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Prospects of DNA-based systems for differentiation and classification of phytoplasmas

Ing-Ming LEE[1], Yan ZHAO[1], Robert E. DAVIS[1], Wei WEI[1], Marta MARTINI[1,2]
[1]*Molecular Plant Pathology Laboratory, Beltsville, MD, USA*
[2]*Dipartimento di Biologia Applicata alla Difesa delle Piante, University of Udine, Italy*

Abstract

During the last two decades, over 800 phytoplasma strains have been reported in association with several hundred plant diseases and numerous insect vectors. Research has yielded new knowledge about phytoplasma ecology and phylogenetic relationships. A taxonomic system has emerged, and perspectives on phytoplasma speciation have changed. Guidelines for naming new '*Candidatus* Phytoplasma' species have been proposed; the16S rRNA gene was employed as the sole phylogenetic marker for species delineation. To date, 26 '*Ca.* Phytoplasma' species have been named, forming the core framework of the emerging phytoplasma taxonomy. A classification system based on restriction fragment length polymorphism (RFLP) analysis of 16S rRNA gene sequences provides a simple, reliable, and rapid means to classify phytoplasmas on a large scale without a need to sequence the gene. The capacity of this classification system has been recently upgraded by the development of a computer-simulated RFLP analysis method. This approach has led to construction of the most comprehensive classification system for phytoplasmas to date. The concept of multi-gene sequence analysis for distinguishing phytoplasma species has emerged with the aim of overcoming deficiencies of the highly conserved 16S rRNA gene for delineating closely related phytoplasma species. Less conserved genes, such as ribosomal protein or *secY*, serve as phylogenetic markers for finer distinctions among phytoplasmas.

Key words: 16S rRNA, genome, mollicute, multi-locus sequence analysis, mycoplasma, PCR, RFLP analysis, ribosomal protein genes, *secY*, taxonomy, *tuf*, virtual RFLP.

Introduction

Phytoplasmas, formerly termed mycoplasma-like organisms (MLOs), are minute cell wall-less prokaryotes that are associated with diseases in several hundred plant species (Lee *et al*., 2000). Since the discovery of these unique plant pathogens four decades ago, attempts to culture them in cell-free media have failed, making it difficult to determine the taxonomic status of phytoplasmas by the traditional methods applied to cultured prokaryotes. Despite their resemblance to animal and human mycoplasmas in morphology and ultrastucture, it remained uncertain whether phytoplasma were members of the class *Mollicutes* until the late 1980's. In 1989 Lim and Sears showed that a MLO (now known as the aster yellows phytoplasma) represented a new member of the class *Mollicutes*. The trivial name "phytoplasma" was officially adopted by the International Phytoplasma Working Team in 1994 to replace the term "mycoplasma-like organism". Due to the inability to culture phytoplasmas *in vitro*, many of the phenotypic criteria traditionally used for the taxonomic classification of cultured mollicutes cannot be applied. Prior to the last two decades, differentiation and classification of phytoplasmas relied primarily on their biological properties, such as similarities and differences in symptoms that they induced in infected plants, plant host ranges, and insect vectors. The determination of these biological properties was laborious and time-consuming, and experimental results were often inconsistent. Advances in molecular biology and phylogeny of bacteria have changed the concept of mollicute taxonomy (Razin *et al.*, 1998). A polyphasic taxonomy system based on phenotypic, genotypic, and phylogenetic criteria has become a consensus approach to modern bacterial systematic (Weisburg *et al.*, 1989; Murray *et al.*, 1990; Vandamme *et al.*, 1996; Razin *et al.*, 1998). However, because of the paucity of accessible phenotypic criteria, it was inevitable that phytoplasma taxonomy would be based heavily on molecular characteristics and molecular phylogeny. Molecular-based analyses introduced during the last two decades have proven to be more accurate and reliable than biological criteria long used previously for phytoplasma identification (Lee *et al.*, 2000; Bertaccini, 2007). Availability of molecular probes, cloned phytoplasma DNA, and monoclonal antibodies has made it possible to classify phytoplasmas on the basis of DNA-DNA homology and serological reactions. Results from application of these approaches revealed new insights into the diversity and genetic interrelationships of phytoplasmas.

PCR-based assays developed in the late 1980's and early 1990's further facilitated detection and classification of phytoplasmas by providing a much more sensitive means than serological tests or DNA-DNA hybridization assays for phytoplasma detection (see references in Lee *et al.,* 2000). Moreover, the design of phytoplasma universal (generic) or phytoplasma group-specific oligonucleotide primers based on highly conserved 16S rRNA gene sequences enabled amplification of 16S rDNA sequences from strains belonging to a broad spectrum of phytoplasma groups and from diverse strains within a given group. For the first time, researchers were able to detect and study the whole spectrum of phytoplasma strains worldwide. Universal and group-specific primers were also developed on the basis of 16S – 23S intergenic spacer region, ribosomal protein (rp) gene, elongation factor EF-Tu (*tuf*), and other gene

sequences (Smart *et al.*, 1996; Schneider *et al.*, 1997; Marcone *et al.*, 2000; Lee *et al*., 2004a; 2004b; Martini *et al.*, 2007;). Analyses of PCR-amplified sequences of these conserved genes or of a specific DNA fragment provided a simple and rapid mean for phytoplasma classification (Lee *et al.*, 1993; Schneider *et al*., 1997; Lee *et al.*, 1998; Marcone *et al.*, 2000; Lee *et al.*, 2004a; 2004b; Streten and Gibb, 2005). This advance in phytoplasma research made it feasible to construct a comprehensive classification scheme for phytoplasmas and to establish a provisional taxonomic system for uncultured phytoplasmas (Lee *et al.*, 2000). This brief review will summarize recent advances and discuss future prospects for DNA-based differentiation and classification of phytoplasmas (Zhao *et al.*, 2005).

Classification systems based on RFLP analysis of 16S rDNA sequences

Classification based on actual RFLP analysis

In the late 1980's and early 1990's, extensive phylogenetic analyses of 16S rRNA and/or ribosomal protein (rp) gene sequences revealed the phylogenetic position of phytoplasmas, definitively placing them as members of the class *Mollicutes* (Gundersen *et al.*, 1994; Namba *et al.*, 1993; Seemüller *et al.*, 1998). Global phylogenetic analyses of 16S rRNA by Gundersen *et al.* (1994) showed that phytoplasmas formed a large discrete monophyletic clade within the expanded *Anaeroplasma* clade. The phytoplasma clade is paraphyletic to *Acholeplasma* species, which are the closest known relatives of phytoplasmas. Within the phytoplasma clade, 11 distinct subclades were identified. To date, more than 20 subclades have been delineated based on 16S rRNA gene sequences (Seemüller *et al.,* 1998). These comprehensive phylogenies have formed a basis for phytoplasma classification. For classification of phytoplasmas on a large scale, restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA sequences with a number of restriction enzymes was used by Lee *et al.* (1993) and Schneider *et al*. (1993) to differentiate various phytoplasmas by their distinct RFLP patterns. This approach enabled a simple, reliable, and rapid way to identify many phytoplasmas of interest without a need to sequence the gene. Based on RFLP analyses with 15 restriction enzymes of a 1.25 kb 16S rDNA fragment amplified from phytoplasma strains associated with numerous diseases, Lee *et al.* (1993) proposed a classification scheme that comprised 10 phytoplasma groups (termed 16S rRNA or 16Sr groups) and 15 subgroups. The similarity coefficients of RFLP patterns between two distinct groups were 90% or less.

The 16S rDNA RFLP classification scheme has been expanded over the last decade. Currently, it comprises 18 groups and more than 50 subgroups and is the most comprehensive classification scheme for phytoplasmas (Lee *et al*., 2000; Montano *et al*., 2001; Arocha *et al.*, 2005; Lee *et al.*, 2006b). The phylogenetic subclades coincided with 16S rRNA phytoplasma groups identified by distinct RFLP patterns, validating the classification schemes that were based on RFLP analysis of 16S rDNA sequences (Gundersen *et al*., 1994; Lee *et al.*, 1998). As a consequence of the congruent phylogenies and RFLP-based groupings, it was proposed that each subclade or 16S rRNA group represents at least one distinct phytoplasma species. This comprehensive classification scheme, combined with illustrative RFLP patterns, continues to provide a simple, reliable, and practical mean to identify and classify unknown phytoplasmas without the need to sequence the 16S rRNA gene. The system has been widely used, resulting in identification of several hundred phytoplasma strains over the last decade.

This system is ideal for preliminary characterization of unknown phytoplasmas that may be associated with a given disease. Such characterization typically involves the analysis of numerous samples. Its accuracy depends on the quality of phytoplasma template DNA and PCR-amplified 16S rDNA. To avoid amplification of non-specific DNA fragments, and to increase sensitivity of phytoplasma detection, a nested PCR reaction is often performed. Alternatively, if feasible, cloned 16S rDNA fragments should be used as template. The capacity of this system depends on the completeness of reference RFLP patterns. Thus, periodic updates of RFLP patterns to include newly discovered phytoplasma strains are absolutely necessary. Unfortunately, this aspect is potentially a major drawback for this system. As numerous new phytoplasma strains are discovered, updating the collection of phytoplasma strains and of complete sets of RFLP patterns could become problematic, but recent availability of a new tool minimizes this obstacle.

Classification based on computer-simulated RFLP analysis

Recent advances in DNA cloning and nucleotide sequencing have drastically reduced the cost of sequencing and have improved the accuracy of sequencing data. In addition, novel bioinformatic approaches for handling nucleotide sequencing data have emerged. To date, more than 800 phytoplasma 16S rRNA gene sequences are available in the National Center for Biotechnology Information's (NCBI) nucleotide database. The majority of sequences were deposited during the past five years. Availability of high-quality sequence data makes it possible to simulate restriction digestions *in silico* and to generate virtual RFLP patterns that are consistent with those obtained by actual RFLP analysis. The computer-simulated method permits high throughput analysis, identification, and classification of diverse phytoplasmas. Recently Wei *et al.* (2007) developed a streamlined computer-simulated RFLP analysis method for rapid identification and classification of phytoplasmas. This work resulted in an expanded classification scheme in which ten new phytoplasma 16S rRNA groups and numerous subgroups were identified (table 1). As applied in the classification based on actual enzymatic RFLP analysis of DNA, the same 1.25 kb 16S rDNA fragment was used for analysis and a threshold similarity coefficient value of 90% or less was used for separation of two distinct groups. The groups delineated by these *in silico* analyses were consistent with those delineated in the classification scheme based on actual RFLP analysis of DNA.

Table 1. Classification based on *in silico* RFLP analysis.

16Sr group/subgroup/strain	GenBank no.
16SrI: Aster yellows group	
I-B '*Ca*. P. asteris'	M30790
16SrII: Peanut witches' broom group	
II-A Peanut witches' broom phytoplasma	L33765
II-B '*Ca*. P. aurantifolia'	U15442
II-D Papaya yellow crinkle phytoplasma	Y10097
16SrIII: X-disease group	
III-A Western X-disease phytoplasma	L04682
16SrIV: Coconut lethal yellows group	
IV-A Coconut lethal yellowing phytoplasma	AF498307
16SrV: Elm yellows group	
V-A '*Ca*. P. ulmi'	AY197655
V-B '*Ca*. P. ziziphi'	AB052876
16SrVI: Clover proliferation group	
VI-A '*Ca*. P. trifolii'	AY390261
16SrVII: Ash yellows group	
VII-A '*Ca*. P. fraxini'	AF092209
16SrVIII: Loofah witches' broom group	
VIII-A Loofah witches' broom phytoplasma	AF353090
16SrIX: Pigeon pea witches' broom group	
IX-A Pigeon pea witches' broom phytoplasma	AF248957
IX-D '*Ca*. P. phoenicium'	AF515636
16SrX: Apple proliferation group	
X-A '*Ca*. P. mali'	AJ542541
X-B '*Ca*. P. prunorum'	AJ542544
X-C '*Ca*. P. pyri'	AJ542543
X-D '*Ca*. P. spartii'	X92869
16SrXI: Rice yellow dwarf group	
XI-A '*Ca*. P. oryzae'	AB052873
16SrXII: Stolbur group	
XII-A Stolbur phytoplasma	AJ964960
XII-B '*Ca*. P. australiense'	L76865
XII-D '*Ca*. P. japonicum'	AB010425
XII-E '*Ca*. P. fragariae'	DQ086423
16SrXIII: Mexican periwinkle virescence group	
XIII-A Mexican periwinkle virescence phytoplasma	AF248960
16SrXIV: Bermudagrass white leaf group	
XIV-A '*Ca*. P. cynodontis'	AJ550984
16SrXV: Hibiscus witches' broom group	
XV-A '*Ca*. P. brasiliense'	AF147708
16SrXVI: Sugarcane yellow leaf syndrome group	
XVI-A '*Ca*. P. graminis'	AY725228
16SrXVII: Papaya bunchy top group	
XVII-A '*Ca*. P. caricae'	AY725234
16SrXVIII: American potato purple top wilt group	
XVIII-A '*Ca*. P. americanum'	DQ174122
16SrXIX: Japanese chestnut witches'-broom group	
XIX-A '*Ca*. P. castaneae'	AB054986
16SrXX: Buckthorn witches' broom group	
XX-A '*Ca*. P. rhamni'	X76431
16SrXXI: Pine shoot proliferation group	
XXI-A '*Ca*. P. pini'	AJ632155
16SrXXII: Nigerian coconut lethal decline (LDN) group	
XXII-A Nigerian coconut lethal decline phytoplasma	Y14175
16SrXXIII: Buckland Valley grapevine yellows group	
XXIII-A Buckland valley grapevine yellows phytoplasma	AY083605
16SrXXIV: Sorghum bunchy shoot group	
XXIV-A Sorghum bunchy shoot phytoplasma	AF509322
16SrXXV: Weeping tea tree witches' broom group	
XXV-A Weeping tea witches' broom phytoplasma	AF521672
16SrXXVI: Mauritius sugarcane yellows D3T1 group	
XXVI-A Sugarcane phytoplasma D3T1	AJ539179
16SrXXVII: Mauritius sugarcane yellows D3T2 group	
XXVII-A Sugarcane phytoplasma D3T2	AJ539180
16SrXXVIII: Havana derbid phytoplasma group	
XXVIII-A Derbid phytoplasma	AY744945

Whereas, actual RFLP patterns characterizing the ten newly identified groups were not available, the virtual RFLP patterns generated can serve as bench top references for classification of an unknown phytoplasma into a 16Sr group following actual RFLP analysis of isolated DNA.

Overall, the computer-simulated RFLP analysis system is compatible with the traditional RFLP analysis system. However, due to greater resolution of banding patterns generated by the computer-simulated system, the similarity coefficient threshold for separation of subgroups may be adjusted accordingly. The accuracy of the computer-simulated system completely depends on 16S rDNA sequence accuracy. False identification may be due to sequence error. If feasible, the patterns should be verified by actual RFLP analysis. The potential of the computer-simulated analysis system reaches beyond the 16S rRNA gene. This system can be easily adapted for analysis of other phylogenetic markers.

Provisional phytoplasma taxonomy and nomenclature

A bacterial species, as recommended and defined by the International Committee on Systematic Bacteriology, includes strains that share at least 70% DNA homology (70% - 85% homology for strains within a subspecies) based on a complete sequence of the bacterial genome and a ΔTm (melting or midpoint temperature) ≤ 5°C (Razin *et al.,* 1998). The naming of new species in the class *Mollicutes* requires description of species in pure culture, but the DNA homology and phenotypic characteristics used to describe mollicute species are unattainable for uncultured phytoplasmas. Therefore, a provisional classification system using the '*Candidatus*' convention has been adopted for phytoplasmas. The provisional '*Candidatus* Phytoplasma' species have been arbitrarily defined based on analysis of 16S rRNA gene sequences in accordance with concepts articulated by Murray and Schleifer (1994). According to recommendations by the International Research Program for Comparative Mycoplasmology, Phytoplasma/Spiroplasma Working Team (IRPCM, 2004), "a '*Candidatus* Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence (>1200bp)" and "a strain can be recognized as a novel '*Ca*. Phytoplasma' species if its 16S rRNA gene sequence has < 97.5% similarity to that of any previously described '*Ca.* Phytoplasma' species". Two phytoplasmas that share more that 97.5% 16S rRNA gene sequence similarity, but clearly represent ecologically separated populations, can be designated as separate '*Ca.* Phytoplasma' species if they meet the following three criteria: "(i) they are transmitted by different vectors; (ii) the two phytoplasmas have a different natural plant host(s); and (iii) there is evidence of molecular diversity between the two phytoplasmas". Based on these proposed criteria, 26 '*Ca.* Phytoplasma' species have been described to date (see list in IRPCM 2004; Lee *et al*., 2006a; Valiunas *et al.*, 2006).

The proposal of 97.5% 16S rRNA gene sequence similarity to separate two phytoplasma species is arbitrary. However, study by Stackebrandt and Goebel

(1994) indicated that organisms sharing less than 97% 16S rRNA sequence similarity will not give a DNA reassociation of more than 60% regardless of which DNA-DNA hybridization methods are used. This indicates the potential of replacing DNA homology with 16S rRNA sequence similarity in the description of new species. However, because of the highly conserved nature of 16S rRNA gene sequences, there is no defined threshold of 16S rRNA sequence similarity for assigning a species rank among bacteria. There are examples of bacterial strains that are readily classified as distinct species by conventional approaches based on DNA homology and phenotypic characters, but cannot be readily distinguished by analysis of 16S rRNA sequences (Fox *et al.*, 1992). Some species share 99% or higher 16S rRNA sequence similarity. The deficiency of 16S rRNA in defining closely related species underscores a need to include phylogenetic markers that are less conserved and permit finer differentiation among closely related but distinct species.

Prospects for multi-locus phylogenetic analysis, species delineation, and strain differentiation

Undoubtedly, many biologically or ecologically distinct phytoplasmas warrant recognition as new taxa but fail to meet the requirement of sharing < 97.5% sequence similarity with existing '*Candidatus* Phytoplasma' species. Thus, unique biological properties as well as additional molecular criteria need to be designated for species definitions. Ribosomal protein (rp) and *secY* genes are more variable than the 16S rRNA gene and proved to be useful for finer differentiation of phytoplasma strains in groups 16SrV and 16SrI (Martini *et al.*, 2002; Lee *et al.*, 2004a; 2004b; 2006b). Analysis of rp or *secY* gene sequences delineated biologically and/or ecologically distinct strains that often cannot be readily resolved based on the 16S rRNA gene alone. Recently, Martini *et al.* (2007) constructed a comprehensive phylogenetic tree based on the analysis of two ribosomal protein genes, *rplV* (*rpl22*) and *rpsC* (*rps3*), from 46 phytoplasma strains representing 12 16Sr groups. This rp gene-based phylogenetic tree, which was congruent with that inferred from the 16S rRNA gene, yielded more clearly defined phylogenetic interrelationships among phytoplasma strains and delineated more distinct phytoplasma subclades and distinct lineages than those resolved by the 16S rRNA gene-based tree. The average rp gene sequence similarity between two given 16Sr phytoplasma groups ranged from 50.4 – 83.5% in comparison with 85 – 96.9% in the case of the 16S rRNA gene. This greater sequence variation makes rp genes a better molecular tool for phytoplasma classification. For example, three '*Ca.* Phytoplasma' species ('*Ca.* P. mali', '*Ca.* P. pyri', and '*Ca.* P. prunorum') that share 98.9 - 99.1 % 16S rDNA sequence similarity, shared 94.3 - 94.6 % rp gene sequence similarity and were readily delineated by analysis of rp gene sequences. Recently, multi-locus sequence typing using *secY*, *map*, and *uvrB-degV* gene sequences was employed for differentiation of three distinct flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe (Arnaud *et al.*, 2007). Less conserved gene sequences clearly amplify the resolving power for delineating phylogenetic relationships among diverse phytoplasma strains. Phylogenies constructed from sets of multi-gene sequences (multiple phylogenetic markers), having varying degrees of sequence conservation, should better represent the overall genome of the organisms and provide a basis to better define phytoplasma species or strains. Over recent decades, advances in genome sequencing and bioinformatic tools have resulted in completion of more than 500 annotated bacterial genomes including two phytoplasma strains (Bai *et al.*, 2006; Oshima *et al.*, 2004). Comparative genomics of these bacteria should reveal genes with varying degrees of sequence variability and different resolving powers for use in the differentiation of phytoplasmas at the taxonomic rank of genus, species, and strain.

Issues and future outlook

Molecular tools such as monoclonal antibodies, DNA-based probes, and PCR-based sensitive detection procedures have largely replaced traditional procedures based on biological properties, greatly advancing phytoplasmal disease diagnostics and facilitating phytoplasma characterization. Nevertheless, a number of important issues may be raised in relation to future progress in phytoplasma research. For example, the issue of criteria for delineation of phytoplasmas at 16Sr subgroup and species level is already rising in prominence as reports of phytoplasmas in previously unknown host plants and little studied geographic regions rapidly increase. Ultimately, phytoplasma differentiation, by whatever methodology, and phytoplasma classification will place further pressure on phytoplasma taxonomy and issues of phytoplasma species nomenclature. Progress toward a formal taxonomic system and eventual abandonment of the '*Candidatus*' species convention has been expressed as a broad goal by members of the International Phytoplasma Working Group, but numerous important issues need to be resolved before this goal can be reached. The central issue relates to what criteria will replace those currently used for delineation and description of '*Candidatus* Phytoplasma' species. It is widely acknowledged that multi-locus DNA marker systems will make enhanced strain and species delineations possible; choices of the most useful sequences are yet to be made. Phytoplasma genome sequencing, both whole genome and targeted segment sequencing, will play an increasingly important role in phytoplasma classification, in part by providing suites of genes from which sets will be chosen for phytoplasma delineations and descriptions at strain, species, genus, and population levels. If the '*Candidatus*' convention is abandoned completely or in part for phytoplasma taxonomy, assuming that molecular criteria continue to provide the major basis for species delineations and descriptions, a set or sets of genes for species descriptions will have to be agreed upon internationally, very likely a difficult task in the

short term. Even in the case of the 16S rRNA gene sequence, used currently as the major basis for recognition of '*Candidatus* Phytoplasma' species, resolution has not been achieved in determining whether one or both rRNA operon sequences should be used -- and if only one, which one.

It is axiomatic that evolutionary speciation is driven by genes other than those, such as 16S rRNA and ribosomal protein genes, that are commonly used for phylogenetic analyses, classification, and delineation and description of '*Candidatus* Phytoplasma' species. In our view, emergence of distinct phytoplasmal lineages results from evolutionary adaptation to distinct ecological niches occupied by these obligate parasites. In this view, niche adaptation and formation of distinct, species level gene pools may depend upon horizontal gene acquisition and/or mutations within genes and regulatory sequences that govern phytoplasma-host interactions.

While conserved genes are useful in phytoplasma strain classification and species delineation, their utility is due to sequence variability that is correlated with, but not determinant of, species evolution. Thus, it is sequence drift in niche-isolated or niche-unique strain populations that results in conserved gene sequence variability. This implies that some closely related species may not be resolved by analyses of conserved genes and that incipient species tend to remain unrecognized. For these reasons and for practicality, it is important at this stage of phytoplasma taxonomy to link phenotypic or biological characteristics with molecular criteria in definition and description of phytoplasma taxa, especially at species level. Ultimately, as more is learned about factors involved in host-pathogen interactions and nucleotide sequences controlling these interactions, it should be possible to bridge molecular criteria and biological properties through judicious choices of suites of nucleotide sequences applicable for distinguishing and describing phytoplasma species.

This brief article summarizes some of the most significant advances in phytoplasma differentiation and classification during the past decade, and we only briefly touch upon just a few of the weighty issues that in our opinion will face phytoplasma researchers over the next five to 10 years. In our view, use of multigene sequences with varying degrees of variability will eventually afford definitions of phytoplasmas at strain, species, or higher level consistent with phylogenies theoretically based on complete genome sequences. This outlook engenders the concept that formal genus *Phytoplasma* taxonomy will become a reality, in spite of inability to isolate these intriguing microbes in artificial cultures, and will one day be based entirely upon gene information.

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Corresponding author: Ing-Ming LEE (e-mail: ingming.lee@ars.usda.gov), USDA-ARS, Molecular Plant Pathology Laboratory, Beltsville, MD 20705, USA.