Members of the class *Mollicutes* exhibit differing life styles, including: obligate parasitism of vertebrate animals and humans; a saprophytic, free-living existence; obligately anaerobic parasitism of animals; the parasitism of insects and other arthropods and an obligately parasitic life style, which alternates between insect vectors and plant hosts. Phytoplasmas, which are characterized by living a transkingdom parasitic life and possessing small, AT-rich genomes, are non-helical members of the class, and are responsible for diseases in numerous plant species (Lee et al., 2000). The available genomic data indicates that phytoplasmas have limited metabolic capacities and, consequently, obligate host dependency. Despite numerous efforts, the isolation and axenic cultivation of phytoplasmas remains a serious challenge. While over recent decades several reports have claimed success, such claims have not been substantiated by independent confirmation. Therefore, for phytoplasmas, many of the phenotypic characteristics required for polyphasic species delineation are difficult to assess directly. According to the convention established for recording properties of uncultured organisms (Murray & Schleifer, 1994; Murray & Stackebrandt, 1995), a provisional genus *Candidatus Phytoplasma* was erected to accommodate phytoplasmas (Firrao, 2004). To date, 37 species of the provisional genus *Ca. Phytoplasma* have been described in the class *Mollicutes* due to difficulties in establishing axenic culture, phytoplasmas were assigned to a provisional genus, *Candidatus Phytoplasma*, and the genus was embraced within the order *Acholeplasmatales*. However, phytoplasmas differ significantly from species of the genus *Acholeplasma* and all other taxa in the class *Mollicutes*.

Since such distinguishing properties of phytoplasmas are not referable to the descriptions of the order *Acholeplasmatales* and of all other existing orders, namely *Mycoplasmales, Entomoplasmales* and *Anaeroplasmatales*, this communication raises the question of whether *Candidatus Phytoplasma* should be retained in the order *Acholeplasmatales* or whether a novel provisional order and family should be created to accommodate the genus *Ca. Phytoplasma*.

Phytoplasmas are a diverse but phylogenetically coherent group of cell-wall-less bacteria affiliated with the class *Mollicutes*. Due to difficulties in establishing axenic culture, phytoplasmas were assigned to a provisional genus, *Candidatus Phytoplasma*, and the genus was embraced within the order *Acholeplasmatales*. However, phytoplasmas differ significantly from species of the genus *Acholeplasma* in their habitat specificities, modes of life, metabolic capabilities, genomic architectures, and phylogenetic positions. This communication describes the unique ecological, nutritional, biochemical, genomic and phylogenetic properties that distinguish phytoplasmas from species of the genus *Acholeplasma* and all other taxa in the class *Mollicutes*. Since such distinguishing properties of phytoplasmas are not referable to the descriptions of the order *Acholeplasmatales* and of all other existing orders, namely *Mycoplasmales, Entomoplasmales* and *Anaeroplasmatales*, this communication raises the question of whether *Candidatus Phytoplasma* should be retained in the order *Acholeplasmatales* or whether a novel provisional order and family should be created to accommodate the genus *Ca. Phytoplasma*.

Abbreviations: APSD, average pairwise sequence distance; PTS, phosphotransferase system; SVM, sequence variable mosaics.

One supplementary table is available with the online Supplementary Material.

International Journal of Systematic and Evolutionary Microbiology (formerly International Journal of Systematic Bacteriology; Harrison et al., 2014), and an additional 12 have been suggested on the basis of their distinct 16S rRNA gene sequences (Wei et al., 2007). The provisional genus is currently placed under an *insertae sedis* within the order *Acholeplasmatales* (Krieg et al., 2010), but the analysis provided in the present communication questions the validity of this taxonomic placement.

Although phytoplasmas likely descended from a Gram-positive, low DNA G+C content, walled bacterium (Gundersen et al., 1994) and are most closely related to species of the genus *Acholeplasma* among the class *Mollicutes* (Davis et al., 1997), the ecological, nutritional, biochemical, physiological, genomic architectural and phylogenetic properties highlighted below clearly distinguish phytoplasmas from acholeplasmas and all other taxa in each of the four existing orders of the class *Mollicutes*. With such distinguishing properties, extant phytoplasmas are not referable to the precise descriptions of the order *Acholeplasmatales* (Edward and Freundt, 1970; Freundt et al., 1984) or to any other existing order and family of the class *Mollicutes*.

**Habitat specificity**

Previous work has established the existence of four orders within the class *Mollicutes* (Krieg et al., 2010). Whilst not being expressed explicitly, microbial habitat is a *de facto* property separating the four orders established previously (Razin, 1992), viz., *Mycoplasmales* (Freundt, 1955; Edward...
and from obligately anaerobic bovine/ovine luminal anaeroplasmas and asteroplasmas (Anaeroplasmatales). Habitat clearly obviates inclusion of phytoplasmas within any of these three orders.

The order Entomoplasmatales accommodates two arthropod-associated families, Entomoplasmataceae and Spiroplasmataceae. Members of the family Entomoplasmataceae inhabit insects exclusively. However, most of the species in the family Spiroplasmataceae inhabit crustaceans or insects, and do not infect plants, except for three species (Spiroplasma kunkelii, Spiroplasma citri and Spiroplasma phoenicium), which are insect-transmitted plant pathogens. While these three species of the genus Spiroplasma share the plant phloem–insect vector habitat with phytoplasmas, they are distinctly different from phytoplasmas in their morphology (Davis et al., 1972), phylogenetic position (Zhao et al., 2005; Gasparich, 2010), as well as in their physiological and genomic properties, as presented in the

Table 1. Taxonomy and properties of the class Mollicutes

Updated and modified from Razin (1992) and Krieg et al. (2010), as phytoplasmas possess distinctive properties that are not referable to the descriptions of any of the four previously established orders. This communication is part of a discussion about whether a novel order and family should be erected to accommodate the genus ‘Ca. Phytoplasma’.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Habitat</th>
<th>Sterol requirement</th>
<th>Distinctive properties*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order I: Mycoplasmatales</td>
<td>Parasites of humans and animals</td>
<td>+</td>
<td>Surface parasites</td>
</tr>
<tr>
<td>Family I: Mycoplasmataceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus I: Mycoplasma</td>
<td></td>
<td></td>
<td>Urease-negative</td>
</tr>
<tr>
<td>Genus II: Ureaplasma</td>
<td></td>
<td></td>
<td>Urease-positive</td>
</tr>
<tr>
<td>Order II: Entomoplasmatales</td>
<td>Inhabitants of arthropod gut, a few species also infect plants</td>
<td>+</td>
<td>Non-motile, non-helical, do not infect plants</td>
</tr>
<tr>
<td>Family I: Entomoplasmataceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus I: Entomoplasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus II: Mesoplasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family II: Spiroplasmataceae</td>
<td></td>
<td></td>
<td>Motile, helical filaments, three species are known to infect both plants and insects</td>
</tr>
<tr>
<td>Genus I: Spiroplasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Order III: Acholeplasmatales</td>
<td>Saprophytic, free-living</td>
<td>−</td>
<td>No parasitic life stage</td>
</tr>
<tr>
<td>Family I: Acholeplasmataceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus I: Acholeplasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Order IV: Anaeroplasmatales</td>
<td>Parasites in bovine and ovine rumen</td>
<td>±</td>
<td>Obligately anaerobic</td>
</tr>
<tr>
<td>Family I: Anaeroplasmataceae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus I: Anaeroplasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genus II: Asteroleplasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Order V: ‘Candidatus Phytoplasmatales’?</td>
<td>Obligate parasites of plants and phloem-feeding insects</td>
<td>+</td>
<td>Lack PTS &amp; redox self-regulating capability</td>
</tr>
<tr>
<td>Family I: ‘Candidatus Phytoplasmataceae’?</td>
<td></td>
<td></td>
<td>Possess SVM genome architecture</td>
</tr>
<tr>
<td>Genus I: ‘Candidatus Phytoplasma’</td>
<td></td>
<td></td>
<td>Transkingdom parasites</td>
</tr>
</tbody>
</table>

*See the in-text citations with respect to the distinctive properties referred to in the table.
following sections. Phytoplasmas cannot be appropriately accommodated within the family Spiroplasmataceae, as a helical morphology is a distinguishing feature of this family (Skripal, 1983).

**Distinguishing nutritional, biochemical, physiological and architectural genomic characteristics inferred from genomic data**

Since a habitat dictates the life style of a given mollicute, the nutritional requirements and other physiological properties of the mollicute are largely determined by habitat; such properties can be inferred from genomics and other -omics data. As described below, fundamental distinctions in metabolic pathways and physiological processes set phytoplasmas apart from other mollicutes. Some characteristics are so strikingly different that they are well beyond the criteria that have been used to separate previously recognized orders in the class Mollicutes.

**Sterol requirement.** In the history of mollicutes systematics, requirement vs non-requirement for sterol in axenic culture media has been an important criterion used to distinguish between taxonomic categories within the class Mollicutes. While this criterion of exogenous sterol-dependence was initially used to differentiate taxa at the generic level (Acholeplasma vs Mycoplasma; Edward & Freundt, 1970), it eventually became a determining factor for separating mollicutes at the ordinal level (Acholeplasmatales vs Mycoplasmatales and Entomoplasmas; Freundt et al., 1984; Tully et al., 1993). As implicated by its name, the order Acholeplasmatales encompasses only species that can grow in sterol-free media. Ordinal assignment of the family Anaeroplasmataceae was once a dilemma as it contained both a sterol-requiring genus, Anaeroplasma, and a sterol-nonrequiring genus, Asterolesplasma; consequently, a new order, Anaeroplasmatales, had to be erected to accommodate the family (Robinson & Freundt, 1987). Biochemical, genomic and proteomic studies have revealed that growth of acholeplasmas in sterol-free media is due to the presence in the acholeplasma genomes of a comprehensive set of genes encoding enzymes for the biosynthesis of fatty acids, isoprenoids, and carotenoids (Lazarev et al., 2011; Kube et al., 2014). In contrast, none of the five completely sequenced phytoplasma genomes possesses anabolic pathway genes leading to fatty acid, isoprenoid or carotenoid formation (Oshima et al., 2004; Bai et al., 2006; Kube et al., 2008; Tran-Nguyen et al., 2008; Andersen et al., 2013). Based on genomic data, sterol would be required in media to achieve the axenic culture of phytoplasmas; such a requirement for growth would exclude phytoplasmas from the order Acholeplasmatales.

**Transport and metabolism of sugar molecules.** Genes encoding components of the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) and hexokinase are absent from the genomes of completely sequenced phytoplasmas (Oshima et al.; 2004; Bai et al., 2006; Kube et al., 2008; Tran-Nguyen et al., 2008; Andersen et al., 2013). In other bacteria, PTS utilizes energy stored in phosphoenolpyruvate, powering cross-membrane transport of glucose, mannose, fructose and other sugar molecules; the PTS system is essential to the minimal genome of a free-living bacterium (Glass et al. 2006). Hexokinase catalyses the phosphorylation of hexoses, mainly glucose, yielding glucose 6-phosphate; the latter is the entry point into glycolysis and other glucose utilization pathways (Nelson & Cox, 2008). The evident lack of both PTS and hexokinase in phytoplasmas indicates that other form(s) of sugar or carbohydrate have to be imported, converted and metabolized by phytoplasma cells (Kube et al., 2012). However, phytoplasma genomes possess genes encoding malate/citrate symporters and enzymes that convert malate to pyruvate, highlighting the likelihood that malate is a carbon source for phytoplasmas (Kube et al., 2012). In sharp contrast, the genomes of all the mollicutes studied, including acholeplasmas, mycoplasmas, ureaplasmas, spiroplasmas and mesoplasmas, have genes encoding PTS components and hexokinases, but do not have genes encoding malate/citrate symporters (http://services.cbib.u-bordeaux2.fr/molligen/). Such distinctions relating to the presence of genes associated with specific sugar transporters and metabolic pathways clearly sets phytoplasmas apart from acholeplasmas and other mollicutes.

**Maintenance of redox homeostasis.** The genomes of species of the genus ‘Ca. Phytoplasma’ lack a suite of genes encoding enzymes essential for regulating cellular redox potentials (Zhao et al., 2014). Nicotinamide adenine dinucleotide (NAD+/NADH) is a coenzyme pair that exists in all living cells studied to date. By transferring electrons from one metabolite to another, the coenzyme participates in numerous metabolic redox reactions (Belenky et al. 2007; Pollak et al. 2007). Additionally, some NAD+ is converted to nicotinamide adenine dinucleotide phosphate (NADP+), another ubiquitous coenzyme performing electron-transfer functions in living cells. The ratios of NAD+ / NADH and NADP+ /NADPH are vital factors in maintaining cellular redox homeostasis (Schafer & Buettner, 2001). NH3/glutamine-dependent NAD+ synthetase, nicotinate nucleotide adenyltransferase, and nicotinate phosphoribosyltransferase are among key enzymes required for the biosynthesis of NAD+ (Olland et al., 2002; Magni et al. 2009; Bi et al. 2011) and inorganic polyphosphate/ATP-NAD kinase is involved in the conversion of NAD+ to NADP+ and in the balance of NADH/NAD+ and NADPH/NADP+ ratios (Kawai & Murata, 2008). However, the genomes of phytoplasmas lack genes encoding these important proteins. Thioredoxin is a ubiquitous redox protein supposedly present in all eukaryotic and prokaryotic cells (Buchanan et al., 2012). It also exists in two forms: an oxidized form (Trx-S2) and a reduced form [Trx(SH)2]. Trx-S2 contains a disulfide bridge that can be reduced by NADPH with thioredoxin reductase; Trx(SH)2 is a powerful hydrogen/electron donor that reduces disulfide in various substrate proteins. In addition to its function in nucleotide metabolism and DNA synthesis, thioredoxin plays an
important role in redox signalling, cell defence against oxidative stress and in the maintenance of redox homeostasis (Hanschmann et al., 2013). Each of the five completely sequenced phytoplasmas has a gene encoding thioredoxin (trxA); however, none of them has a gene encoding thioredoxin reductase (trxB). Since both thioredoxin reductase and NADPH are required to maintain the reducing potential of thioredoxin, the lack of both a thioredoxin reductase gene and an ATP-NAD kinase gene (and therefore NADPH) in the genomes of phytoplasmas raises the question of how oxidized thioredoxin is reduced in phytoplasma cells and, ultimately, how redox homeostasis is achieved in phytoplasma cells. Compounding the situation further, phytoplasma genomes also lack genes encoding glutaredoxin and glutathione reductase. Zhao et al. (2014) hypothesized that oxidized thioredoxin molecules in phytoplasma cells may be exported to host cells, where they are reduced by host thioredoxin reductase and NADPH, and ‘recharged’ thioredoxin molecules are then shuttled back into phytoplasma cells. No other mollicutes exhibit the genetic predisposition to be unable to self-regulate cellular redox homeostasis.

**Distinctive genomic architecture**

Phytoplasmas possess a genome architecture that is unique among all members of the class *Mollicutes*. While mobile genetic elements have significantly influenced biological evolution across all studied organisms, perhaps nowhere is this phenomenon more remarkable than in the case of phytoplasma emergence and evolution (Jomantiene & Davis, 2006; Jomantiene et al., 2007; Wei et al., 2008). Unlike the compact genomes of other mollicutes, phytoplasma genomes are unique in possessing densely clustered repetitive sequences termed sequence variable mosaics (SVM; Jomantiene & Davis, 2006). These SVMs were likely formed by recurrent and targeted attacks by ancient phages at the root of phytoplasma evolution (Wei et al., 2008). Repeated sequences in phytoplasma genomes also include insertion-like sequences (Lee et al., 2005) and putative mobile units (Bai et al., 2006). It has been hypothesized that the phytoplasma genomes were evolved through two mutually complementary genome-sculpting mechanisms: gene loss and horizontal acquisition (Zhao et al., 2014). Thus, the evolutionary shrinkage of phytoplasma genomes...
Fig. 2. Phylogenetic relationships between phytoplasmas. The phytoplasma subtree has been expanded from the condensed phylogenetic tree shown in Fig. 1. The subtree topology indicates that the phytoplasma clade is divided into three distinct subclades. The phytoplasma taxa included in the phylogenetic analysis are reference strains of 37 species described as ‘Ca. Phytoplasma’, four incidentally cited species (marked with an asterisk *‘), and eight potentially new, but yet to be described species (marked with two asterisks **‘). Bar, number of nucleotide substitutions per site. Numbers at branch nodes indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test. Taxon names are labelled as follows: for formally described and incidentally cited ‘Ca. Phytoplasma’ species, only the specific epithet is given; BVGYP, Buckland valley grapevine yellows phytoplasma; CbYP, chinaberry yellows phytoplasma; DP, derbid phytoplasma; MPVP, Mexican periwinkle virescence phytoplasma; SBSP, sorghum bunchy shoot phytoplasma; SP-D3T1, sugar cane phytoplasma D3T1; SP-D3T2, sugar cane phytoplasma D3T2; WTWBP, weeping tea witches-broom phytoplasma.
was partially countered by repeated and targeted chromosomal integration of phage genomes, and by further gene acquisition through targeted insertion of mobile gene cassette-like elements (Jomantiene et al., 2007; Wei et al., 2008). While the loss of genes encoding diverse metabolic pathways led to increased host dependence, new capabilities were acquired that enabled and enhanced phytoplasma-host interactions (Zhao et al., 2014). No SVM-like structures have been found in any other prokaryotic genomes including those of walled and wall-less bacteria.

**Phylogenetic separation of phytoplasmas from other mollicutes**

To date, the evolutionarily conserved 16S rRNA gene remains the most powerful phylogenetic marker for taxonomic resolution of prokaryotes, and the 16S rRNA gene-derived phylogeny continues to serve as the backbone of prokaryotic systematics (Ludwig & Klenk, 2010). For this reason, the polyphasic delineation of bacterial taxa is often guided by the results of comparative analysis of 16S rRNA gene sequences (Stackebrandt, 2009). In the 1990s, based on comparative and phylogenetic analyses of the then available 16S rRNA gene sequences of known phytoplasma strains, a consensus was reached among researchers that phytoplasmas comprise a phylogenetically coherent group that is distinct from other prokaryotes including mycoplasmas and acholplasmas (Gundersen et al., 1994). As more and more phytoplasmas were discovered over the past two decades, several updated analyses were conducted to include sequences of newly identified strains of species of the provisional genus ‘Ca. Phytoplasma’; it was concluded that the monophyletic status of phytoplasmas remained concrete with strong support from high bootstrap values. Fig. 1 presents a condensed 16S rRNA gene tree that encompasses all species of the genus ‘Ca. Phytoplasma’ and other species of the class Mollicutes. In the tree, phytoplasmas form a monophyletic clade, which is discrete from all other mollicutes, with the acholplasma clade and the anaeroplasma clade being the closest neighbouring clades. The separation between the phytoplasma clade and the acholplasma clade is as compelling as the separation between the anaeroplasma clade and the acholplasma clade.

In addition to phylogenetic relationships, the average pairwise sequence distance (APSD) scores also support the separation of the phytoplasma clade from the acholplasma clade and the anaeroplasma clade: the APSD score between the species in the phytoplasma clade and the species in the acholplasma clade is 14.94 ± 1.63 %, and the APSD score between the species in the phytoplasma clade and the anaeroplasma clade is 17.97 ± 0.99 %. Such APSD scores are comparable to the APSD score between the anaeroplasma clade and the acholplasma clade, 15.43 ± 1.00 %. The pairwise sequence distance scores were calculated in the present study using Lasergene sequence analysis software (DNASTAR).

Recently, Martini et al. (2014) suggested that ‘Ca. Phytoplasma’ should be tentatively classified in the family Acholeplasmataceae for two reasons: (i) a persistent inability to grow the phytoplasmas in vitro hinders the identification of their distinctive phenotypic traits, and (ii) the two genera Acholeplasma and ‘Ca. Phytoplasma’ are phylogenetically related and form a distinct clade within the class Mollicutes. Our reasoning differs from this assessment: firstly, various unique properties distinguishing phytoplasmas and acholplasmas are accessible through readily observable phenotypic characteristics, such as natural habitat and life cycle, and others can be deduced by thoughtful, in depth analysis of the available genomic data, as described in some of the previous sections of the present communication. Secondly, although phytoplasmas and acholplasmas shared a common ancestor, the phytoplasmas have formed a phylogenetically distinct clade, which is divergent from acholplasmas (Fig. 1).

In summary, phytoplasmas possess distinctive ecological, nutritional, biochemical, physiological, genomic architectural and phylogenetic properties that are not referable to the descriptions of the order Acholeplasmatales or any other existing order of the class Mollicutes. The analysis and reasoning presented in this communication illuminate the future prospect of establishing a novel ordinal level taxon to host the phytoplasmas. We anticipate that, under the future phytoplasma-embracing order, a novel family will also be erected. The need for erection of a familial level taxon to accommodate phytoplasmas has been reasoned previously (Zhao et al., 2010, 2014), as the phylogenetic relationships (Fig. 2) and other properties of diverse phytoplasmas can be interpreted to favour the delineation of multiple genera.

While the current International Code of Nomenclature of Prokaryotes (ICNP) lacks a means to incorporate Candidatus organisms into the formal hierarchical classification system, a consensus has emerged within the scientific community that, ‘given the genomic era we are in, there is a greater need than ever to determine a means for the ICNP to embrace yet-to-be cultured species’ (personal communication from IJSEM Editor Professor Richard Birtles). We envision that a formal taxonomy of phytoplasmas, as well as other Candidatus organisms, will eventually be established through in-depth genomic analysis and logical reasoning, as attempted in this communication.

**References**


