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Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups

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Phytoplasmas are cell wall-less bacteria that cause numerous plant diseases. As no phytoplasma has been cultured in cell-free medium, phytoplasmas cannot be differentiated and classified by the traditional methods which are applied to culturable prokaryotes. Over the past decade, the establishment of a phytoplasma classification scheme based on 16S rRNA restriction fragment length polymorphism (RFLP) patterns has enabled the accurate and reliable identification and classification of a wide range of phytoplasmas. In the present study, we expanded this classification scheme through the use of computer-simulated RFLP analysis, achieving rapid differentiation and classification of phytoplasmas. Over 800 publicly available phytoplasma 16S rRNA gene sequences were aligned using the CLUSTAL_X program and the aligned 1.25 kb fragments were exported to pDRAW32 software for *in silico* restriction digestion and virtual gel plotting. Based on distinctive virtual RFLP patterns and calculated similarity coefficients, phytoplasma strains were classified into 28 groups. The results included the classification of hundreds of previously unclassified phytoplasmas and the delineation of 10 new phytoplasma groups representing three recently described and seven novel putative '*Candidatus* Phytoplasma' taxa.

INTRODUCTION

Phytoplasmas, previously referred to as mycoplasma-like organisms (Doi *et al.*, 1967), are small, cell wall-less prokaryotes that descended from an ancestral low G+C Gram-positive bacterium, possibly a *Clostridium*-like member of the *Lactobacillus* lineage (Woese, 1987; Weisburg *et al.*, 1989). Along with mycoplasmas, spiroplasmas, acholeplasmas and other cell wall-less bacteria, phytoplasmas are classified in the class *Mollicutes*. Phytoplasmas are obligate intracellular parasites that reside in the sieve cells of plant phloem tissue and cause diseases in hundreds of plant species worldwide (McCoy *et al.*, 1989; Lee *et al.*, 2000). In nature, phytoplasmas are transmitted from diseased to healthy plants by phloem-feeding insect vectors, mainly leafhoppers and psyllids (Tsai, 1979). To date, no phytoplasma culture has been established in a cell-free medium; thus, differentiation and classification of phytoplasmas by means of the biophysical- and biochemical-based phenotypic criteria that are routinely used for culturable micro-organisms has been impossible. When the aetiological agents of phytoplasmal diseases (yellows diseases) were mistakenly believed to be viruses, differentiation of these presumed 'viruses' was based on the symptoms exhibited by diseased plants and on the identity of specific plant hosts and insect vectors (Chiykowski, 1962; Freitag, 1964; Granados & Chapman, 1968; Chiykowski & Sinha, 1989; McCoy *et al.*, 1989). Given that the same phytoplasma strain may induce different symptoms in different hosts and different phytoplasma strains may share a common vector(s) or cause diseases characterized by similar symptoms, this 'guilty by affiliation' approach could not provide an accurate means for phytoplasma classification.

In the 1980s and early 1990s, the employment of serological (Lin & Chen, 1985; Lee *et al.*, 1993a) and nucleic acid-based (Lee & Davis, 1988; Lee *et al.*, 1992a, b; Griffiths *et al.*, 1994) assay techniques revealed new insights into the diversity and genetic interrelationships of phytoplasmas. In particular, based on restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S rRNA, Lee and colleagues constructed the first comprehensive phytoplasma classification scheme (Lee *et al.*, 1993b, 1998, 2000), providing a reliable means for the differentiation of a broad array of phytoplasmas. To date, this system has classified phytoplasmas in 18 groups and more than 40 subgroups and has become the most comprehensive and widely accepted phytoplasma classification system (Lee *et al.*, 1998, 2004a, b; Arocha *et al.*, 2005; Lee *et al.*, 2006). Over the last few years, numerous and diverse phytoplasmas have been discovered

Abbreviation: CNI, close neighbour interchange.

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at an increasingly rapid pace in emerging diseases worldwide. These developments have raised expectations that the number of 16S rRNA RFLP groups (16Sr groups) and subgroups could rise considerably, warranting expansion of the existing phytoplasma classification scheme. However, attempts to update the classification scheme using conventional RFLP analysis have been hindered by the lack of a complete, or near-complete, collection of phytoplasma strains as sources of DNA, emphasizing the need for a method to circumvent this obstacle.

Recent technological advancements now make possible an alternative approach for updating the phytoplasma classification scheme: the cost of DNA sequencing has dramatically reduced while the accuracy of the sequencing data has significantly improved and novel bioinformatic approaches for handling nucleotide sequence data have emerged. At the time of writing, more than 800 phytoplasma 16S rRNA gene sequences have been deposited into the National Center for Biotechnology Information's (NCBI) nucleotide sequence database. The availability of high-quality sequence data makes it possible to simulate restriction digestions *in silico* and to generate virtual RFLP patterns for high throughput identification and classification of diverse phytoplasmas. Here, we report the exploitation of a computer-simulated RFLP analysis method for classification of phytoplasma strains that resulted in the identification of new phytoplasma groups, significantly expanding the 16S RNA gene sequence-based phytoplasma classification scheme and unveiling putative novel phytoplasma species.

METHODS

Retrieval of 16S rRNA gene sequences. Phytoplasma 16S rRNA gene sequences were retrieved online from the NCBI's nucleotide sequence database at http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi using the Entrez search and retrieval tool (Wheeler *et al.*, 2005). The retrieved sequences were kept in a Microsoft Excel-based mini-database, in which sequence accessions were organized into groups according to the existing 16S rRNA RFLP-based phytoplasma classification scheme as outlined in the '*Candidatus* Phytoplasma' Taxonomy Browser (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Tree&id=33926). For the purpose of cladistic analysis, 16S rRNA gene sequences from 90 non-phytoplasma bacterial taxa and two cyanobacterial taxa were also retrieved from the nucleotide sequence database at the NCBI. Of the 90 non-phytoplasma eubacterial taxa, 64 were cell wall-less bacteria from eight genera in the class *Mollicutes* (*Acholeplasma*, *Anaeroplasma*, *Asteroleplasma*, *Entomoplasma*, *Mesoplasma*, *Mycoplasma*, *Spiroplasma* and *Ureaplasma*), 18 were Gram-positive low G+C walled bacteria from three representative groups (orders *Bacillales*, '*Clostridia*' and '*Lactobacillales*') and eight were Gram-positive high G+C bacteria in the class *Actinobacteria*. The two cyanobacterial taxa were *Synechocystis* sp. and *Nostoc* sp.

Alignment of 16S rRNA gene sequences and cladistic analysis. For multiple sequence alignment, nucleotide sequences were compiled in FASTA format. Compiled sequences were aligned using CLUSTAL_X (version 1.83) by selecting the 'do complete alignment' option with default parameters (gap opening penalty 15.00, gap extension penalty 6.66, delay divergent sequences 30 %, DNA transition weight 0.5) (Thompson *et al.*, 1997; Jeanmougin *et al.*, 1998). Each aligned sequence was trimmed to an approximately 1.25 kb fragment (termed the F2nR2 region hereafter) that was bounded by the two conserved nucleotide blocks corresponding to the annealing sites for the phytoplasma-universal 16S rRNA primer pair R16F2n/R16R2 (Gundersen & Lee, 1996). Accessions not encompassing the full F2nR2 region and accessions containing two or more consecutive undetermined nucleotides were considered inadmissible and were excluded from further analyses. The trimmed sequences were realigned and the final alignment was converted to MEGA format for cladistic analyses.

Maximum-parsimony cladistic analysis was conducted with MEGA3 software (Kumar *et al.*, 2004) using the close neighbour interchange (CNI) algorithm. The initial tree for the CNI search was created by random addition for 10 replications. The reliability of the analysis was subjected to a bootstrap test with 100 replicates. The choice of these settings was a compromise because the present study was comparing up to 616 sequences which made an exhaustive search by heuristic algorithms prohibitive. In phylogenetic tree reconstruction, the two cyanobacterial taxa served as an out-group.

***In silico* restriction enzyme digestions and virtual gel plotting.** The aligned and trimmed sequences were exported to the *in silico* restriction analysis and virtual gel plotting program pDRAW32, developed by AcaClone Software (http://www.acaclone.com). Each aligned DNA fragment was digested *in silico* with 17 distinct restriction enzymes that have been routinely used for phytoplasma 16S rRNA gene RFLP analysis (Lee *et al.*, 1998). These enzymes were *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I. After *in silico* restriction digestion, a virtual 3.0 % agarose gel electrophoresis image was plotted automatically to the computer screen. The virtual gel image was then captured as a device-independent file in PDF format for subsequent RFLP pattern comparisons.

Comparison of virtual RFLP patterns and calculation of similarity coefficients. Virtual RFLP patterns, i.e. the sum result from *in silico* digestions with 17 enzymes, were compared using the multiple layer function of the Photoshop graphics editing software (Adobe Systems). A similarity coefficient (F) was calculated for each pair of phytoplasma strains according to the formula described previously (Nei & Li, 1979; Lee *et al.*, 1998), $F=2N_{xy}/(N_x+N_y)$, in which x and y are two given strains under investigation, N_x and N_y are the total number of DNA fragments (bands) resulting from digestions by 17 enzymes for strains x and y, respectively, and N_{xy} is the number of fragments shared by the two strains.

RESULTS AND DISCUSSION

By mimicking actual restriction enzyme digestions and subsequent gel electrophoresis, the computer-simulated 16S rRNA gene analysis produced virtual RFLP patterns, allowing high throughput differentiation and identification of phytoplasma strains. Based on the distinctive RFLP pattern types, all available phytoplasma sequence accessions were classified into 28 16Sr RFLP groups and the classification status of more than 250 previously unclassified phytoplasma strains was determined.

Sequence data validation and cladistic analysis

As of the end of August 2006, a total of 829 phytoplasma 16S rRNA gene sequence accessions had been deposited in the nucleotide databases of the DNA DataBank of Japan

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(DDBJ), the European Molecular Biology Laboratory (EMBL) and GenBank at the NCBI. The lengths of these registered sequences ranged from a few hundred bases to full-length rRNA operons. To remain consistent with the well-established, actual gel-based (conventional) phytoplasma 16S rRNA gene RFLP classification scheme (Lee *et al.*, 1998), an admissible sequence accession for the present study had to encompass the complete F2nR2 region. This sequence admissibility test validated a total of 524 accessions. For an overwhelming majority of the 524 admissible accessions, the F2nR2 region varied from 1235 to 1254 bp. Nine accessions had an exceptionally long F2nR2 region (1371–1377 bp), whereas seven accessions had an exceptionally short F2nR2 region (1142–1225 bp).

The F2nR2 regions of the 524 phytoplasma 16S rRNA gene sequence accessions were used to reconstruct a maximum-parsimony phylogenetic tree (Fig. 1a). As indicated by the topology of the parsimony tree, the 524 phytoplasma accessions under investigation constituted a monophyletic clade (with a bootstrap value of 100 %) that subsumed 16 accessions that had either an exceptionally long or an exceptionally short F2nR2 region. The phytoplasma clade was paraphyletic to the clade formed by acholeplasmas, the closest known relatives of phytoplasmas.

Within the phytoplasma clade, three major branches and at least 14 distinct subclades were evident according to the topology of the phytoplasma parsimony tree (Fig. 1b). Previously classified phytoplasma strains, each having been assigned to one of the 18 (16SrI to 16SrXVIII) already delineated 16Sr groups (Lee *et al.*, 1998, 2004a, b, 2006; Arocha *et al.*, 2005), fell into nine of the 14 subclades seen in the phytoplasma tree (Fig. 1b). The results of this cladistic analysis strongly suggested that, among the 524 phytoplasma accessions studied in the present work, there were previously unrecognized phytoplasma groups occupying at least five subclades.

Virtual RFLP analysis and expansion of the phytoplasma classification scheme

The F2nR2 regions from 524 phytoplasma 16S rRNA gene sequence accessions were each digested *in silico* with 17 restriction enzymes. Virtual RFLP analyses of the resulting DNA fragments generated 250 distinct pattern types that were sorted into 28 groups and around 100 subgroups. Delineation of groups was based on the previously established convention in which coefficients of 16S rRNA gene RFLP pattern similarity between two distinct groups were equal to or less than 90 % (Lee *et al.*, 1998). The criteria used for the delineation of the rapidly growing number of subgroups will be addressed in a separate communication.

The virtual RFLP patterns of 53 16S rRNA gene sequence accessions from 51 phytoplasma strains representing 28

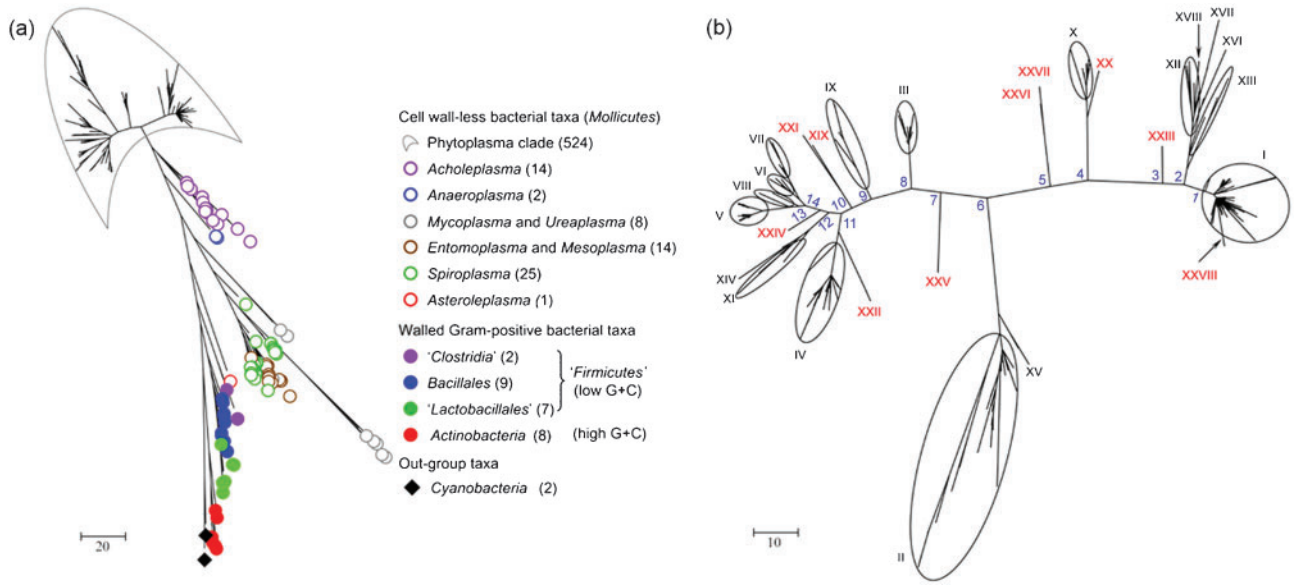

Fig. 1. Maximum-parsimony cladistic analysis of phytoplasmas based on the F2nR2 region of 16S rRNA gene sequences. (a) A global phylogenetic tree reconstructed from 524 phytoplasma sequence accessions and 92 non-phytoplasma bacterial taxa. Cell wall-less bacterial taxa are marked with colour-coded circles. Walled Gram-positive bacterial taxa are marked with colour-coded dots. Two cyanobacterial taxa, used as an out-group, are marked with diamonds. Phytoplasmas formed a monophyletic clade. Bar, 20 nucleotide substitutions. (b) A tree topology reconstructed from 524 phytoplasma sequence accessions. Three major branches and at least 14 subclades (indicated by Arabic numerals) are evident within the phytoplasma clade. Group affiliation is indicated by Roman numerals; circles encompass group members. New 16Sr groups delineated in the present study are shown in red. Bar, 10 nucleotide substitutions.

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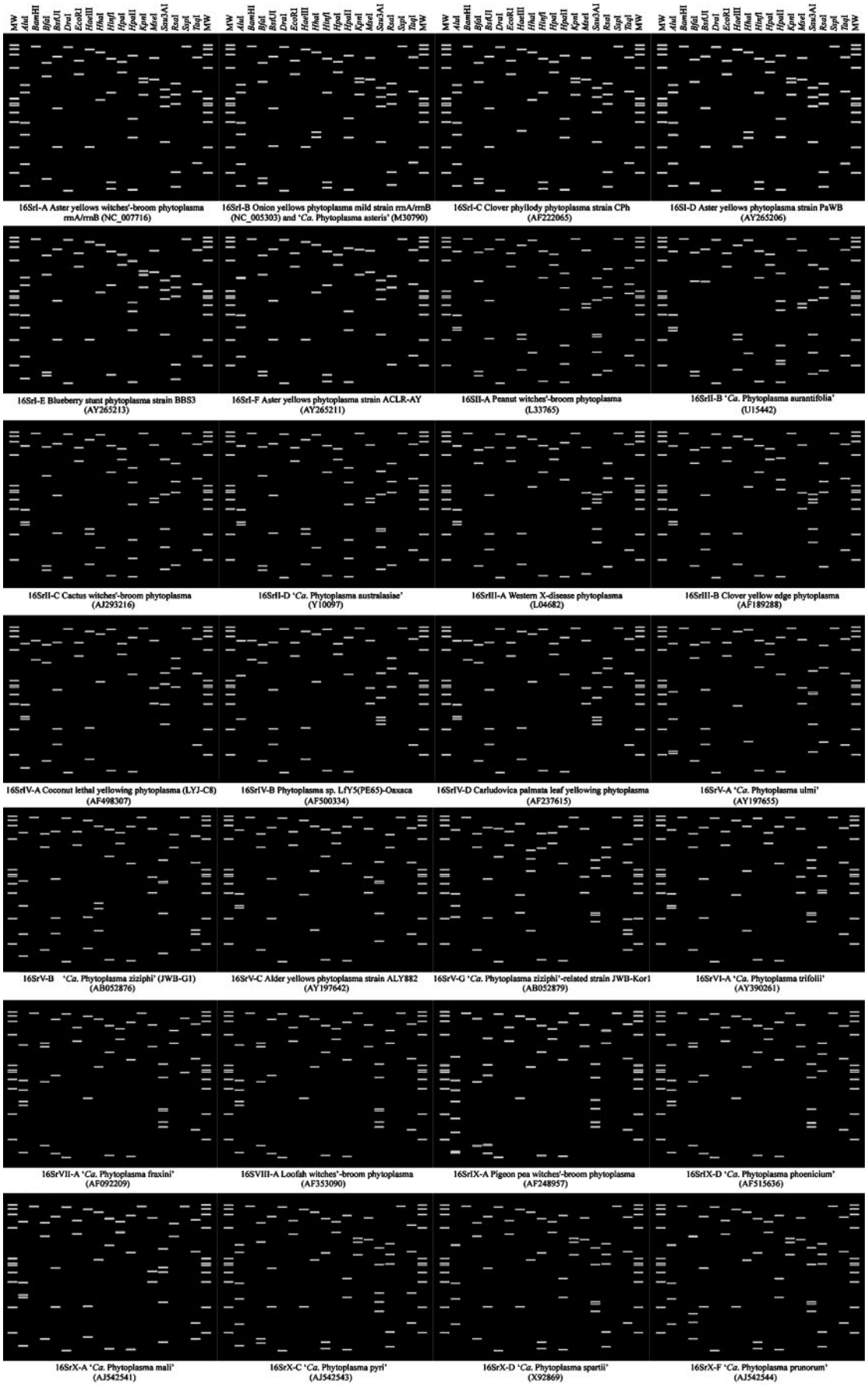

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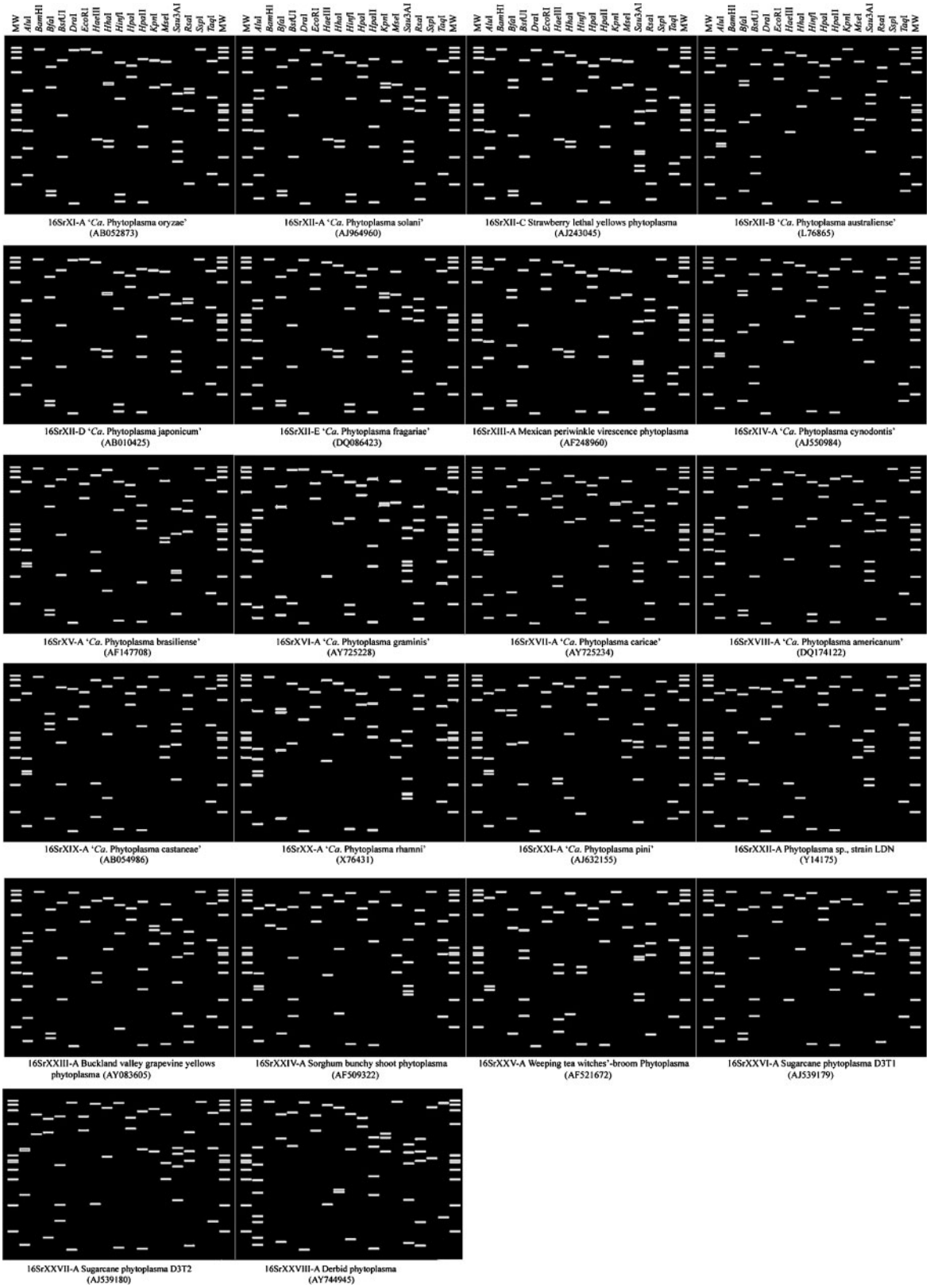

Fig. 2. Virtual RFLP patterns from *in silico* digestions of 16S rRNA gene F2nR2 fragments from 51 phytoplasma strains representing 28 groups. Recognition sites for the following 17 restriction enzymes were used in the simulated digestions: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. MW, φX174DNA-*Hae*III digestion.

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Table 1. Classification of phytoplasmas based on *in silico* RFLP analysis of 16S rRNA gene sequences

Fifty-one representative strains are listed in the table. Among them are 25 formally described '*Candidatus* Phytoplasma' taxa and representative strains for each 16Sr group. '*Ca.* Phytoplasma allocasuarinae' is not included because the available 16S rRNA gene sequence does not cover the complete F2nR2 region.

16Sr group	Strain	GenBank accession no.	Reference
16SrI: Aster yellows group			
I-A	Aster yellows witches'-broom phytoplasma (AYWB) *rrn*A	NC_007716	Bai *et al.* (2006)
I-A	Aster yellows witches'-broom phytoplasma (AYWB) *rrn*B	NC_007716	Bai *et al.* (2006)
I-B	Onion yellows phytoplasma mild strain (OY-M) *rrn*A	NC_005303	Oshima *et al.* (2004)
I-B	Onion yellows phytoplasma mild strain (OY-M) *rrn*B	NC_005303	Oshima *et al.* (2004)
I-B	'*Ca.* Phytoplasma asteris'	M30790	Lee *et al.* (2004a)
I-C	Clover phyllody phytoplasma strain CPh	AF222065	2000 (GenBank submission)
I-D	Aster yellows phytoplasma strain PaWB	AY265206	2003 (GenBank submission)
I-E	Blueberry stunt phytoplasma strain BBS3	AY265213	2003 (GenBank submission)
I-F	Aster yellows phytoplasma strain ACLR-AY	AY265211	2003 (GenBank submission)
16SrII: Peanut WB group			
II-A	Peanut witches'-broom phytoplasma	L33765	Gundersen *et al.* (1994)
II-B	'*Ca.* Phytoplasma aurantifolia'	U15442	Zreik *et al.* (1995)
II-C	Cactus witches'-broom phytoplasma	AJ293216	Cai *et al.* (2002)
II-D	'*Ca.* Phytoplasma australasiae'	Y10097	White *et al.* (1998)
16SrIII: X-disease group			
III-A	Western X-disease phytoplasma	L04682	1999 (GenBank submission)
III-B	Clover yellow edge phytoplasma	AF189288	1999 (GenBank submission)
16SrIV: Coconut lethal yellows group			
IV-A	Coconut lethal yellowing phytoplasma (LYJ-C8)	AF498307	Harrison *et al.* (2002a)
IV-B	Phytoplasma sp. LfY5(PE65)-Oaxaca	AF500334	Harrison *et al.* (2002b)
IV-D	*Carludovica palmata* leaf yellowing phytoplasma	AF237615	Cordova *et al.* (2000)
16SrV: Elm yellows group			
V-A	'*Ca.* Phytoplasma ulmi'	AY197655	Lee *et al.* (2004b)
V-B	'*Ca.* Phytoplasma ziziphi' strain JWB-G1	AB052876	Jung *et al.* (2003a)
V-C	Alder yellows phytoplasma strain ALY882	AY197642	Lee *et al.* (2004b)
V-G	'*Ca.* Phytoplasma ziziphi'-related strain JWB-Kor1	AB052879	Jung *et al.* (2003a)
16SrVI: Clover proliferation group			
VI-A	'*Ca.* Phytoplasma trifolii'	AY390261	Hiruki & Wang (2004)
16SrVII: Ash yellows group			
VII-A	'*Ca.* Phytoplasma fraxini'	AF092209	Griffiths *et al.* (1999)
16SrVIII: Loofah witches'-broom group			
VIII-A	Loofah witches'-broom phytoplasma	AF353090	2001 (GenBank submission)
16SrIX: Pigeon pea witches'-broom group			
IX-A	Pigeon pea witches'-broom phytoplasma	AF248957	2000 (GenBank submission)
IX-D	'*Ca.* Phytoplasma phoenicium'	AF515636	Verdin *et al.* (2003)
16SrX: Apple proliferation group			
X-A	'*Ca.* Phytoplasma mali'	AJ542541	Seemüller & Schneider (2004)
X-C	'*Ca.* Phytoplasma pyri'	AJ542543	Seemüller & Schneider (2004)
X-D	'*Ca.* Phytoplasma spartii'	X92869	Marcone *et al.* (2004a)
X-F	'*Ca.* Phytoplasma prunorum'	AJ542544	Seemüller & Schneider (2004)
16SrXI: Rice yellow dwarf group			
XI-A	'*Ca.* Phytoplasma oryzae'	AB052873	Jung *et al.* (2003b)
16SrXII: Stolbur group			

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Table 1. cont.

16Sr group	Strain	GenBank accession no.	Reference
XII-A	'*Ca.* Phytoplasma solani'	AJ964960	Firrao *et al.* (2005) (Incidental citation)
XII-B	'*Ca.* Phytoplasma australiense'	L76865	Davis *et al.* (1997)
XII-C	Strawberry lethal yellows phytoplasma	AJ243045	Padovan *et al.* (2000b)
XII-D	'*Ca.* Phytoplasma japonicum'	AB010425	Sawayanagi *et al.* (1999)
XII-E	'*Ca.* Phytoplasma fragariae'	DQ086423	Valiunas *et al.* (2006)
16SrXIII: Mexican periwinkle virescence group			
XIII-A	Mexican periwinkle virescence phytoplasma	AF248960	2000 (GenBank submission)
16SrXIV: Bermudagrass white leaf group			
XIV-A	'*Ca.* Phytoplasma cynodontis'	AJ550984	Marcone *et al.* (2004b)
16SrXV: Hibiscus witches'-broom group			
XV-A	'*Ca.* Phytoplasma brasiliense'	AF147708	Montano *et al.* (2001)
16SrXVI: Sugar cane yellow leaf syndrome group			
XVI-A	'*Ca.* Phytoplasma graminis'	AY725228	Arocha *et al.* (2005)
16SrXVII: Papaya bunchy top group			
XVII-A	'*Ca.* Phytoplasma caricae'	AY725234	Arocha *et al.* (2005)
16SrXVIII: American (TX+NE) potato purple top wilt group			
XVIII-A	'*Ca.* Phytoplasma americanum'	DQ174122	Lee *et al.* (2006)
16SrXIX: Japanese chestnut witches'-broom group			
XIX-A	'*Ca.* Phytoplasma castaneae'	AB054986	Jung *et al.* (2002)
16SrXX: Buckthorn witches' broom group			
XX-A	'*Ca.* Phytoplasma rhamni'	X76431	Marcone *et al.* (2004a)
16SrXXI: Pine shoot proliferation group			
XXI-A	'*Ca.* Phytoplasma pini'	AJ632155	Schneider *et al.* (2005)
16SrXXII: Nigerian coconut lethal decline (LDN) group			
XXII-A	Phytoplasma sp. strain LDN	Y14175	Tymon *et al.* (1998)
16SrXXIII: Buckland Valley grapevine yellows group			
XXIII-A	Buckland valley grapevine yellows phytoplasma	AY083605	Constable *et al.* (2002)
16SrXXIV: Sorghum bunchy shoot group			
XXIV-A	Sorghum bunchy shoot phytoplasma	AF509322	Blanche *et al.* (2003)
16SrXXV: Weeping tea tree witches'-broom group			
XXV-A	Weeping tea witches'-broom phytoplasma	AF521672	2002 (GenBank submission)
16SrXXVI: Mauritius sugar cane yellows D3T1 group			
XXVI-A	Sugar cane phytoplasma D3T1	AJ539179	2003 (GenBank submission)
16SrXXVII: Mauritius sugar cane yellows D3T2 group			
XXVII-A	Sugar cane phytoplasma D3T2	AJ539180	2003 (GenBank submission)
16SrXXVIII: Havana derbid phytoplasma group			
XXVIII-A	Derbid phytoplasma	AY744945	2004 (GenBank submission)

groups are shown in Fig. 2. Of the 51 phytoplasma strains (Table 1), 41 had previously been classified by means of the conventional phytoplasma 16S rRNA gene RFLP analysis (Lee *et al.*, 1998, 2006; Arocha *et al.*, 2005). The virtual 16S rRNA gene RFLP patterns of previously classified strains matched the RFLP patterns on real gels perfectly (data not shown). These results indicated that virtual RFLP analysis could serve as a convenient and reliable alternative to conventional RFLP analysis.

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Prior to the present study, over 40 16S rRNA gene RFLP pattern types had been documented in the literature, characterizing phytoplasma strains from a total of 18 groups (16SrI to 16SrXVIII) (Lee *et al.*, 1998, 2006; Arocha *et al.*, 2005). The results from the present study revealed that at least 10 previously unrecognized phytoplasma 16Sr groups can also be delineated. The expansion of the existing phytoplasma classification scheme to include these 10 new groups (16SrXIX to 16SrXXVIII) is justified by their distinct 16S rRNA gene RFLP patterns (Fig. 2) and by their lower-than-threshold coefficients of similarity with other groups. From the data presented in Table 2, it can be seen that, for each strain of a new group, the similarity coefficient is less than 0.85 with all strains in previously delineated groups. Recognition of the new groups is strengthened by their distinct cladistic positions in the maximum-parsimony phylogenetic tree [with one exception, where 16SrXXVIII (GenBank accession number AY744945) clustered together with 16SrI] (Fig. 1b).

Significantly, each of the 10 new 16Sr groups is represented by at least one either formally described or potential '*Candidatus* Phytoplasma' taxon. This result is consistent with the earlier proposal that each 16Sr group represents at least one species (Lee *et al.*, 1998). The new groups established in the present work, 16SrXIX, 16SrXX, and 16SrXXI, contain three recently named '*Candidatus* Phytoplasma', '*Candidatus* Phytoplasma castaneae' (Jung *et al.*, 2002), '*Candidatus* Phytoplasma rhamni' (Marcone *et al.*, 2004a) and '*Candidatus* Phytoplasma pini' (Schneider *et al.*, 2005), respectively. Phytoplasma strains representing the other seven new 16Sr groups are Nigerian coconut lethal decline phytoplasma strain LDN (GenBank accession number Y14175, 16SrXXII) (Tymon *et al.*, 1998), Buckland valley grapevine yellows phytoplasma (AY083605, 16SrXXIII) (Constable *et al.*, 2002), sorghum bunchy shoot phytoplasma (AF509322, 16SrXXIV) (Blanche *et al.*, 2003), weeping tea witches'-broom phytoplasma (AF521672, 16SrXXV), Mauritius sugar cane yellows phytoplasma strain D3T1 (diversity group 3, type 1) (AJ539179, 16SrXXVI), Mauritius sugar cane yellows phytoplasma strain D3T2 (diversity group 3, type 2) (AJ539180, 16SrXXVII) and Havana derbid phytoplasma (AY744945, 16SrXXVIII). All strains share less than 97.5 % 16S rRNA gene sequence similarity with each other and with any previously described '*Candidatus* Phytoplasma' taxa; therefore, each may be recognized as a novel '*Candidatus* Phytoplasma' taxon, in accordance with the concept put forward by Murray & Schleifer (1994) and the recommendation made by the Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group of the International Research Program on Comparative Mycoplasmology (IRPCM, 2004).

Thus far, 26 '*Candidatus* Phytoplasma' taxa have been described (Lee *et al.*, 2000, 2006; IRPCM, 2004; Arocha *et al.*, 2005; Firrao *et al.*, 2005; Valiunas *et al.*, 2006). The results from the present study, which point to an additional seven '*Candidatus*' taxa yet to be described, underscore the diversity within the phytoplasma clade. Conceivably, as more phytoplasma strains are discovered and become quickly characterized by virtual RFLP analysis, the total number of phytoplasma 16S rRNA gene RFLP pattern types will rise rapidly. The present work's accurate classification of 18 previously identified groups and delineation of 10 new groups has demonstrated that any phytoplasma strain can be readily classified based on RFLP patterns produced by *in silico* digestion. In fact, group level classification can be achieved by comparison of virtual RFLP patterns generated by digestion using three key restriction enzymes, namely, *Mse*I, *Rsa*I and *Hin*fI. As shown in Fig. 3, 19 of the 28 groups could be sufficiently differentiated by *Mse*I digestion alone and the remaining nine groups could be separated by comparison of *Mse*I and *Rsa*I digestion profiles or *Mse*I and *Hin*fI digestion profiles.

16S rRNA gene accessions with an exceptionally long or short F2nR2 region

During the process of delineating new phytoplasma groups, we paid special attention to those phytoplasma 16S rRNA gene accessions that had an unusually long or an unusually short F2nR2 region. In the cladistic analysis, these accessions clustered well with 16S rRNA gene sequences from phytoplasma strains in existing 16Sr groups 16SrI (GenBank accession number AY787141), 16SrII (AY787140, DQ286948, DQ286949, DQ286950, DQ286951, DQ286952, DQ387052, DQ868531, DQ868532 and DQ868533) and 16SrXII (AY725212, AY725230, AY725231, AY725232 and AY725233). However, due to a large (>100 bp) insertion or deletion within the F2nR2 region, these accessions each yielded an RFLP pattern type significantly different from those of any other 16Sr groups, including the 10 new 16Sr groups delineated in the present study. Low coefficients of 16S rRNA gene RFLP pattern similarity per se would qualify these accessions as belonging to at least three additional groups. Nevertheless, we would prefer to defer adding such new groups until the sequences are further verified, as such a large insertion or deletion within the conserved region of the 16S rRNA gene would inevitably alter the structure of the ribosome and, therefore, would be of biological significance.

Issues of *rrn* interoperon sequence heterogeneity

Many phytoplasma strains have two rRNA operons, *rrn*A and *rrn*B (Schneider & Seemüller, 1994; Firrao *et al.*, 1996b; Lauer & Seemüller, 2000; Padovan *et al.*, 2000a; Marcone & Seemüller, 2001). While *rrn*A and *rrn*B may be identical or nearly identical in some phytoplasma strains, apparent *rrn* interoperon sequence heterogeneity exists in other strains (Lee *et al.*, 1993b; Firrao *et al.*, 1996a; Liefting *et al.*, 1996; Davis & Sinclair, 1998; Jomantiene *et al.*, 2002). If sequence variations between two heterogeneous 16S rRNA genes affect the restriction sites in the F2nR2 region, in an actual gel an RFLP pattern may be a composite of the patterns from two sequence-heterogeneous rRNA operons. A

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Table 2. Similarity coefficients derived from analysis of virtual RFLP patterns of 16S rRNA genes from 51 representative phytoplasma strains

Representative strains of the 10 new groups (16SrXIX to 16SrXXVIII) are shaded. Designations and representative strains of groups 16SrI through 16SrXVIII are given in accordance with prior literature.

	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
1	I-A (AYWB)	1.00
2	I-B (OY-M & M30790)	0.94	1.00
3	I-C (AF222065)	0.93	0.93	1.00
4	I-D (AY265206)	0.91	0.97	0.90	1.00
5	I-E (AY265213)	0.93	0.93	0.92	0.92	1.00
6	I-F (AY265211)	0.92	0.90	0.91	0.91	0.91	1.00
7	II-A (L33765)	0.50	0.51	0.48	0.52	0.48	0.48	1.00
8	II-B (U15442)	0.50	0.49	0.47	0.51	0.47	0.53	0.90	1.00
9	II-C (AJ293216)	0.50	0.51	0.48	0.51	0.48	0.53	0.93	0.93	1.00
10	II-D (Y10097)	0.53	0.54	0.50	0.54	0.50	0.56	0.95	0.93	0.98	1.00
11	III-A (L04682)	0.57	0.52	0.55	0.51	0.53	0.58	0.59	0.59	0.61	0.62	1.00
12	III-B (AF189288)	0.59	0.54	0.57	0.53	0.57	0.60	0.63	0.63	0.65	0.66	0.92	1.00
13	IV-A (AF498307)	0.54	0.51	0.52	0.49	0.49	0.49	0.48	0.47	0.50	0.51	0.74	0.72	1.00
14	IV-B (AF500334)	0.48	0.45	0.46	0.44	0.44	0.45	0.47	0.46	0.48	0.49	0.75	0.73	0.88	1.00
15	IV-D (AF237615)	0.52	0.49	0.49	0.47	0.47	0.49	0.51	0.49	0.52	0.53	0.79	0.77	0.91	0.97	1.00
16	V-A (AY197655))	0.52	0.47	0.49	0.45	0.43	0.40	0.48	0.47	0.50	0.51	0.68	0.68	0.63	0.62	0.61	1.00
17	V-B (AB052876)	0.50	0.45	0.47	0.43	0.43	0.47	0.53	0.52	0.54	0.55	0.68	0.70	0.61	0.59	0.59	0.93	1.00
18	V-C (AY197642)	0.49	0.44	0.47	0.45	0.41	0.46	0.48	0.47	0.49	0.51	0.65	0.65	0.60	0.59	0.58	0.97	0.90	1.00
19	V-G (AB052879)	0.54	0.48	0.51	0.47	0.47	0.48	0.50	0.49	0.52	0.53	0.65	0.67	0.60	0.57	0.56	0.90	0.97	0.87	1.00
20	VI-A (AY390261)	0.57	0.50	0.55	0.48	0.34	0.39	0.52	0.51	0.53	0.54	0.76	0.76	0.69	0.65	0.67	0.89	0.89	0.86	0.86	1.00
21	VII-A (AF092209)	0.56	0.55	0.51	0.53	0.36	0.39	0.52	0.50	0.52	0.53	0.69	0.69	0.65	0.59	0.60	0.77	0.77	0.74	0.80	0.85	1.00
22	VIII-A (AF353090)	0.60	0.54	0.59	0.55	0.55	0.50	0.58	0.57	0.60	0.60	0.76	0.76	0.67	0.65	0.69	0.80	0.80	0.77	0.77	0.89	0.74	1.00
23	IX-A (AF248957)	0.52	0.45	0.48	0.44	0.40	0.41	0.52	0.50	0.54	0.53	0.59	0.59	0.56	0.55	0.58	0.58	0.56	0.56	0.56	0.64	0.60	0.68	1.00
24	IX-D (AF515636)	0.62	0.54	0.57	0.53	0.46	0.46	0.56	0.55	0.60	0.58	0.72	0.72	0.69	0.67	0.71	0.69	0.69	0.66	0.66	0.80	0.66	0.84	0.77	1.00
25	X-A (AJ542541)	0.58	0.47	0.58	0.54	0.52	0.55	0.36	0.43	0.46	0.45	0.57	0.57	0.46	0.40	0.43	0.43	0.52	0.56	0.52	0.51	0.46	0.53	0.42	0.49	1.00
26	X-C (AJ542543)	0.61	0.47	0.60	0.56	0.54	0.58	0.37	0.44	0.46	0.45	0.58	0.58	0.46	0.40	0.44	0.44	0.53	0.57	0.52	0.52	0.46	0.54	0.42	0.49	0.97	1.00
27	X-D (X92869)	0.59	0.50	0.61	0.57	0.55	0.56	0.32	0.46	0.49	0.48	0.50	0.58	0.45	0.37	0.40	0.36	0.45	0.46	0.51	0.43	0.45	0.46	0.37	0.41	0.83	0.86	1.00
28	X-F (AJ542544)	0.60	0.46	0.59	0.55	0.53	0.55	0.38	0.43	0.41	0.42	0.59	0.59	0.47	0.41	0.45	0.41	0.47	0.51	0.47	0.48	0.43	0.51	0.41	0.48	0.90	0.93	0.80	1.00
29	XI-A (AB052873)	0.63	0.56	0.58	0.54	0.54	0.60	0.61	0.58	0.63	0.62	0.75	0.75	0.68	0.60	0.64	0.68	0.68	0.65	0.67	0.76	0.78	0.76	0.61	0.70	0.66	0.69	0.62	0.65	1.00
30	XII-A (AJ964960)	0.87	0.88	0.88	0.87	0.87	0.81	0.47	0.46	0.47	0.48	0.53	0.55	0.53	0.49	0.51	0.48	0.46	0.46	0.48	0.45	0.47	0.54	0.41	0.47	0.51	0.53	0.53	0.56	0.59	1.00
31	XII-C (AJ243045)	0.92	0.88	0.89	0.87	0.87	0.86	0.49	0.50	0.50	0.51	0.59	0.61	0.54	0.53	0.54	0.54	0.52	0.52	0.52	0.51	0.46	0.60	0.44	0.53	0.54	0.57	0.53	0.58	0.61	0.93	1.00
32	XII-B (L76865)	0.88	0.85	0.85	0.85	0.83	0.82	0.48	0.49	0.49	0.50	0.56	0.58	0.51	0.49	0.51	0.51	0.49	0.51	0.48	0.48	0.43	0.56	0.41	0.50	0.55	0.58	0.52	0.59	0.58	0.90	0.96	1.00
33	XII-D (AB010425)	0.72	0.78	0.77	0.77	0.71	0.78	0.41	0.42	0.42	0.43	0.47	0.47	0.46	0.42	0.44	0.48	0.46	0.45	0.45	0.49	0.48	0.51	0.42	0.51	0.46	0.48	0.49	0.52	0.48	0.78	0.78	0.75	1.00
34	XII-E (DQ086423)	0.85	0.90	0.88	0.88	0.83	0.77	0.45	0.46	0.47	0.48	0.51	0.53	0.55	0.49	0.51	0.51	0.48	0.48	0.50	0.52	0.52	0.56	0.43	0.54	0.53	0.55	0.55	0.56	0.55	0.89	0.85	0.82	0.83	1.00
35	XIII-A (AF248960)	0.69	0.73	0.72	0.74	0.66	0.68	0.51	0.50	0.51	0.52	0.54	0.56	0.47	0.45	0.47	0.49	0.47	0.48	0.46	0.44	0.45	0.52	0.37	0.42	0.57	0.60	0.56	0.63	0.56	0.72	0.71	0.69	0.69	0.76	1.00
36	XIV-A (AJ550984)	0.59	0.52	0.56	0.52	0.50	0.54	0.51	0.48	0.53	0.52	0.71	0.71	0.66	0.58	0.62	0.73	0.73	0.76	0.72	0.81	0.74	0.74	0.61	0.70	0.66	0.67	0.67	0.63	0.87	0.51	0.53	0.52	0.42	0.49	0.49	1.00
37	XV-A (AF147708)	0.53	0.53	0.54	0.52	0.52	0.58	0.80	0.78	0.83	0.85	0.64	0.68	0.57	0.55	0.59	0.63	0.65	0.60	0.63	0.65	0.57	0.71	0.65	0.69	0.46	0.47	0.43	0.46	0.66	0.53	0.55	0.51	0.53	0.55	0.51	0.60	1.00
38	XVI-A (AY725228)	0.54	0.55	0.54	0.54	0.54	0.46	0.25	0.27	0.25	0.27	0.28	0.30	0.28	0.24	0.26	0.24	0.24	0.24	0.26	0.25	0.29	0.27	0.21	0.23	0.28	0.29	0.30	0.32	0.31	0.62	0.52	0.50	0.45	0.58	0.44	0.27	0.27	1.00
39	XVII-A (AY725234)	0.52	0.55	0.54	0.54	0.56	0.46	0.25	0.29	0.25	0.27	0.22	0.26	0.22	0.20	0.22	0.20	0.20	0.18	0.24	0.21	0.27	0.25	0.21	0.23	0.20	0.20	0.24	0.24	0.27	0.62	0.52	0.51	0.45	0.55	0.36	0.20	0.27	0.57	1.00
40	XVIII-A (DQ174122)	0.83	0.83	0.88	0.82	0.82	0.79	0.50	0.51	0.51	0.52	0.59	0.61	0.58	0.52	0.56	0.52	0.52	0.49	0.53	0.59	0.55	0.63	0.51	0.61	0.60	0.63	0.65	0.63	0.63	0.85	0.81	0.78	0.71	0.83	0.66	0.58	0.58	0.54	0.54	1.00
41	XIX-A (AB054986)	0.52	0.49	0.49	0.49	0.47	0.51	0.51	0.49	0.52	0.53	0.62	0.60	0.74	0.66	0.70	0.59	0.59	0.58	0.58	0.69	0.65	0.71	0.56	0.67	0.54	0.55	0.57	0.52	0.68	0.46	0.48	0.47	0.40	0.46	0.41	0.66	0.51	0.24	0.20	0.52	1.00
42	XX-A (X76431)	0.49	0.40	0.46	0.44	0.44	0.46	0.32	0.42	0.47	0.46	0.56	0.69	0.47	0.41	0.45	0.38	0.40	0.42	0.40	0.46	0.39	0.54	0.49	0.50	0.81	0.84	0.71	0.80	0.65	0.44	0.45	0.44	0.35	0.42	0.48	0.73	0.37	0.26	0.20	0.46	0.62	1.00
43	XXI-A (AJ632155)	0.41	0.40	0.40	0.40	0.40	0.42	0.48	0.44	0.47	0.48	0.52	0.50	0.64	0.56	0.60	0.47	0.47	0.46	0.46	0.57	0.57	0.59	0.47	0.52	0.38	0.39	0.35	0.38	0.62	0.40	0.37	0.36	0.31	0.38	0.36	0.56	0.49	0.28	0.20	0.42	0.62	0.35	1.00
44	XXII-A (Y14175)	0.51	0.48	0.51	0.46	0.44	0.44	0.46	0.44	0.47	0.48	0.65	0.63	0.74	0.71	0.72	0.60	0.60	0.57	0.59	0.67	0.63	0.70	0.57	0.67	0.38	0.39	0.42	0.38	0.62	0.46	0.47	0.44	0.41	0.46	0.40	0.60	0.47	0.26	0.24	0.48	0.79	0.50	0.65	1.00
45	XXIII-A (AY083605)	0.78	0.77	0.82	0.76	0.80	0.72	0.48	0.50	0.51	0.50	0.52	0.54	0.47	0.41	0.45	0.45	0.43	0.42	0.46	0.50	0.47	0.56	0.41	0.48	0.47	0.47	0.54	0.48	0.56	0.74	0.73	0.69	0.61	0.76	0.67	0.49	0.51	0.50	0.48	0.72	0.43	0.44	0.42	0.42	1.00
46	XXIV-A (AF509322)	0.46	0.43	0.47	0.41	0.41	0.45	0.42	0.39	0.44	0.43	0.64	0.62	0.80	0.77	0.80	0.65	0.65	0.62	0.65	0.76	0.67	0.73	0.56	0.69	0.48	0.48	0.53	0.45	0.66	0.46	0.44	0.41	0.40	0.46	0.39	0.70	0.48	0.26	0.20	0.49	0.72	0.62	0.68	0.74	0.39	1.00
47	XXV-A (AF521672)	0.55	0.53	0.52	0.52	0.50	0.56	0.59	0.58	0.61	0.61	0.64	0.66	0.57	0.55	0.57	0.61	0.61	0.60	0.58	0.65	0.59	0.65	0.51	0.60	0.57	0.60	0.54	0.58	0.57	0.47	0.51	0.48	0.44	0.49	0.46	0.57	0.59	0.25	0.22	0.52	0.57	0.49	0.41	0.54	0.48	0.51	1.00
48	XXVI-A (AJ539179)	0.64	0.59	0.59	0.57	0.57	0.51	0.40	0.39	0.41	0.42	0.63	0.63	0.75	0.76	0.73	0.56	0.56	0.53	0.55	0.62	0.56	0.59	0.47	0.59	0.45	0.46	0.42	0.47	0.57	0.58	0.58	0.55	0.39	0.58	0.46	0.59	0.42	0.36	0.32	0.63	0.62	0.44	0.55	0.65	0.53	0.67	0.54	1.00
49	XXVII-A (AJ539180)	0.73	0.72	0.73	0.71	0.71	0.69	0.50	0.51	0.51	0.52	0.69	0.71	0.77	0.69	0.72	0.57	0.57	0.55	0.61	0.65	0.63	0.65	0.55	0.67	0.51	0.54	0.56	0.55	0.62	0.69	0.71	0.68	0.57	0.67	0.56	0.62	0.52	0.44	0.42	0.77	0.60	0.52	0.50	0.58	0.64	0.62	0.54	0.74	1.00
50	XXVIII-A (AY744945)	0.45	0.50	0.45	0.50	0.49	0.42	0.27	0.29	0.25	0.27	0.24	0.26	0.24	0.19	0.22	0.16	0.16	0.16	0.20	0.17	0.27	0.21	0.18	0.19	0.22	0.21	0.24	0.24	0.27	0.44	0.41	0.40	0.35	0.42	0.35	0.21	0.26	0.46	0.51	0.47	0.24	0.20	0.26	0.22	0.35	0.20	0.20	0.26	0.40	1.00

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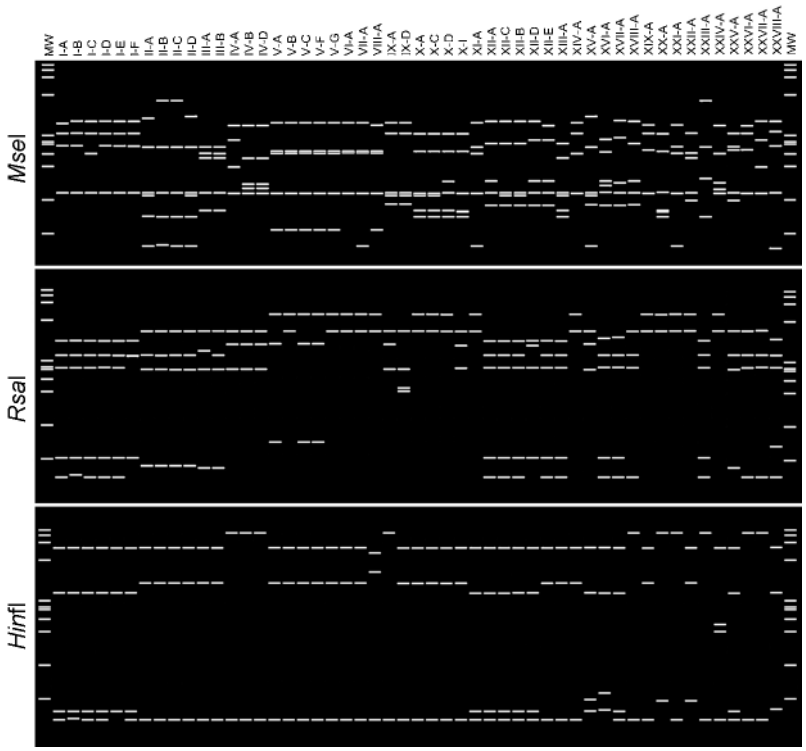

Fig. 3. Key restriction enzymes that distinguish phytoplasma groups. Nineteen of the 28 phytoplasma groups can be differentiated by *Mse*I digestion alone. The remaining nine groups can be distinguished by digestion using additional enzymes as follows: separation of 16SrV from 16SrVIII, 16SrIV from 16SrXXIV and 16SrXIX from16SrXXVI can be achieved by comparisons of *Mse*I *and Rsa*I banding patterns; groups 16SrVII, 16SrXI and 16SXXI can be separated from each other by *Mse*I and *Hinf*I digestions. MW, φX174DNA-*Hae*III digestion.

composite pattern is suspected when the sum of the sizes of DNA fragments is greater than the expected size of the F2nR2 region (1.25 kb). Furthermore, RFLP analysis of a phytoplasma's sequence-heterogeneous rRNA operons in mutual isolation could result in erroneous assignment of the same phytoplasma to two different 16S rRNA subgroups, or putative taxa, in classification schemes that are based on RFLP patterns (Davis *et al.*, 2003). Although such a composite banding pattern may not be encountered in virtual RFLP analysis, a 'chimaeric' banding pattern could arise due to nucleotide sequencing of an uncloned PCR product if the analysed sequence is a consensus that contains bases from two sequence-heterogeneous operons. Thus, for accurate classification of a phytoplasma strain, it is preferable to sequence 16S rRNA genes after separation of *rrn* operons by cloning.

We examined 16S rRNA gene sequences from both *rrn*A and *rrn*B operons of 17 phytoplasma strains. Of the 17 strains, four yielded identical virtual 16S rRNA gene RFLP patterns for the *rrn*A and *rrn*B operons (AYWB, OY-M, *Carludovica palmata* leaf yellowing phytoplasma and loofah witches'-broom phytoplasma strain LfWB). The remaining 13 strains yielded discrete 16S rRNA gene banding patterns for the *rrn*A and *rrn*B operons. However, the differences in the patterns were minor and did not affect the group classification of those phytoplasma strains.

Conclusion

The availability of a comprehensive set of phytoplasma 16S rRNA gene RFLP pattern types (Lee *et al.*, 1993b, 1998, 2000) has made possible the accurate and reliable identification, differentiation and classification of a broad array of phytoplasmas and has greatly stimulated and expanded phytoplasma research over the past decade. Typically, RFLP analysis of DNA segments has been done in the absence of prior nucleotide sequence information. Nowadays, as sequence information has become readily available, either by database retrieval or by *de novo* determination, one may ask whether RFLP analysis still remains a useful tool for phytoplasma identification, differentiation, and classification. We suggest that it does. First, the already established phytoplasma 16S rRNA gene RFLP patterns have become authoritative expositions for scientists in the phytoplasma research community and have served as standard keys for phytoplasma strain identification and classification. Second, although sequence information-based analyses such as pairwise sequence comparisons and phylogenetic analyses can be used to assess the relationships among phytoplasma strains, neither percentage sequence similarities from pairwise comparisons nor tree topologies from phylogenetic analyses directly reveal informative sites along the sequences or 'visible' genetic footprints provided by RFLP analysis. While RFLP analysis remains a valuable tool for studying microbial diversity and classification, the method by which RFLP analysis is carried out has evolved (Moyer *et al.*, 1996; Edwards & Turco, 2005; Ricke *et al.*, 2005; Abdo *et al.*, 2006). The virtual RFLP analysis method implemented in the present study simulates laboratory restriction enzyme digestions and subsequent gel electrophoresis, quickly generating reproducible RFLP patterns. These

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computer-generated patterns not only faithfully replicate the classical, authoritative pattern types that have been established by conventional RFLP analysis but also reveal new pattern types that have not been recognized previously, providing additional standard keys for future identification and classification of the rapidly growing numbers of phytoplasmas by either computer-simulated or conventional RFLP analyses. The value of virtual RFLP analysis was evident in the delineation of 10 new phytoplasma groups, in the elucidation of candidates for novel species descriptions and in the recognition of about 50 new, potentially significant subgroup lineages. The virtual 16S rRNA gene RFLP pattern types generated from 51 representative phytoplasma strains will be accessible online at http://www.ba.ars.usda.gov/data/mppl/virtual-gel.html as reference patterns. A web interface will soon be developed for users to enter sequences, create and compare RFLP patterns and update the classification scheme as new pattern types are identified.

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