroplasma corruscae* was originally isolated from an overwintered lampyrid beetle, which has raised the issue of transmission and maintenance of the spiroplasma during winter (Hackett *et al.*, 1996b). It is possible that beetles provide an overwintering reservoir for *S. corruscae* and that tabanids acquire the spiroplasma in the spring at feeding sites that are shared with the beetles (Gasparich *et al.*, 1998).

Status of *S. apis* and *Spiroplasma montanense* as sister species. These sister species formed a clade (apis–montanense clade) that was separated from other tabanid spiroplasma clades by relatively small evolutionary distances. Both species have been isolated from tabanids, but

S. apis causes May Disease of honey bees in France and has been isolated from other insect host species.

Sister lineage status of serogroups XXI (strain W115) and XXX (strain BIUS-1) flower spiroplasmas. These two spiroplasmas were sister species in most trees. Strains W115 and BIUS-1 were respectively isolated from flower surfaces in Oklahoma and Maryland, USA, so the actual (presumed) insect hosts of these species is unknown.

Position of *S. lampyridicola* and *S. leptinotarsae* as sisters. These sister species are both beetle spiroplasmas. *S. leptinotarsae* is a specialist that is associated with the Colorado potato beetle (Clark, 1982) and *S. lampyridicola* is a specialist that is associated with firefly beetles (Coleoptera: Lampyridae), respectively (Clark *et al.*, 1987; Hackett *et al.*, 1992; Stevens *et al.*, 1997). *S. leptinotarsae* is transmitted from beetle to beetle on leaf surfaces (Clark, 1984). The unusual morphology of this species is discussed in the Morphology section. *S. lampyridicola* has been isolated from both larvae and adult fireflies, but the transmission mechanism is unknown. Cells of this spiroplasma are unusual in that, when tested as an antigen, they react serologically in both deformation (DF) (Williamson *et al.*, 1978) and metabolism inhibition (MI) (Williamson *et al.*, 1979a) tests with the majority of spiroplasma antisera. Cells of *S. leptinotarsae*, when tested as antigens, also react with multiple spiroplasma antisera. Whether this property is synapomorphic for the leptinotarsae–lampyridicola clade is unknown.

Position of *Spiroplasma sabaudiense* (serogroup XIII), *Spiroplasma alleghenense* (serogroup XXVI) and *Spiroplasma* sp. strain TIUS-1 (serogroup XXVIII) as a monophyletic clade. The sabaudiense–alleghenense–TIUS-1 clade, which appeared in most trees, makes less ecological sense than most of the other spiroplasma clades. It consists of serogroups XIII, XXVI and XXIX, which have been isolated from a diverse range of insects, including mosquitoes (postulated to feed on flowers) (Chastel & Humphery-Smith, 1991) and flower-visiting tiphiid wasps, as well as scorpionflies.

Phylogenetic classification and phenotype

Phylogenetic reconstructions have at least three practical uses. The first is to predict or anticipate the properties of organisms that have yet to be determined. The second is to shed light on the evolutionary process itself, which must be understood in order to make sense of biology. The third is guidance in construction of Linnaean classifications, so that the latter may reflect, as closely as possible, the natural relationships among taxa.

Previous authors, after examination of the relationship between mollicute phylogeny and phenotype, painted a rather bleak picture. Weisburg *et al.* (1989), whilst acknowledging that some correlations between phylogeny and phenotype were apparent *ex post facto*, felt that phenotype was not well predicted by phylogeny. Also, Dodge *et al.* (1998) felt that their phenetic tree showed that serological analysis of spiroplasmas was unreliable. Our trees and phenotypic data support neither of these views.

The study of Weisburg *et al.* (1989), which still stands as a defining paper on mollicute phylogeny, dealt with a relatively small sample of available organisms, which were chosen because they were perceived to represent a wide spectrum of mollicute biodiversity. Thus, they were chosen because they were likely to be members of diverse clades that were phylogenetically remote from one another and, hence, less likely to be phenotypically similar than species that were more closely related. Many microbial phenotypic properties are apomorphic traits that are only shared with closely related species and often converge despite phylogenetic disparity.

In the analysis of Dodge *et al.* (1998), weight was given to a sequence whose placement was suspiciously anomalous. The value of serology was discounted in large part, as one unsubstantiated sequence for strain TAUS-1 (*Spiroplasma tabanidicola*) appeared to be related closely to sequences of spiroplasmas in the chrysopicola–syrphidicola–TAAS-1 clade. If this relationship were genuine, it would not only fly in the face of strong serological evidence, but also would contradict the seemingly unerring congruence of spiroplasma serology with DNA–DNA reassociation data (Junca *et al.*, 1980; Bové *et al.*, 1983). Further, it would imply that extremely short and narrow organisms with a relatively high DNA G+C content and the ability to catabolize arginine aggressively were related extremely closely to spiroplasmas that were relatively long and wide with a considerably lower G+C content and with no capability to catabolize arginine. Thus, the rejection of serological evidence in the systematics of these organisms on the basis of an aberrant placement of a single sequence would require one to ignore a suite of robust characters that separate the chrysopicola–syrphidicola–TAAS-1 clade from the Apis clade *sensu latu*.

Morphology. Morphology is a primary descriptor in most classifications of animals and plants; it is also important among microbes. Spiroplasma morphologies were not analysed herein as characters, because some distinctions are somewhat arbitrary. However, the consistency of morphology with cladistic topology is striking and must be discussed, even if it is not analysed rigorously. It is common for spiroplasma clades to share a common apomorphic – and in many cases, autapomorphic – set of morphological attributes. The following discussion refers to morphology as viewed microscopically during growth of the organisms in culture. The following morphotypes can be recognized.

(i) In the Citri–Poulsonii (serogroup I–serogroup II) clade, organisms are seen, after elongation of short forms during very early exponential phase, as long helices (10 or more turns) (Garnier *et al.*, 1984). This morphology remains

stable throughout the exponential and early stationary growth phases.

(ii) In the chrysopicola–syrphidicola–TAAS-1 (serogroup VIII) clade, organisms grow very fast to titres as high as 10^{11}. Cells are short and narrow and can pass quantitatively through 220 nm filters. This morphology and the high titre to which cultures grow are diagnostic and, in routine screening, it is often possible to test candidate serogroup VIII strains against the bank of serogroup VIII antisera without preliminary screening against antiserum to organisms of other spiroplasma groups (Whitcomb & Hackett, 1996). Also, the vast majority of the serogroup VIII isolates (>100) have short cells, although in a few strains, longer cells have been observed (F. E. French, unpublished data).

(iii) Most spiroplasmas of the Apis clade *sensu strictu* tend to resemble those of the Citri–Poulsonii clade in their helicity, but are usually shorter. This morphology is presumably plesiomorphic in the clade and perhaps even in the genus (see Evolution). Some of the members of the clade (e.g. *Spiroplasma helicoides*) exhibit robust helical morphology that remains uncompromised throughout the exponential and early stationary growth phases.

(iv) Some species of the Apis clade *sensu latu* show autapomorphic modifications of simple helicity. In *S. leptinotarsae*, the cells are funnel-shaped, in that they are broad at one end but narrow to a tip at the other, with increasing amplitude from the tip to the posterior end of the cell when observed in early passages. *In vitro*, these cells exhibit extremely rapid translational motility. *In vivo*, in the gut of the host (the Colorado potato beetle), the helical cells flatten into a single plane, so that transverse sections show coiled tubules. Under dark-field microscopy, these structures appear to be coin-shaped. When these flattened coils are released into fresh culture medium, the cells regain their funnel (or spring-like) morphology and are again capable of rapid translational motility. As spectacular as this morphological adaptation is, it is ephemeral. In primary culture, *S. leptinotarsae* grows very poorly in artificial media, but cultures can be established in co-cultures with insect cells. After a relatively short number of passages in broth media, the ability to form helical cells with continuously varying helical amplitudes disappears and the cells then revert to simple helicity [see (iii) above].

(v) Some members of the BIUS-1–Floricola–Diabroticae–Monobiae clade of the Apis clade *sensu strictu* have short cells that may be helical at only certain phases of the growth cycle. *Spiroplasma floricola* (serogroup III) grows very rapidly in the exponential phase as short helices, but as the medium acidifies and is deprived of nutrients by the rapidly growing organisms, the cells become non-helical. For a short period, these non-helical cells, if they are reinoculated into fresh medium, are able to grow and helical morphology reappears (Whitcomb & Coan, 1980). In serogroup XXX (strain BIUS-1), the morphological cycle *in vitro* is somewhat similar, although helices appear for a short time only in mid-exponential phase. Cells of either *S. floricola* or strain BIUS-1, if examined superficially and viewed only in stationary phase, might appear to be non-helical and could conceivably be mistaken for entomoplasmas. In fact, when strain BNR1 of *S. floricola* was first isolated and passed in the laboratory, it was mistaken for a non-helical organism, but when examined in exponential phase, it was found to exhibit swarms of short helical cells (D. L. Williamson and R. F. Whitcomb, unpublished data). *S. diminutum*, although allied closely to the CB-1–Ar 1357 clade, in which cellular morphology is unremarkable, grows prolifically as extremely short cells, with perhaps no more than two or three turns per cell. *Spiroplasma culicicola*, a species of the Apis clade *sensu strictu* that has no close sister species, also grows as very short cells with few turns. As all spiroplasmas that have very short cells have rapid growth rates, it is possible that the tendency for short cells may be explained by the rapidity of cellular growth.

(vi) Strain TIUS-1 (serogroup XXIX) exhibits such poor helicity in culture that it could easily be mistaken for an entomoplasma. Helical forms are rarely seen in culture. This species is basal to the apis clade *sensu latu* and, evolutionarily, may represent a transitional morphological stage in the loss of helicity.

Three spiroplasma species show autapomorphic adaptations that involve a departure from simple generic modal helicity: (vii) *S. ixodetis* has cells that are extremely variable in culture. Some of them are not helical and some have extremely tightly coiled helices – so much so that under dark-field microscopy, they may not appear helical at all. (viii) Serogroup XV spiroplasmas, the 16S rDNA of which was not sequenced, lose helicity and motility and become stiff and rod-like as the growth medium acidifies in late exponential phase. However, if the pH of the medium is raised to neutral, helicity and motility return. (ix) *Spiroplasma platyhelix*, the 16S rDNA of which was also not sequenced, displays a low-pitch helical morphology and, along with serogroup XV spiroplasmas, exhibits a unique motility, in which tightly coiled segments move in waves along the length of the filament.

In summary, there are many modifications of the simple helicity that is exhibited by species of the Apis clade *sensu strictu*. In many cases, these modifications are synapomorphic for clades of closely related species. However, several are autapomorphies and thus do not provide phylogenetic information.

Serology. Careful examination of the totality of spiroplasma data indicates a strong correlation between serology and molecular phylogeny. Serology of spiroplasmas has been studied in great detail [summarized by Williamson *et al.* (1998)] by the spiroplasma DF (Williamson *et al.*, 1978) and MI (Williamson *et al.*, 1979a) tests, and by growth inhibition (Whitcomb *et al.*, 1982) for serogroups I–XI. Serogroup classification of

spiroplasmas (Junca *et al.*, 1980; Whitcomb *et al.*, 1987) is based to a considerable degree on serological data. Minimum standards have been established for the serological evidence that is required to establish spiroplasma serogroups (Whitcomb *et al.*, 1987). In these criteria, it was recognized that reciprocal cross-reactivity was required to establish serogroups, as one-way crosses, which can be of considerable magnitude in some cases, are not unusual in mollicute serology. Every combination of *Spiroplasma* antigen/antibody reaction has been tested by DF and MI serology in the course of defining the 34 serogroups and 14 subgroups of spiroplasmas (Williamson *et al.*, 1998). Every one of the >5000 reciprocal cross-reactions that are observed in these tests is consistent with the topologies in our phylogenetic reconstructions. Invariably, reciprocal serological cross-reactions are predictive of close phylogenetic affinity. Pairs of species that shared reciprocal serological cross-reactivities were invariably separated by very small evolutionary distances and were members of the same clade. For example, sequences that represented the seven sequenced subgroups of serogroup I (Citri clade) had sequence similarities of no less than 0·986. The three subgroups of the chrysopicola–syrphidicola–TAAS-1 clade (serogroup VIII) reported here, and an assemblage of other serogroup VIII strains that were not included in our reconstruction, had much higher similarity values (>0·99) than serogroup I spiroplasmas, and all members of both serogroups I and VIII shared a high degree of intragroup serological reactivity (Williamson *et al.*, 1998). When the 16S rDNA similarity between species is 97 % or more, Stackebrandt & Goebel (1994) recommend that DNA–DNA reassociation studies should be performed to assist in the decision concerning possible species identity of the paired strains. In the case of spiroplasma subgroups, DNA–DNA reassociation studies have already been correlated with serological reactivities in species designations (Junca *et al.*, 1980; Bové *et al.*, 1983). Later, DNA–DNA reassociation studies were performed on the chrysopicola–syrphidicola–TAAS-1 clade (serogroup VIII; Gasparich *et al.*, 1993) and the CB-1–Ar 1357 clade (serogroup XVI; Abalain-Colloc *et al.*, 1993). Cross-reactions among strains in serogroups I, VIII and XVI represent the vast majority of spiroplasma intrasubgroup serological cross-reactivities. However, the sister species *S. apis* and *S. montanense* share a very low level of reciprocal cross-reactivity (DF reciprocal cross-reactions of 20 or 40). No other reciprocal serological cross-reactivities were noted. Thus, results from *Spiroplasma* phylogeny and serology are congruent and serology – DF in particular – remains a practical means for identification of isolates from particular ecological situations.

It is important to point out that members of one subgroup may fail to cross-react serologically with one or more members of other subgroups. For example, subgroup VIII-2 does not cross-react with subgroup VIII-1, a circumstance that originally led to its being designated as a separate serogroup (Tully *et al.*, 1987). However, both of these strains share numerous cross-reactions with other members of serogroup VIII. Strain BARC 2649 was also originally thought, on the basis of its failure to react with any of the designated subgroups, to represent a novel spiroplasma serogroup. However, its characteristically short, narrow helices, its ability to grow to very high titres in media and its aggressive utilization of arginine suggested that it might nevertheless belong to serogroup VIII. And indeed, 'bridge strains' – ones that cross-react with each of two non-cross-reactive strains – were eventually found and strain BARC 2649 is now recognized – on the basis of its phenotypic properties and the close similarity of its 16S rDNA sequence to those of other serogroup VIII spiroplasmas – to represent an undescribed subgroup of serogroup VIII (Williamson *et al.*, 1998).

Genome size. At one time, genome size – like sterol requirement – was considered to be a highly conserved trait among mollicutes and was therefore regarded as a useful character at higher levels of taxonomy (Freundt & Razin, 1984). Earlier phylogenetic studies (Rogers *et al.*, 1985; Weisburg *et al.*, 1989; Maniloff, 1992) indicated that there had been several independent genome size reductions in mollicute evolution. The work of Pyle *et al.* (1988) and, later, of Neimark & Lange (1990) and Carle *et al.* (1995), showed that mollicute genomes varied widely in size and could apparently change over short periods of evolutionary time. In the genus *Spiroplasma*, genomes vary widely in size, as they do in other mollicutes, and can even vary within species (Robertson *et al.*, 1990). Genome-size change in *S. citri* has been observed in as few as 100 generations (Ye *et al.*, 1995, 1996). As noted, the large size of 2220 kbp for the *S. ixodetis* genome appears to represent a plesiomorphically large genome, from which size reductions may have occurred throughout the genus. Of course, we do not know the fraction of coding DNA in this species, so until the genome is sequenced, the question remains unanswered. In the large assemblage of spiroplasmas that exists in the arthropod/leaf surface habitat (largely in the Apis clade *sensu strictu*), genome size appears to be in the range of about 1100–1500 kbp. Relatively low genome sizes in the mirum (1300 kbp) and chrysopicola–syrphidicola–TAAS-1 clades (1170–1270 kbp) may simply reflect a normal complement of potential coding regions without the surplus baggage from plasmid, virus and/or repeat sequences. In contrast, members of the Citri–Poulsonii clade (serogroups I and II) have genome sizes that range from 1460 to 2040 kbp. In some species, extreme genome-size reduction has occurred, particularly in the Apis clade. In this clade, there appear to be autapomorphic reductions for *Spiroplasma* sp. TIUS-1 (840 kbp), *Spiroplasma monobiae* (940 kbp), *Spiroplasma* sp. W115 (960 kbp), *S. diminutum* (1080 kbp) and *S. leptinotarsae* (1085 kbp). As serogroup XXVIII (*S. platyhelix*) has not yet been sequenced, we cannot assess the phylogenetic significance of its genome size of 780 kbp, but it may represent yet another autapomorphic genome size reduction in *Spiroplasma*. Therefore,

genome size reduction in mollicutes, which was once thought to be a rare event (Rogers *et al.*, 1985), is, instead, frequent and distributed polyphyletically. In general, genome sizes in clades whose species have high similarity values (e.g. serogroup I, serogroup VIII and the apis–montanense clade) tend to provide evidence for the use of genome size in spiroplasma classification. Whereas it was common for sister species and members of small clades to have very similar genome sizes, the total intrageneric range of genome sizes in *Spiroplasma* is 760–2220 kbp (Carle *et al.*, 1995).

G+C content of spiroplasmal DNA. As with genome size, the degree to which G+C content of chromosomal DNA is informative depends on the evolutionary distances between or among the species to be compared. In all spiroplasma serogroups in which constituent members have been divided into subgroups, DNA G+C content, like genome size, is an important clade property. In general, DNA G+C content is more stable than genome size in this respect. For example, in the diminutum–CB-1–Ar 1357 clade, all three of the constituent members have DNA G+C contents of 26 mol%. In the chrysopicola–syrphidicola–TAAS-1 (serogroup VIII) clade, DNA G+C contents are 28–31 mol%. In the nine species or subgroups of serogroups I and II, the values are 26 mol%, with the exception of *S. insolitum*, which has a DNA G+C content of 28 mol%, an anomaly that led to the specific species epithet. Although the central tendency and the plesiomorphic state of DNA G+C content appear, on the basis of the DNA G+C content (26 mol%) of *S. ixodetis*, to be 25–27 mol%, values higher than this might be considered to be apomorphic. In the apis–montanense clade, the DNA G+C content is 28–30 mol%. In the TIUS-1 clade, the DNA G+C content is 28 mol% and in *Spiroplasma clarkii*, there is an autapomorphic value of 29 mol%. Throughout the spiroplasmas and their non-helical descendants, there is a strong suggestion that close association with a host may be linked to lowering of the DNA G+C content. This is suggested strongly by the dramatic reduction in DNA G+C content when the *Entomoplasmataceae* lineage diverged and one lineage became associated with ruminants. Finally, it is only fair, while advocating a role for DNA G+C content in spiroplasma taxonomy, to acknowledge that there are sister species whose DNA G+C contents are rather discrepant. For example, strains W115 and BIUS-1 have DNA G+C contents of 24 and 28 mol%, respectively; similarly, *Spiroplasma velocicrescens* and *Spiroplasma chinense* have values of 26 and 29 mol%, respectively. In summary, we believe that DNA G+C content, like genome size, provides valuable taxonomic information for species that are closely related. Of course, as the study of genomics proceeds, this information and DNA–DNA reassociation data will be replaced with whole-genome sequencing data (e.g. Persson, 2002).

Hosts. Many of the phenotypic and genotypic characteristics of the Citri–Poulsonii clade appear to be related directly to their close association with insect hosts and, in some cases, to the requirement for an obligate insect/plant phloem cycle. The tiny, arginine-consuming serogroup VIII spiroplasmas of the chrysopicola–syrphidicola–TAAS-1 clade coexist in their (largely) tabanid/plant surface cycles with a large assemblage of long spiroplasmas (members of the Apis clade *sensu strictu*), most of which do not catabolize arginine. These two types of tabanid spiroplasma appear to occupy different niches in their insect hosts. If this is true, requirements for optimization in these niches may have provided the selective forces that maintain these two very different phenotypic character suites. Also, as discussed in the following section, obligate affiliation with a particular host or biological cycle is usually associated with a decreased growth rate.

Sterol requirements. Unlike many other phenotypic and genotypic properties, sterol requirements in *Entomoplasmatales* and the ability to grow in 0·04 % PES are distributed polyphyletically. Rose *et al.* (1993) showed that *S. floricola* grew in serum-free medium with and without PES. This supplement, which was used at the time in the context of sterol requirements, turned out to be inhibitory to some mollicutes, including four of eight acholeplasmas studied. In the *Entomoplasmataceae*, the genera *Entomoplasma* and *Mesoplasma* are polyphyletic, as discussed below. Thus, unlike other phenotypic and genotypic characters, phylogeny did not predict or explain sterol requirements or the ability to grow in sterol-free media in the presence of PES in the *Entomoplasmataceae* or, for that matter, in the *Entomoplasmatales*.

Evolutionary considerations

Spiroplasma evolution. Earlier phylogenetic analyses (Woese *et al.*, 1980; Weisburg *et al.*, 1989; Maniloff, 1992; Gundersen *et al.*, 1994) and the cladograms herein indicate that the roots of *Mollicutes* lie in low-G+C, Gram-positive bacteria, including certain clostridial lineages, and *Erysipelothrix* (Maidak *et al.*, 2001). Evidence points strongly to polyphyly of *Mollicutes* in at least one respect: *Asteroleplasma* appears to be related more closely to certain Gram-positive bacteria than to any mollicute lineage. Monophyly of other mollicutes – specifically the Acholeplasma–Anaeroplasma–Phytoplasma and the Entomoplasmatales–PHUH clades – has not been established unequivocally. Woese *et al.* (1980) were non-committal on this point and little relevant supplemental information has been added since that time. Rather, the discovery of differential codon usage between the Acholeplasma–Anaeroplasma–Phytoplasma and Entomoplasmatales–PHUH clades (Renaudin *et al.*, 1986) has re-emphasized the evolutionary gulf between the two clades. Noting the admonition of Siddall & Whiting (1999) that hypotheses must be tested by additional data and not by other hypotheses, we believe that as large a sample as possible of Gram-positive bacteria must be assembled to provide an adequate test of this hypothesis. It is very easy for distantly related taxa to look

like sisters if no intermediate taxa are available or, if available, are not included in the analysis. Although the two lineages may have been polyphyletic, extinction of walled relatives whose divergences were located between those of acholeplasmas and mycoplasmas may now obscure the real relationships between them.

It is clear that the plesiomorphic morphology of the mollicutes involved walled cells. Recent work on the cytoskeletal elements of Gram-positive bacteria, such as *Bacillus* (Jones *et al.*, 2001), has given new insights into the evolution of *Mollicutes*. For example, *Bacillus* has a set of helical fibrils as part of its interior cytoskeletal structure (Jones *et al.*, 2001). It is likely that the *Spiroplasma–Mycoplasma* lineage possessed a substantial suite of cytoskeletal elements that conferred a (plesiomorphic) ability to form filaments, which may have been capable of some kind of movement. There appears to be continuity in the presence of actin throughout bacterial ancestral lineages (van den Ent *et al.*, 2001) and this material has been deduced to be present in some spiroplasmas (Williamson *et al.*, 1979b; Mouches *et al.*, 1982; Simoneau & Laberère, 1990). Some members of the Pneumoniae clade (e.g. *M. iowae*) have the capacity to form filaments in culture. To our knowledge, helicity has never been observed in the PHUH clade. However, it is possible that unknown ancestors of this lineage may have been helical, even after the *Spiroplasma–Mycoplasma* split. In *Spiroplasma*, helicity (albeit atypical) is present in the ixodetis clade and helicity, as a general property (Skripal, 1983), is synapomorphic for the genus. It is not known whether the unusual tightly coiled helicity of *S. ixodetis* cells is a plesiomorphic state, reflecting an early stage in the development of helices. However, Trachtenberg & Gilad (2001) (S. Trachtenberg, personal communication) suggest that the major evolutionary step is the development of helicity itself and that variations, such as tightness of coiling, might be caused relatively easily without substantial genotypic change. As noted, the status of some hypothetically related, non-culturable organisms that are associated with various sex-ratio conditions in insects is unclear, as none of the organisms have in fact been envisioned. It is possible that other mollicutes of the spiroplasma lineage that are even more plesiomorphic than *S. ixodetis* may be discovered.

At least by the time of the divergence leading to the Mirum–Citri–Chrysopicola clade, helicity and motility, as they exist in a great majority of spiroplasmas species, had become firmly established. The only described species in the ixodetis clade is *S. ixodetis* and the large evolutionary distance between this organism and more apomorphic *Spiroplasma* species makes it difficult to determine evolutionary directionality of phenotypic and genotypic properties at the time of its divergence. Both the Citri–Poulsonii clade and the chrysopicola–syrphidicola–TAAS-1 clade have apomorphic traits. In serogroup I spiroplasmas, the large amounts of DNA that occur as repeat sequences and integrated viral and plasmid DNA apparently inflate the genome size greatly, without adding to the organism's coding capacity. If the basal position of the phloem-inhabiting strain LB-12 in the Citri–Poulsonii clade reflects an ancestral habitat, the flower-surface habitat of *S. insolitum* (subgroup I-6) would represent an apomorphic return to the insect gut/flower surface habitat. The apomorphic nature of such a shift is in accord with the growth rate of *S. insolitum*, which is significantly slower than that of other plant surface-dwelling mollicutes. This species is not suited as well to existence on plant surfaces as those species in the Apis clade *sensu strictu* that appeared to have evolved and speciated in association with that strategy. The fact that the DNA G+C content that accompanied this shift increased significantly is intriguing.

A more or less modal helical morphology was maintained in members of serogroups I and II, but was modified substantially in serogroup VIII by cell-size reduction, as described above. With morphological innovation in serogroup VIII came other modifications. The DNA G+C content of organisms in serogroup VIII is substantially higher than that of serogroup I organisms and the genome size is considerably smaller. Serogroup VIII appears to be radiating rapidly in dipterous insects – particularly horse flies – and is distributed worldwide. In contrast, members of the Citri–Poulsonii clade are associated with specific insect or insect/plant habitats.

Subsequent to the divergence that led to the Citri–Chrysopicola–Mirum clade, a major divergence occurred that led to the Apis clade *sensu latu*, which consists of the original Apis clade *sensu strictu* of Weisburg *et al.* (1989), but which now, as a result of the data herein, contains five novel species. These five species are, on the one hand, basal to the Apis clade *sensu latu*, and to the Entomoplasmataceae clade on the other. These basal members of the Apis clade *sensu latu* include *Spiroplasma* sp. TIUS-1, an organism with extremely poor helicity, *S. alleghenense*, another poorly helical organism, the uniquely funnel-shaped *S. leptinotarsae* and yet another organism with flawed helicity, *S. lampyridicola*, which is associated with firefly beetles. The fifth basal species is *S. sabaudiense*, the helical morphology of which is unremarkable.

The Apis clade *sensu strictu* contains many spiroplasmas that are acquired by their hosts from plant surfaces and then reside in the insect gut. At first inspection, the presence of this serogroup as a (sister) terminus in mollicute evolution is surprising, as Clark (1984) had proposed that the leaf surface/insect gut cycle is a primitive cycle from which other more complex maintenance cycles had evolved. Why, if we are to assume the existence of a modal tendency toward simple helicity and a simple insect gut/leaf surface existence, should the Apis clade *sensu strictu* be a terminus, rather than a basal clade? There is a simple hypothesis by which this conundrum can be explained. The hypothesis rests on the assumption, mentioned above, that host association confers a greater longevity on genotypes with close host associations than that experienced by genotypes in simple maintenance

cycles. Once the accrual of large amounts of non-coding DNA in serogroup I and cell-size reduction in serogroup VIII are seen to be apomorphic, and once it is recognized that the tight cellular coiling of *S. ixodetis* could be a simple modification of basic helicity (and therefore also apomorphic), all apparent 'plesiomorphic' states, 'from which simple helices have been derived', vanish. There remains, then, only the simple helicity of *S. mirum*, whose morphology, DNA G+C content and genome size are very similar to those of the majority of members of the Apis clade *sensu strictu*.

There are several modifications within the Apis clade *sensu strictu* that indicate that evolutionary exploration continues in *Spiroplasma*. There is at least one specialist, *S. clarkii*, that is associated with a particular insect host (*Cotinus nitida*, the green june beetle). One of the clades in the Apis clade *sensu latu*, the BIUS-1–Floricola–Diabroticae–Monobiae clade, which was often, but not always, a feature of our trees, appears to be associated with insect/flower cycles, if we assume that fast-growing spiroplasma species isolated from insects that visit flowers regularly are in fact transmitted from insect to insect on flower surfaces. By the same token, spiroplasma species associated with tabanid flies – and presumptively passed (Wedincamp *et al.*, 1996) horizontally from fly to fly, at least during the summer season, at common feeding sites [perhaps honeydew (Schutz & Gaugler, 1989; Janzen & Hunter, 1998)] – also cluster, but in different clades than those that contain flower-dwellers.

The question of whether spiroplasmas that are associated with tabanids are monophyletic remains unanswered. The phylogenetic reconstructions presented here do not resolve this question. In a dynamically evolving matrix of micro-organisms that pass from plant surfaces to insect guts and vice versa, it would be expected that numerous exploratory host transfers would occur, most of which would fail. However, if one should succeed, there may be nothing to prevent that lineage from returning to its plesiomorphic host over a short period of time. Futuyma (1995) has discussed this type of reversion in terms of insect host specificities in plants. Suppose a 'monophyletic clade of tabanid specialists' transferred to another type of maintenance cycle over a brief period of evolutionary time. If there were a divergence that resulted in a secondary lineage that returned to the tabanid cycle, whether or not tabanid spiroplasmas were perceived to be monophyletic would depend on whether the intermediate lineage became extinct or, if it survived, whether it would be discovered. Since the genotypic extinction rates in *Mollicutes* (or any microbial taxon) are enormous, almost all 'monophyletic' groups must in reality be at least paraphyletic. Examined in this light, the question of monophyly of the tabanid spiroplasmas is seen to be (i) indeterminable and (ii) effectively irrelevant.

Spiroplasmas that are associated with mosquitoes occur in several clades within the Apis clade *sensu latu*. Some are associated with spiroplasmas that are transmitted on flower surfaces. This is not surprising, as a study of mosquito spiroplasmas (Chastel & Humphery-Smith, 1991) assumed that these organisms are transmitted from mosquito to mosquito when the insects feed on flower surfaces. It is remarkable that no tabanid spiroplasma has ever been isolated from a mosquito, or vice versa. It is also remarkable that the tabanid spiroplasma clades do not contain any mosquito spiroplasmas. The small clade that consists of the sister species *S. apis* and *S. montanense* is distinguishable from other clades of the Apis clade *sensu strictu* in several respects. *S. apis* has been recovered from tabanids, honeybees and a number of insect species, and from flower surfaces. Thus it is more of a generalist than other spiroplasmas that are isolated from tabanids. *S. montanense* occurs in the northern United States and Canada and is often isolated from *Hybomitra* horse flies. Recent attention has been focused on the biogeography of micro-organisms (Staley & Gosink, 1999). Thus, it is of interest to note that this species is one of several tabanid spiroplasmas that appear to have a clear biogeography (Whitcomb *et al.*, 1990).

Scenario for divergence of the mycoides group from *Spiroplasma*. In general, the older a lineage, the more opportunity it has had to radiate into other hosts and/or habitats. Mycoplasmas of the PHUH clade are found in many vertebrate and invertebrate animals, including tortoises (Brown *et al.*, 1995), alligators (Brown *et al.*, 2001), seals (Heldtander Königsson, 2001) and crocodiles (Kirchhoff *et al.*, 1997), as well as primates [including man (Somerson & Cole, 1979)] and the usual ruminant animals. As the search among non-vertebrate hosts has been peremptory at best, it is reasonable to presume that *Mycoplasma* species of the Hominis and Pneumoniae clades are spread widely throughout the animal kingdom. In contrast, the *M. mycoides* clade occurs only in ruminants and the evolutionary distances among the species are small. All trees, including those developed in the sophisticated analyses of K.-E. Johansson's group [reviewed by Johansson & Pettersson (2002)] suggest that entomoplasmas (and/or mesoplasmas) arose first and that the Mycoides clade arose from an entomoplasmal ancestor. When this is considered, studies that suggest a possible interaction with fleas of bovine animals (DaMassa *et al.*, 1994) can be seen in a new light. Ruminants first arose, according to fossil evidence, only in the mid-Eocene, perhaps 40 million years ago. If the Mycoides clade has radiated only in ruminants, it must therefore be a taxon that has evolved relatively recently. On the other hand, *Mycoplasma* species of the Hominis and Pneumoniae clades may have arisen several hundred million years ago.

Just as the Mycoides clade arose from an ancestral root in the *Entomoplasmataceae*, so species of the *Entomoplasmataceae* themselves appear to have arisen from ancestors that are shared with the Apis clade *sensu latu*. In the basal divergences of the Apis clade *sensu latu*, two clades of spiroplasmas – the lampyridicola–leptinotarsae clade and the alleghenense–sabaudiense–TIUS-1 clade – show

especially labile morphology. This contrasts with the more stable morphology in members of the Apis clade *sensu strictu*, whose cells generally retain helicity and motility throughout cultural manipulations. In particular, serogroup XXVIII strain TIUS-1, whilst exhibiting occasional helical forms, is seen mostly in culture as a non-helical mollicute – one that, without an isolation history that includes extensive microscopic observations, could easily be mistaken for a member of the *Entomoplasmaceae*. The deterioration of helicity in species such as TIUS-1, which are related more closely to the Mycoides–Entomoplasmataceae clade than most other spiroplasmas, suggests a scenario for the evolution of this clade of non-helical entomoplasmas. Thus, the concept of a relatively recent origin of the Mycoides clade from an entomoplasmal ancestor is entirely consistent with existing data and evolutionary constraints.

Phylogenetic and Linnaean classification of *Mollicutes*

Phylogenetic versus Linnaean classifications. The phylogenetic classification outlined, in which nested sets (clades) of organisms that are likely to have had common ancestors are identified, contrasts sharply with conventional Linnaean classifications, in which species, genera, families and orders are delineated. In these classifications, whatever the clade structure of the dataset, existing biodiversity is made to fit into a predetermined template with a fixed number of hierarchical levels. The advantage of this kind of classification is its sharp, although possibly artificial, boundaries. When organisms are of practical importance (as *M. mycoides* certainly is), the binomial system offers a means for establishment of identification and quarantine procedures and the scientific community outside the walls of systematics laboratories is generally able to count on the classification being relatively stable.

On the other hand, phylogenetic classifications are unconstrained by templates. Given the discrepancy in taxic numbers and/or sampling among clades in many phylogenetic reconstructions, it may be awkward to shoehorn the data into a Linnaean structure. Also, even if the dataset of taxa and characters remains stable, phylogenetic classifications may change, depending on the outcome of arguments among systematists. Furthermore, in the era of molecular systematics, as new technologies are introduced and new characters become available, it is inevitable, no matter what technique is replacing what, that the new phylogenetic classification based on an enlarged set of characters will differ from the previous versions and if new taxa are added, the fundamental clade structure will be altered. For example, species that are perceived at first to be sister species may turn out to be separated by a considerable evolutionary distance, in comparison to newly discovered species that are related more closely to either one of them.

When changes in phylogenetic reconstructions occur, it is very common for molecular systematists to demand immediately that binomial nomenclature should be revised to accommodate the latest phylogenetic groupings. It is insufficiently appreciated that all phylogenetic analyses are only hypotheses and should be tested before they can be implemented properly. Kluge (1997) argues forcefully that the best test of a hypothesis is to make every attempt to falsify it, rather than to verify it. What is the best way to test a hypothesis in microbial taxonomy? Eukaryote systematists, often stuck with a dataset that is difficult or impossible to enlarge, tend to become embroiled in controversy over which algorithms should be used for analysis of the same dataset. Microbiologists, who have much less trouble expanding the scope of their taxic sampling, are more apt to be attracted to the admonition of Siddall & Whiting (1999) that – as noted earlier – hypotheses cannot be tested by formulating hypotheses and that they can be tested only by additional data (specifically, more characters and/or more taxa).

Phylogenetic classification of *Entomoplasmatales* and Linnaean taxonomy of the genus *Mycoplasma*. In the case of *Mollicutes*, the initial deep-branching patterns observed by Woese *et al.* (1980), Weisburg *et al.* (1989), Maniloff (1992), Johansson & Pettersson (2002; many trees summarized), Maidak *et al.* (2001) and in this paper are mutually supportive. With two exceptions, species in *Entomoplasmatales* and the genus *Mycoplasma* are classified in Linnaean nomenclature in a way that is consistent with phylogeny.

The *Mesoplasma* problem. The simplest anomaly (from the standpoint of available resolution) in present *Mollicutes* classification is that phylogenetic classifications show that the genera *Entomoplasma* and *Mesoplasma* are polyphyletic. Actually, at the time of the designation of *Entomoplasmataceae*, the authors were aware that the trees of Weisburg *et al.* (1989) showed that *Mesoplasma* and *Entomoplasma* might be related rather closely. It was, nonetheless, logical for both genera to be proposed. To understand why both these genera were designated, it is important to realize that the results of Weisburg *et al.* (1989) were based on distance analyses, which have been criticized by many systematists. At that time, neighbour-joining (Saitou & Nei, 1987) was beginning to be employed by microbiologists, but it was only recently that maximum-likelihood (Felsenstein, 1981; Saitou & Imanishi, 1989) and maximum-parsimony (Swofford, 1983; Gundersen *et al.*, 1994) have begun to see widespread use in microbial taxonomy. On these grounds alone, it would have been – retrospectively – premature to have placed all the entomoplasmas and mesoplasmas into a single genus. In 1993, it seemed to be a giant step to remove the species that are now considered to be mesoplasmas from *Acholeplasma*, and species that are now considered to be entomoplasmas from *Mycoplasma*, where they had been respectively placed. To have taken not only this large a step, but, in addition, to have combined the

two taxa into a single novel genus on the basis of limited phylogenetic information, was contraindicated.

A second and even more important factor was involved in the decision to propose two genera within the *Entomoplasmataceae*. It has only been in the past decade that it became clear that sterol requirement (or non-requirement) is not a profound and significant character at higher taxonomic levels, as had been thought, but is instead a character that may vary among species in a single genus. It was the extensive studies of Rose *et al.* (1993), performed soon after the 1993 revision of *Mollicutes*, that revealed the difficulties with taxonomic tests concerning the sterol requirement in *Mollicutes*. These authors found that the genus *Spiroplasma* contained both sterol-requiring and -non-requiring species. Also, although Rose *et al.* (1993) studied the ability of mollicutes to grow in PES-supplemented medium, a definitive tie to sterol metabolism could not be demonstrated. This continues to be the case, so the basis for the test remains uncertain.

Today, with accumulated evidence from many different methods of phylogenetic analysis and with additional sequences now available, the original suggestion of polyphyly has been confirmed repeatedly. It now seems anomalous to retain both genera and, indeed, there have already been calls for them to be combined into a single genus – presumably *Entomoplasma* (Johansson & Pettersson, 2002).

Polyphyly of *Mycoplasma*. There is no 'quick fix' for the second anomaly in the classification of *Mollicutes*. It is now clear that the type species of the genus *Mycoplasma* and a small group of related mycoplasmal inhabitants of ruminants are derived not from an ancestor that is shared with the >100 *Mycoplasma* species of the Hominis and Pneumoniae clades, but instead from an ancestor in the *Entomoplasmataceae*. Species of *Entomoplasmataceae*, in turn, have an ancestral root in the genus *Spiroplasma*. Thus, the genus *Mycoplasma*, as currently defined (Freundt & Razin, 1984), is polyphyletic.

If the mycoplasmas were simply environmental isolates with no practical baggage, the problem could be remedied easily; the genus name would be retained for *M. mycoides* and its allies and a novel genus name would be proposed for the remaining mycoplasmas. However, species of the PHUH clade are associated with >50 disease conditions (Freundt & Razin, 1984; J. G. Tully, personal communication) and thousands of papers have been published under the existing names. If one were to change the genus name of one of the two *Mycoplasma* clades, it would be easier to change the genus name of members of the Mycoides clade (Johansson & Pettersson, 2002). However, not only are these organisms, like members of the PHUH clade, associated with a very large body of literature, but the name *M. mycoides* is associated with practical identifications and quarantines of important disease agents. More importantly, *M. mycoides* is the type species of *Mycoplasma*, *Mycoplasmataceae* and *Mycoplasmatales*, so any name-change would have to be approved by the Judicial Commission.

It is our view that, in the case of the polyphyly of the genus *Mycoplasma*, it is sensible for the phylogenetic and Linnaean characterization to disagree on matters that concern genus names for members of the two clades of *Mycoplasma* species. It seems pointless to perform, at great expense to veterinary science and mycoplasmology, a minor fix on a Linnaean classification that contains myriads of other major discrepancies between Linnaean and phylogenetic classifications.

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