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Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences

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RFLP analyses of 16S rDNA nested PCR products from 34 phytoplasma strains with 17 restriction enzymes delineated distinct pattern types. Based on similarity coefficients derived from RFLP analyses, the 34 representative phytoplasma strains were differentiated into 14 major groups (termed 16Sr groups) and 32 sub-groups. The similarity coefficients of RFLP patterns between distinct groups were 90% or below. By including additional groups and sub-groups from which RFLP analyses were not performed but for which 16S rDNA sequence data were available to predict restriction sites, a total of 14 groups and 41 sub-groups were proposed. By combined RFLP analyses of 16S rRNA and ribosomal protein gene sequences, thus far, a total of 46 sub-groups have been recognized. The phytoplasma 16Sr groups were consistent with the phylogenetic groups (subclades) defined by phylogenetic analysis of near-full-length 16S rRNA gene sequences, indicating that the RFLP-based groups are phylogenetically valid. The approach using RFLP analyses of PCR-amplified 16S rDNA (and ribosomal protein gene sequences) provides a simple, reliable and rapid means for differentiation and classification of unknown phytoplasmas.

Keywords: phytoplasma classification, RFLP, 16S rRNA, ribosomal protein, mycoplasma-like organism

INTRODUCTION

Phytoplasmas, formerly called mycoplasma-like organisms, are associated with diseases in several hundred plant species (101, 126). Thus far, none has been cultured *in vitro* (77). Until the last decade, differentiation and classification of uncultured phytoplasmas relied primarily on their biological properties, such as specificity of plant and insect hosts, and symptomatology of affected plants (15–18, 35, 39, 43, 70). The determination of biological properties has often been time-consuming, laborious and sometimes unreliable (77, 78). The less laborious molecular-based analyses introduced in the last decade have proved to be more accurate and reliable for identification of phytoplasmas (13, 77). The use of molecular probes, phytoplasma-specific cloned DNA and monoclonal antibodies have made it possible to classify phytoplasmas on the basis of DNA–DNA homology and serological data (6, 11–14, 19, 20, 25–27, 30, 32, 34, 53, 56, 57, 64, 67, 69, 72, 76–81, 91, 100, 103, 120, 122). For example, based on dot- and Southern-hybridization analyses using cloned phytoplasma DNA probes, several distinct phytoplasma strain clusters have been recognized (32, 53, 56, 67–69, 72, 76, 78–81, 100, 103). However, the sensitivity of these types of molecular probes was insufficient for many phytoplasmas associated with woody plants, in which phytoplasma concentrations are relatively low. The development of PCR assays using specific primers based on cloned phytoplasma DNA sequences allowed a more sensitive means for phytoplasma detection (31, 34, 36, 44, 52, 54, 55, 60, 78, 80, 92, 110, 113).

Recently, phylogenetic analyses based on 16S rRNA and ribosomal protein gene sequences have revealed that the uncultured phytoplasmas form a large discrete

Abbreviations: 16Sr, 16S rRNA; rp, ribosomal protein; ICSB, International Committee on Systematic Bacteriology.

Table 1. Classification of phytoplasmas based on RFLP (or putative restriction site) analyses of 16S rRNA and ribosomal protein gene sequences

16Sr group	Strain	Original source	16Sr-rp sub-group	Accession no. (16Sr)	Accession no. (rp)	Reference
16SrI (Aster yellows group)						
I-A	Tomato big bud BB	Tomato: Arkansas	16SrI-A(rp-A)	L33760	L27004	23, 78
I-A	New Jersey aster yellows NJAY	Lettuce: New Jersey	16SrI-A(rp-A)			78, 91
I-A	Aster yellows AY27	Aster: Canada	16SrI-A(rp-A)			78
I-A	Eastern aster yellows NAY	Lettuce: Canada	16SrI-A(rp-A)			78
I-A	Periwinkle little leaf CN1	Periwinkle: Connecticut	16SrI-A(rp-A)			30, 78
I-A	Oklahoma aster yellows OKAY1	Lettuce: Oklahoma	16SrI-A(rp-A)			35, 78
I-B	Michigan aster yellows MIAY	Evening primrose: Michigan	16SrI-B(rp-B)	M30970	M77470	88, 89
I-B	Maryland aster yellows AY1	Periwinkle: Maryland	16SrI-B(rp-B)	L33767		78
I-B	American aster yellows AAY	Periwinkle: Florida		X68373		121
I-B	Dwarf aster yellows DAY	Clover: California				78
I-B	Western aster yellows SAY	Celery: California	16SrI-B(rp-B)	M86340		78
I-B	Aster yellows OKAY3	Carrot: Okalahoma	16SrI-B(rp-B)			35, 78
I-B	Western aster yellows TLAY	Potato: California				78
I-B	Hydrangea phyllody HyPH1	Hydrangea: Italy	16SrI-B(rp-K)			134
I-B	Chrysanthemum yellows CY	Chrysanthemum: Italy				6, 78
I-B	Onion yellows OAY (OA)	Onion: Japan		D12569		105
I-B	European aster yellows EAY	Aster: Gemany				134
I-B	Aster yellows-Koolsard KD	Cabbage: UK				134
I-B	Aster yellows-Cactus CC	Cactus: UK				134
I-B	Primula yellows PY	Primula: Germany				134
I-B	Gladiolus yellows GLY	Gladiolus: UK				134
I-B	Hydrangea virescence	Hydrangea: Belgium				134
I-B	Mitsuba witches'-broom JHW	*Cryptotaenia*: Japan				107
I-B	Garland chrysanthemum WB GCW	*Chrysanthemum coronarium*: Japan				107
I-B	Eggplant dwarf ED	Eggplant: Japan				107
I-B	Tomato yellows TY	Tomato: Japan				107
I-B	Marguerite yellows MY	*Chrysanthemum frutescens*: Japan				107
I-B	Ipomoea witches'-broom IOB	*Ipomoea* sp.: Taiwan	16SrI-B(rp-F)			83
I-B	Maize bushy stunt MBS	Corn: Ohio, Mexico	16SrI-B(rp-L)			26 52, 83
I-B	Mulberry dwarf MD	Mulberry: Japan, China				105
I-C	Clover phyllody CPh	Clover: Canada	16SrI-C(rp-C)	L33762		78
I-C	Strawberry green petal SGP	Strawberry: Canada	16SrI-C(rp-C)			78
I-C	Ranunculus phyllody RPh	Ranunculus: Italy	16SrI-C(rp-C)			48
I-D	Paulownia witches'-broom PaWB	Paulownia: Taiwan	16SrI-D(rp-D)			78
I-E	Blueberry stunt BBS1 BBS3	Blueberry: Michigan, Arkansas	16SrI-E(rp-E)			78
I-F	Apricot chlorotic leaf roll ACLR-AY	Apricot: Spain		X68383		114
I	Grey dogwood witches'-broom GD1	Grey dogwood: USA	16SrI(rp-M)			46
16SrII (Peanut WB group)						
II-A	Peanut witches'-broom PnWB	Peanut: Taiwan		L33765		83
II-A	Sweet potato witches'-broom SPWB	Sweet potato: Taiwan		L33770		83
II-A	Sunhemp witches'-broom SUNHP	Sunhemp: Thailand		X76433		115
II-B	Witches'-broom of lime WBDL	Lime: Arabic Peninsula		U15442		145
	'*Cadidatus* Phytoplasma aurantifolia'					
II-C	Faba bean phyllody FBP	Faba bean: Sudan		X83432		115
II-D	Sweet potato little leaf SPLL	Sweet potato: Australia				41
16SrIII (X-disease group)						
III-A	X-disease CX	Peach: Canada	16SrIII-A(rp-A)	L33733	L27016	81, 83
III-A	X-disease WX	Peach: California	16SrIII-A(rp-B)	L04682	L27047	81, 83
III-A	Peach yellow leaf roll PYLR	Peach: California	16SrIII-A(rp-B)			48
III-A	X-disease CCX	Choke cherry: New York	16SrIII-A(rp-B)			48
III-B	Clover yellow edge CYE	Clover: Canada	16SrIII-B(rp-C)	L33766	L27019	81, 83
III-B	Vaccinium witches'-broom VAC	Vaccinium: Germany		X76430		114, 121
III-B	Tsuwabuki witches'-broom TW	Farfugium: Japan		D12580		107
III-B	Gentian witches'-broom GW	Gentian: Japan				107
III-C	Pecan bunch PB	Pecan: Georgia	16SrIII-C(rp-G)			48
III-D	Goldenrod yellows GRY (GR1)	Goldenrod: New York	16SrIII-D(rp-E)			45, 48
III-E	Spiraea stunt SP1	Spiraea: New York	16SrIII-E(rp-F)			45, 48
III-F	Milkweed yellows MWY (MW1)	Milkweed: New york	16SrIII-F(rp-D)			45, 48
III-G	Walnut witches'-broom WWB	Walnut: Georgia	16SrIII-G(rp-B)			12, 45
III-H	Poinsettia branch-inducing PoiBI	Poinsettia: US				84
16SrIV (Coconut lethal yellows group)						
IV-A	Coconut lethal yellows LY, LY3	Palm: Florida		U18747		53–55
IV-B	Yucatan coconut lethal decline LDY	Palm: Mexico		U18753		54
IV-C	Tanzanian coconut lethal decline LDT	Palm: Africa		X80117		54
16SrV (Elm yellows group)						
V-A	Elm yellows EY1	Elm: New York	16SrV-A(rp-A)	L33763	L27022	80
V-A	Elm yellows ItaEY	Elm: Italy				75, 80
V-A	Elm witches'-broom ULW	Elm: France		X68376		100, 121
V-B	Cherry lethal yellows CLY	Cherry: China	16SrV-B(rp-B)			86, 144
V-B	Jujube witches'-broom JWB	Jujube: China	16SrV-B(rp-C)			144
V-C	Rubus stunt RS	Rubus: Italy				75
V-C	Alder yellows AlY	Alder: Germany				100

Table 1. *(cont.)*

16Sr group	Strain	Original source	16Sr-rp sub-group	Accession no. (16Sr)	Accession no. (rp)	Reference
V-C	Spartium witches'-broom (EY)	Spartium: Italy				97
V-C	Eucalyptus little leaf	Eucalyptus: Italy				97
V-C	Flavescence dorée FD	Grapevine: France	16SrV-C(rp-D)	X76560		20–22
16SrVI (Clover proliferation group)						
VI-A	Clover proliferation CP	Clover: Canada		L33761	L27011	34, 79
VI-A	Periwinkle virescence VR, BLTVA	Periwinkle: California				79, 122
VI-A	Tomato big bud TBB	Tomato: California				122
VI-A	Potato witches'-broom PWB	Potato: Canada				34, 79
VI-A	Potato yellows	Potato: North Dakota				This study
16SrVII (Ash yellows group)						
VII-A	Ash yellows AshY	Ash: New York		X68339	L26999	32, 127
VII-A	Lilac witches'-broom LiWB	Lilac: New York				57
16SrVIII (Loofah witches'-broom group)						
VIII-A	Loofah witches'-broom LfWB	Loofah: Taiwan		L33764	L27027	83
16SrIX (Pigeon pea witches'-broom group)						
IX-A	Pigeon pea witches'-broom PPWB	Pigeon pea: Florida		U18763	L27036	56
16SrX (Apple proliferation group)						
X-A	Apple proliferation AT, AP-A	Apple: Germany, Italy		X68375	L27994	37, 68, 93
X-B	Apricot chlorotic leaf roll ACLR (Ita)	Apricot: Italy				7, 74
X-B	Plum leptonecrosis PLN	Japanese plum: Italy				74
X-B	European stone fruit yellows PPER	Peach: Germany		X68374		114
X-C	Pear decline PD	Pear: Italy, UK				7, 24, 74
X-D	Spartium witches'-broom SPAR	Spartium: Italy		X92869		97
X-E	Black alder witches'-broom BAWB (Buckthorn witches'-broom BWB)	Black alder (Buckthorn): Germany		X76431		121
16SrXI (Rice yellow dwarf group)						
XI-A	Rice yellow dwarf RYD	Rice: Japan, India		D12581		85, 103, 105
XI-B	Sugarcane white leaf SCWL	Sugarcane: Thailand		X76432		85, 103, 105
XI-B	Sugarcane grassy shoot SCGS	Sugarcane: India				85
XI-C	Leafhopper-borne BVK	*Psammotettix cephalotes*: Germany		X76429		121
16SrXII (Stolbur group)						
XII-A	Stolbur STOL	*Capsicum annum*: Serbia		X76427		121
XII-A	Grapevine yellows	Grapevine: Germany		X76428		94, 121
XII-A	Celery yellows CelY	Celery: Italy				134
XII-B	Australian grapevine yellows AUSGY '*Candidatus* Phytoplasma australiense'	Grapevine: Australia		L76865		28, 109
XII-B	Phormium yellow leaf PYL	New Zealand flax: New Zealand		U43570		87
16SrXIII (Mexican periwinkle virescence group)						
XIII-A	Mexican periwinkle virescence MPV	Periwinkle: Mexico				48
XIII-B	Strawberry green petal (Florida)	Strawberry: Florida				61, 62
16SrXIV (Bermudagrass white leaf group)						
XIV-A	Bermudagrass white leaf BGWL	Bermudagrass: Thailand				85
XIV-A	Annual blue grass white leaf ABGWL	*Poa annua*: Italy				85

monophyletic clade within the class *Mollicutes* (49, 64, 65, 71, 88–90, 105, 115, 119, 121, 130). The phylogenetic interrelationships among representative phytoplasmas provide a basis for establishing a phylogenetically valid classification (49, 105, 121). PCR using phytoplasma group-specific or universal primers derived from conserved 16S rRNA gene sequences has provided, for the first time, a sensitive means for detection of a broad array of phytoplasmas from infected plants or insect vectors (1–3, 24, 29, 33, 46, 47, 51, 74, 83, 93, 104, 114, 128). By direct sequence analysis or RFLP analysis of PCR-amplified 16S rDNA, the phytoplasmas detected can be differentiated and classified (83, 114, 118). Several classification systems have been proposed either directly, based on sequence analysis or indirectly, by RFLP analysis of PCR-amplified 16S rDNA. Classification by RFLP analysis has provided a simple and rapid method that can be used to differentiate and identify a large number of unclarified phytoplasmas in a relatively short period of time. However, in some cases, the phytoplasma groups classified on the basis of RFLP analyses using few restriction enzymes were not always consistent with groups based on phylogenetic analysis of 16S rRNA gene sequences (114, 121).

Our objective was to develop a comprehensive classification scheme based on RFLP analysis of phytoplasma 16S rDNA sequences that is phylogenetically valid. On the basis of similarity coefficients of collective RFLP patterns derived by extensive RFLP analyses of PCR-amplified 16S rDNA with 15 restriction enzymes, we have previously proposed a classification scheme that comprises nine distinct phytoplasma groups (83). The grouping is consistent with the strain clusters previously identified, based on DNA–DNA homology and serological data (32, 53, 56, 67–69, 72, 78–81, 100). Sub-groups within each group were determined, based on the dissimilarity of restriction sites identified by RFLP analyses of 16S rDNA. Fourteen sub-groups

were preliminarily identified. The classification scheme was later expanded to include one additional group and six new sub-groups (28, 45, 48–50, 61, 62, 134). Moreover, combined RFLP analyses of 16S rDNA and ribosomal protein gene sequences were proposed for finer sub-group differentiation (48, 50). The sub-groups delineated by this combined approach are more consistent with the subclusters identified based on DNA–DNA homology.

As many new phytoplasma strains have been identified in the last four years (7–9, 12, 14, 21–22, 27, 28, 40–42, 54, 57, 61, 62, 84–87, 93–100, 107, 109, 110, 115, 116, 133–135, 142, 144, 145), it has been necessary to update and expand the current scheme. The objectives of this study were to revise and further expand the current RFLP-based classification scheme into a comprehensive classification system in which representative strains of known phytoplasmas were included and to validate the phytoplasma classification by parallel phylogenetic analysis using full-length 16S rRNA gene sequences.

METHODS

Sources of phytoplasma strains. Phytoplasma strains used in this study are listed in Table 1. Total nucleic acid was extracted from freshly collected or oven-dried tissues according to a previously described procedure (80).

Primers and PCR conditions. The two universal primer pairs, R16mF2/R16mR1 and R16F2n/R16R2, were previously designed based on 16S rRNA gene sequences and used for amplification of phytoplasma 16S rDNA (47, 83). Nested PCR with the primer pair R16mF2/R16mR1, followed by R16F2n/R16R2, was used to detect putative phytoplasmas from each of the nucleic acid preparations. Nested PCR can also be performed by using the universal primer pair P1/P7 (118) followed by R16F2n/R16R2. The primer pair rpF1/rpR1, designed by Lim & Sears (90) was used to amplify a segment of the ribosomal protein gene operon from members of phytoplasma 16S rRNA (16Sr) group I (aster yellows and related phytoplasmas) and group III (X-disease and related phytoplasmas). Two primer pairs, rp(V)FI/rpR1 and rp(V)F2/rpR1 were used to amplify ribosomal protein gene sequences from members of 16Sr group V (elm yellows and related phytoplasmas). The oligonucleotide sequences of the primers used in this study are: R16mF2, 5′-CATGCAAGTCGAACGA-3′; R16mR1, 5′-CTTAACCCCAATCATCGAC-3′; R16F2n, 5′-GAAACGACTGCTAAGACTGG-3′; R16R2, 5′-TGACGGGCGGTGTGTACAAACCCCG-3′; rpF1, 5′-GGACATAAGTTAGGTGAATTT-3′; rpR1, 5′-ACGATATTTAGTTCTTTTTGG-3′; rp(V)F1, 5′-TCGCGGTCATGCAAAAGGCG-3′; rp(V)F2, 5′-TTGCCTCGTTTATTTCCGAGAGCTA-3′. Semi-nested PCR with rp(V)F1/rpR1 followed by rp(V)F2/rpR1 was used to amplify ribosomal protein gene sequences from members of 16Sr group V.

For PCR amplification, 35 cycles were conducted in an automated thermocycler (Perkin Elmer DNA Thermal Cycler 480) with AmpliTaq or AmpliTaq Gold polymerase. PCR was performed as described previously (82) in mixtures containing 1 μl of diluted nucleic acid preparations (1:30 in sterile water), 200 μM each dNTP and 0·4 μM each primer. The following conditions were used: denaturation at 94 °C for 1 min (2 min with AmpliTaq or 12 min with AmpliTaq Gold for the first cycle), annealing for 2 min at 60 °C (55 °C for second amplification in nested PCR) and primer extension for 3 min (10 min in the final cycle) at 72 °C. One microlitre of diluted (1:30) PCR products from the first amplification was used as the template in the second-round PCR. The PCR products (5–10 μl) were analysed by electrophoresis on a 1% agarose gel followed by staining with ethidium bromide and visualization of the DNA bands with a UV transilluminator.

Phylogenetic analysis. Complete or nearly complete 16S rRNA gene sequences from 42 phytoplasmas, three *Acholeplasma* spp. and three *Anaeroplasma* spp. were aligned separately by using CLUSTAL, version 5, using DNASTAR's LaserGene software (DNASTAR, Madison, WI, USA) and, if necessary, visually inspected for logical placement of gaps and manually adjusted (49). Cladistic analyses were performed with the computer program PAUP (phylogenetic analysis using parsimony), version 3.1, written by D. L. Swofford (University of Illinois), on a Power Macintosh model 8100. Uninformative characters were excluded from analyses. A phylogenetic tree was constructed by a heuristic search via random stepwise addition implementing the tree bisection and reconnection branch-swapping algorithm to find the optimal phylogenetic tree(s). *Anaeroplasma abactoclasticum* was selected as the out-group to root the tree. The analysis was replicated 100 times. Bootstrapping was performed to estimate the stability and support for the inferred clades.

RFLP analysis of PCR products. 16S rDNA fragments from the putative phytoplasmas amplified by PCR with the primer pair R16F2n/R16R2, and ribosomal protein sequences amplified with the primer pair rpF1/rpR1 or rp(V)F2/rpR1 were analysed by restriction endonuclease digestion. Each PCR product (3–5 μl, 100–200 ng DNA) was digested separately with some of the following restriction enzymes according to the instructions of the manufacturer: *Alu*I, *Bam*HI, *Bfa*I, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Rsa*I, *Ssp*I, *Taq*I, *Tha*I and *Tsp*509I (used only for the ribosomal protein gene)(Gibco-BRL), and *Mse*I and *Sau*3A I (New England Biolabs) (50, 83). To ensure the PCR products were fully digested, digestions were performed for a longer time than recommended by manufacturer (up to 48 h for *Alu*I). The restriction products were then separated by electrophoresis through a 5 or 12% (for some restriction products of ribosomal protein DNA sequences) polyacrylamide gel and stained in ethidium bromide. DNA bands were visualized with a UV transilluminator (50).

For constructing a dendrogram, the R16F2n/R16R2 PCR products from 34 representative phytoplasma strains were first digested with each of the 17 restriction endonucleases listed above. The RFLP patterns (the sum result of 17 enzymes) of 34 phytoplasmas were compared and analysed by the method of Nei & Li (106). The similarity coefficient (F) of strains x and y was calculated as $F=2N_{xy}/(N_x+N_y)$, in which N_x and N_y are the number of fragments resulting from digestions by 17 enzymes in strains x and y, respectively, and N_{xy} is the number of fragments shared by the two strains. A dendrogram was derived from a cluster analysis by using the Sahn clustering method (NTSYS-pc program, Exeter Publishing, Setauket, NY).

Putative restriction site analysis of 16S rDNA. Putative restriction site maps of 16S rRNA gene sequences for phytoplasmas from which RFLP analyses were not performed in this study were generated by using the DNASTAR

program MapDraw option (DNASTAR) (50). Sequences acquired from the GenBank database were analysed to identify the restriction recognition sequences for 17 restriction enzymes.

RESULTS AND DISCUSSION

RFLP and phylogenetic analyses of 16S rDNA

RFLP analyses of the 34 phytoplasma 16S rDNAs (R16F2n/R16R2 nested PCR products) with 17 restriction enzymes identified distinct pattern types. Representative patterns of the 34 phytoplasma 16S rDNAs are shown in Fig. 1 and Table 2. By using clean and specific nested-PCR products for RFLP analyses, the RFLP patterns of each representative phytoplasma strain analysed with various restriction enzymes have been shown to be consistent and absolutely reproducible. Based on similarity coefficients derived from RFLP analyses, the 34 representative phytoplasma strains were divided into 14 major groups (termed 16Sr groups) and 32 sub-groups (Fig. 3, Table 2). The similarity coefficients of RFLP patterns between two distinct groups were 90% or below (Table 3). By including the additional groups and sub-groups identified, based on analysis of phylogeny and putative restriction sites of 16S rDNAs (data not shown) from the phytoplasmas of which RFLP analyses were not performed, a total of 14 groups and 41 sub-groups were proposed. By combined RFLP analyses of 16S rRNA and ribosomal protein gene sequences, thus far, a total of 46 sub-groups [16Sr and 16Sr-rp (rp, ribosomal protein)] have been recognized. Phylogenetic analysis of near-full-length 16S rRNA gene sequences from 42 diverse phytoplasmas and representative *Acholeplasma* species and *Anaeroplasma* species yield 10 trees that are equally the most parsimonious, minor differences occurring only at outermost branching taxa, one of which is shown in Fig. 4. As shown in previous studies (49, 118, 121, 130), phytoplasmas formed a large phylogenetic group more closely related to *Acholeplasma palmae* and *Acholeplasma modicum*. Within the phytoplasma clade, a total of 13 distinct phytoplasma monophyletic groups or taxa, which we designate as subclades (using lowercase Roman numerals) were recognized. Two new subclades (xii and xiii) were identified in this and previous studies (28, 87).

Revision and expansion of the phytoplasma classification scheme

In this study, on the basis of comprehensive RFLP or putative-restriction-site analyses of 16S rDNAs (from representatives of the known phytoplasma strains), we have expanded the previous classification scheme (49, 50, 82) to include three new major phytoplasma 16Sr groups (XII, stolbur group; XIII, Mexican periwinkle virescence group; and XIV, bermudagrass white leaf group) and 16 new 16Sr sub-groups (II-B, strain witches'-broom WBDL; II-C, faba bean phyllody FBP; II-D, sweet potato little leaf SPLL; III-G, walnut witches'-broom WWB; III-H, poinsettia branch-inducing PoiBI; IV-B, Yucatan coconut lethal decline LDY; IV-C, Tanzanian coconut lethal decline LDT; V-B, cherry lethal yellows CLY and jujube witches'-broom JWB; V-C, rubus stunt RS, alder yellows AlY, spartium witches'-broom (EY type), eucalyptus little leaf, and flavescence dorée FD; X-C, pear decline PD; X-D spartium witches'-broom SPAR (AP type); X-E, black alder witches'-broom BAWB (new term, buckthorn witches'-broom BWB); XII-A, stolbur STOL and celery yellows CelY; XII-B, Australian grapevine yellows AUSGY and phormium yellow leaf PYL; XIII-A, Mexican periwinkle virescence MPV; and XIV-A, bermudagrass white leaf BGWL and annual blue grass white leaf ABGWL, and five new 16Sr-rp [formerly called 16Sr-(rr-rp)] sub-groups: 16SrI-B(rp-K), hydrangea phyllody HyPH1; 16SrV-A(rp-A), elm yellows EY1 and elm yellows ItaEY; 16SrV-B(rp-B), cherry lethal yellows CLY; 16SrV-B(rp-C), jujube witches'-broom JWB; 16SrV-C(rp-D), flavescence dorée FD based on combined analyses of rr and ribosomal protein gene sequences (Table 1). A new coding system (16Sr-rp) to indicate sub-groups derived from combined analyses was used in this study. Both rRNA and ribosomal protein RFLP pattern types of a given strain were incorporated. The phytoplasma strains whose group or sub-group affiliations were revised and reassigned were: 16SrI-C(rp-C), ranunculus phyllody RPh [formerly I-G(16Sr-rp)]; XII-A, stolbur STOL (formerly I-G) and celery yellows CelY (formerly I-G); XII-B, Australian grapevine yellows AUSGY (formerly I-J); III-F, milkweed yellows MW1 (formerly III-B); III-G, walnut witches'-broom WWB (formerly III-E); X-C, pear decline PD (formerly X-A); XIII-A, Mexican periwinkle virescence MPV (formerly I-I); and XIV-A, bermudagrass white leaf BGWL (formerly XI-C) and annual blue grass white leaf ABGWL (formerly XI-C). To avoid the potential confusion, sub-groups I-G, I-I, I-J and XI-C will not be used for assigning new phytoplasma strains.

Parallel phylogenetic analysis of near-full-length 16S rRNA gene sequences from most of the representative phytoplasma strains indicated that the RFLP-based groups are phylogenetically valid; groups based on extensive RFLP analyses were consistent with phylogenetic groups (subclades) (Fig. 4). The extensive RFLP analyses using 17 restriction enzymes provides sufficient characters (restriction sites) for comparison among phytoplasmas. Each group and sub-group can be defined on the basis of RFLP-pattern type (Fig. 1 and Table 2). The approach, using RFLP analyses of PCR-amplified 16S rDNAs, has provided a simple and reliable means of differentiation and classification of many unknown phytoplasmas in a relatively short period of time. An uncharacterized phytoplasma can be identified and classified preliminarily by comparison of its RFLP pattern type with known pattern types of designated phytoplasma groups and sub-groups. In

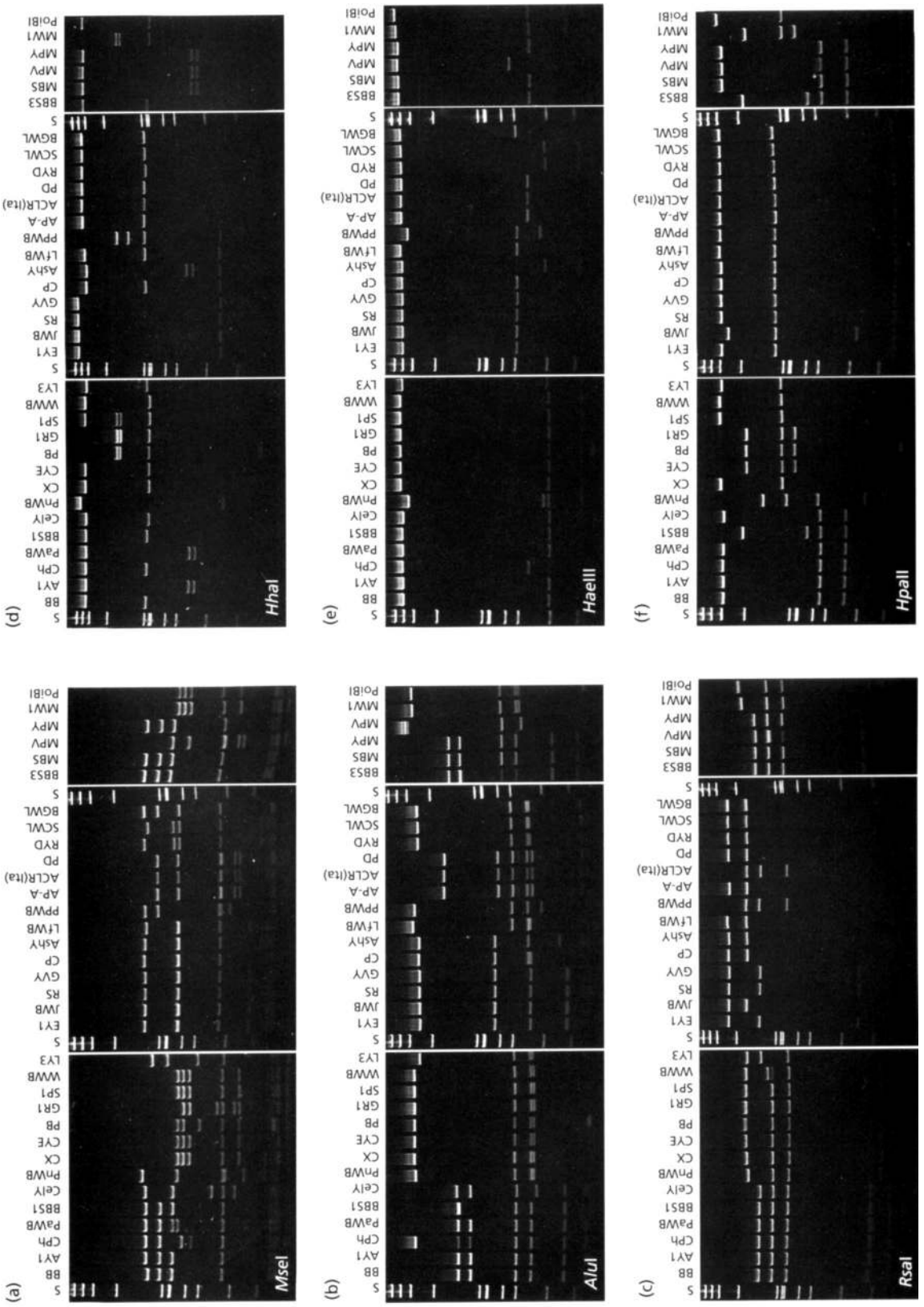

Fig. 1. For legend see facing page.

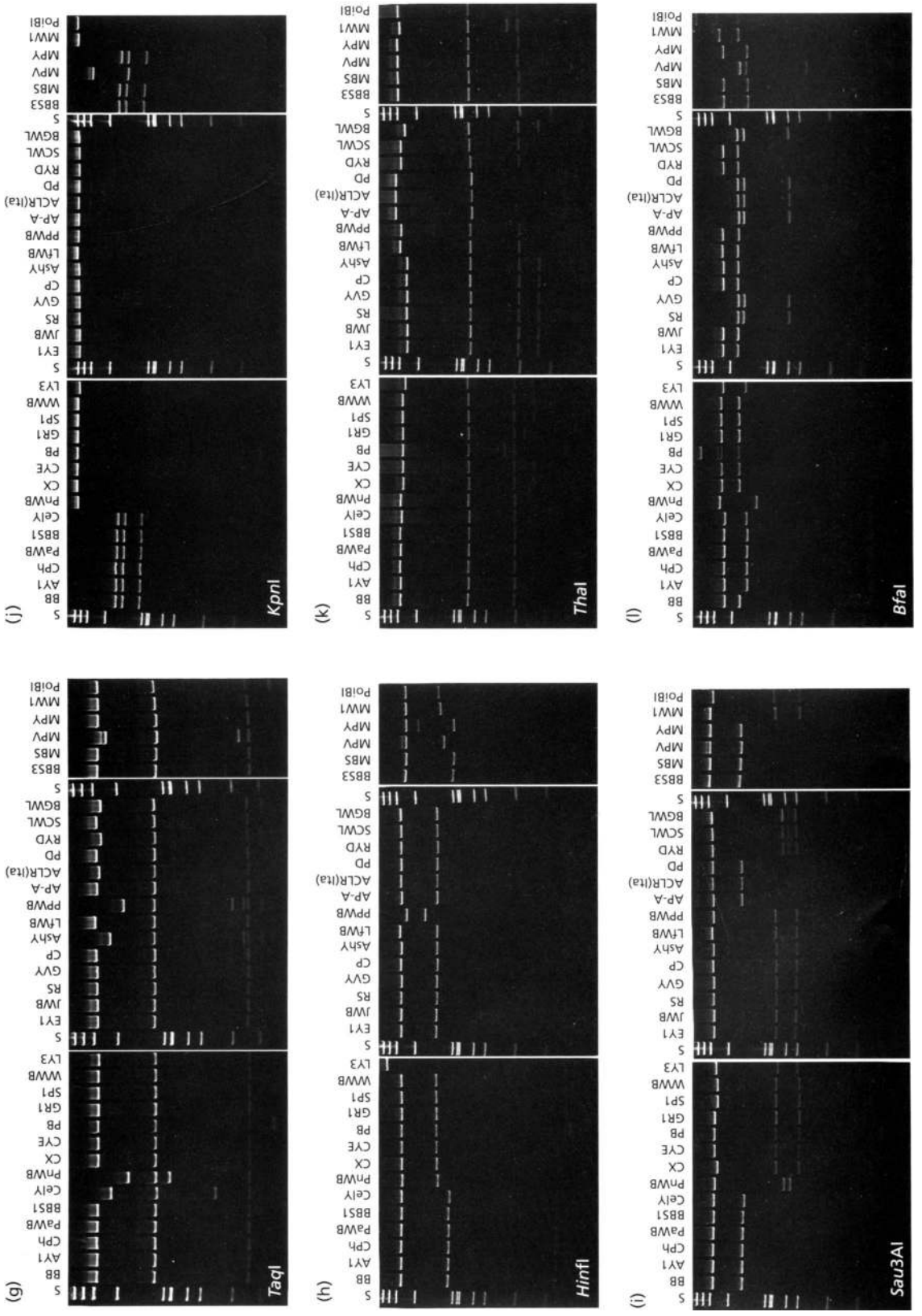

Fig. 1. RFLP analyses of 34 phytoplasma 16S rDNAs (R16F2n/R16R2 nested PCR products). DNA products were digested with *Mse*I (a), *Alu*I (b), *Rsa*I (c), *Hha*I (d), *Hae*III (e), *Hpa*II (f), *Taq*I (g), *Hin*fI (h), *Sau*3AI (i), *Kpn*I (j), *Tha*I (k) or *Bfa*I (l) and separated by electrophoresis through a 5% polyacrylamide gel. The presence of upper two bands seen in lanes PB and PoiBI in (l) were due to incomplete digestion. Lane S, ϕX174 RFI DNA *Hae*III digest, fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. Other abbreviations are defined in Table 1.

Table 2. Summary of pattern groups produced by RFLP analysis of 16S rDNA from representative type strains of phytoplasmas

The numbers in each column represent the number of distinct RFLP types obtained with each restriction endonuclease.

Strain*	16Sr group†	No. RFLP types obtained with restriction enzyme:																
		*Mse*I	*Alu*I	*Rsa*I	*Hha*I	*Hae*III	*Hpa*II	*Taq*I	*Hin*fI	*Sau*3AI	*Kpn*I	*Tha*I	*Bam*HI	*Dra*I	*Eco*RI	*Hpa*I	*Ssp*I	*Bfa*I
BB	I-A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AYI	I-B	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2
MIAY	I-B	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2
MBS	I-B	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2
MPY	I-B	1	1	1	2	1	1	1	4?	1	1	1	1	1	1	1	1	2
CPh	I-C	2	2	1	1	2	1	1	1	1	1	1	1	1	1	1	1	2
PaWB	I-D	3	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	2
BBS1	I-E	1	3	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2
BBS3	I-E	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2
(ACLR-AY)	I-F	4	1	7	1	1	1	1	1	1	2	1	1	1	1	1	1	2
PnWB	II-A	6	4	2	3	3	3	2	2	2	3	1	1	1	1	1	1	3
(WBDL)	II-B	7	4	2	3	3	4	1	2	2	3	1	1	1	1	1	1	3
(FBP)	II-C	7	4	2	3	3	3	1	2	2	3	1	1	1	1	1	1	3
SPLL	II-D	6	5	2	–		3		–	–			–					–
CX	III-A	8	6	2	1	4	5	1	2	3	3	3	1	1	1	1	1	1
CYE	III-B	9	6	2	1	4	6	1	2	4	3	3	1	1	1	1	1	1
PB	III-C	10	6	2	4	4	6	1	2	4	3	4	1	1	1	1	1	4
GR1	III-D	11	6	2	4	4	6	1	2	4	3	5	1	1	1	1	1	1
SP1	III-E	9	6	2	5	4	5	1	2	3	3	3	1	1	1	1	1	1
MW1	III-F	9	6	2	4	4	6	1	2	4	3	4	1	1	1	1	1	1
WWB	III-G	9	6	3	1	4	5	1	2	3	3	3	1	1	1	1	1	1
PoiBI	III-H	9	6	2	1	4	5	1	2	3	3	3	1	1	1	1	1	4
LY3	IV-A	12	7	4	6	4	5	1	3	4	3	3	2	1	1	1	1	2
(LDY)	IV-B	13	8	4	6	4	5	1	3	4	3	3	2	1	1	1	1	2
(LDT)	IV-C	14	7	4	4	2	5	1	3	4	3	3	2	1	1	1	1	2
EY1	V-A	14	9	5	2	5	7	1	2	4	3	3	1	1	1	1	1	1
CLY	V-B	14	9	6	2	5	8	1	2	4	3	3	1	1	1	1	1	1
JWB	V-B	14	9	6	2	5	8	1	2	4	3	3	1	1	1	1	1	1
RS	V-C	14	9	5	2	5	7	1	2	4	3	3	1	1	1	1	1	5
GVY	V-C	14	9	5	2	5	7	1	2	4	3	3	1	1	1	1	1	5
FD	V-C	14	9	5	2	5	7	1	2	4	3	3	1	1	1	1	1	5
CP	VI-A	15	10	6	7	5	7	1	2	4	3	3	1	1	1	1	1	1
AshY	VII-A	16	11	6	8	4	7	3	2	4	3	3	1	1	1	1	1	1
LfWB	VIII-A	14	6	6	7	5	7	1	2	4	3	1	1	1	1	1	1	1
PPWB	IX-A	17	12	4	9	6	7	4	4	4	3	1	1	2	1	1	1	1
AP-A	X-A	18	13	6	1	7	7	1	2	1	3	2	1	1	1	1	2	5
ACLR(Ita)	X-B	19	13	4	1	7	7	5	2	1	3	2	1	1	1	1	1	5
PD	X-C	18	13	6	1	7	7	1	2	1	3	2	1	1	1	1	1	5
(SPAR)	X-D	20	13	6	1	7	7	1	2	1	3	4	1	1	1	1	1	6
(BAWB)	X-E	21	14	6	1	5	9	2	2	1	3	2	1	1	1	1	1	5
RYD	XI-A	22	7	6	1	4	7	6	2	5	3	1	1	1	1	1	1	1
SCWL	XI-B	23	7	6	1	4	7	1	2	5	3	1	1	1	1	1	1	1
(BVK)	XI-C	18	7	6	1	4	7	7	2	5	3	1	1	1	1	1	1	1
STOL	XII-A	24	1	1	1	1	1	7	1	1	1	1	1	1	1	1	1	2
CelY	XII-A	24	1	1	1	1	1	7	1	1	1	1	1	1	1	1	1	2
AUSGY	XII-B	25	15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
MPV	XIII-A	26	16	7	2	2	1	8	5	6	2	1	1	1	1	1	1	5
BGWL	XIV-A	27	7	6	1	5	7	6	2	5	3	3	1	1	1	1	1	5

* See Table 1 for descriptions of phytoplasma strains. Strains in bold represent type species for sub-groups. RFLP patterns of strains in parentheses were based on putative restriction sites. Patterns of strains MIAY, CLY, STOL, SPLL and AUSGY were based on previously published (28, 41, 82) and unpublished (I.-M. Lee) data.

† Letters (A–H) represent sub-groups. Sub-group assignments for strains BBS3 and MPY are tentative.

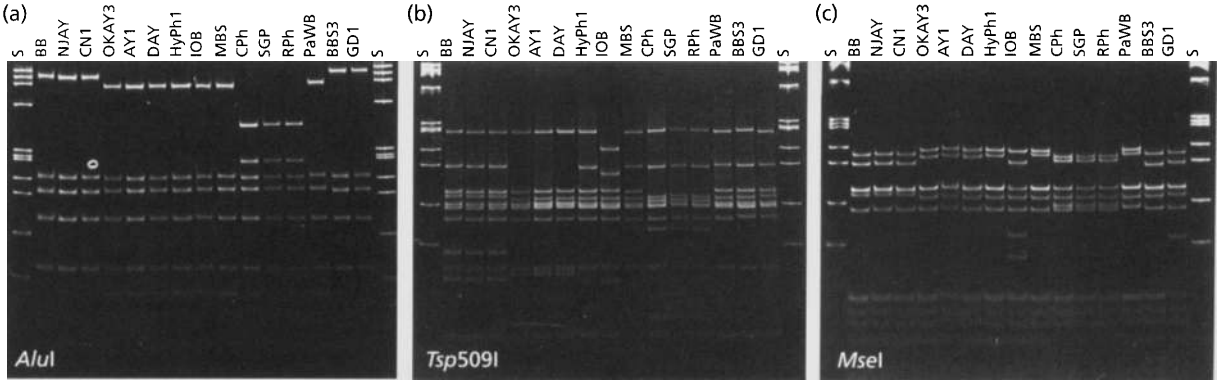

Fig. 2. RFLP analyses of ribosomal protein gene operon sequences amplified by PCR with primers rpF1 and rpR1 from representative phytoplasma strains belonging to aster yellows group (16SrI). The PCR products were digested with restriction enzymes *Alu*I (a), *Tsp*509I (b) or *Mse*I (c). Lane S contained size markers as described in the legend to Fig. 1.

Table 3. Similarity coefficients derived from RFLP analysis of 16S rDNA of representative type strains of phytoplasmas

Each similarity coefficient is based on analyses of RFLP patterns generated by separate digestion of amplified 16S rDNA with 17 different restriction enzymes.

		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
1	BB	1·00																																	
2	AY1	0·95	1·00																																
3	CPh	0·93	0·93	1·00																															
4	PaWB	0·94	0·99	0·92	1·00																														
5	BBSI	0·93	0·93	0·91	0·92	1·00																													
6	CEL	0·87	0·90	0·88	0·89	0·88	1·00																												
7	MPV	0·71	0·73	0·68	0·73	0·67	0·72	1·00																											
8	MBS	0·95	1·00	0·93	0·99	0·92	0·90	0·73	1·00																										
9	BBS3	0·95	0·95	0·93	0·94	0·98	0·90	0·68	0·96	1·00																									
10	MPY	0·93	0·98	0·93	0·97	0·93	0·88	0·72	0·98	0·95	1·00																								
11	PnWB	0·51	0·50	0·50	0·55	0·51	0·48	0·50	0·50	0·50	0·51	1·00																							
12	CX	0·60	0·55	0·59	0·56	0·56	0·52	0·58	0·55	0·55	0·54	0·66	1·00																						
13	CYE	0·60	0·56	0·59	0·57	0·60	0·53	0·60	0·56	0·59	0·55	0·67	0·93	1·00																					
14	PB	0·57	0·52	0·56	0·53	0·57	0·49	0·53	0·54	0·57	0·53	0·65	0·85	0·92	1·00																				
15	GRY	0·57	0·53	0·56	0·52	0·57	0·52	0·52	0·50	0·54	0·49	0·64	0·86	0·92	0·94	1·00																			
16	SPI	0·59	0·55	0·58	0·56	0·56	0·52	0·56	0·53	0·53	0·52	0·64	0·97	0·92	0·90	0·89	1·00																		
17	WWB	0·60	0·55	0·59	0·56	0·56	0·52	0·58	0·55	0·55	0·54	0·64	0·98	0·94	0·88	0·87	0·95	1·00																	
18	MWI	0·57	0·52	0·56	0·53	0·57	0·49	0·57	0·52	0·56	0·51	0·65	0·87	0·94	0·94	0·98	0·90	0·85	1·00																
19	PoiB1	0·60	0·56	0·59	0·57	0·57	0·53	0·57	0·56	0·56	0·55	0·65	0·99	0·94	0·88	0·90	0·98	0·97	0·88	1·00															
20	LY3	0·62	0·57	0·59	0·56	0·56	0·54	0·54	0·60	0·60	0·59	0·54	0·73	0·74	0·72	0·71	0·71	0·73	0·68	0·74	1·00														
21	EY1	0·53	0·54	0·52	0·54	0·53	0·51	0·53	0·54	0·52	0·53	0·52	0·65	0·66	0·64	0·63	0·64	0·65	0·64	0·68	0·67	1·00													
22	JWB	0·51	0·50	0·49	0·50	0·51	0·48	0·50	0·50	0·50	0·50	0·56	0·65	0·68	0·66	0·65	0·64	0·65	0·64	0·66	0·65	0·93	1·00												
23	RS	0·55	0·54	0·52	0·53	0·53	0·51	0·57	0·54	0·52	0·53	0·51	0·65	0·65	0·63	0·62	0·64	0·65	0·63	0·67	0·67	0·97	0·90	1·00											
24	GVY	0·55	0·54	0·52	0·53	0·53	0·51	0·57	0·54	0·52	0·53	0·51	0·65	0·65	0·63	0·62	0·64	0·65	0·63	0·67	0·67	0·97	0·90	1·00	1·00										
25	CP	0·57	0·54	0·54	0·54	0·55	0·51	0·51	0·54	0·54	0·53	0·55	0·73	0·74	0·72	0·71	0·73	0·73	0·70	0·74	0·73	0·89	0·89	0·86	0·86	1·00									
26	AshY	0·56	0·59	0·53	0·58	0·52	0·54	0·54	0·59	0·53	0·58	0·56	0·69	0·72	0·66	0·65	0·70	0·67	0·64	0·68	0·69	0·83	0·83	0·80	0·80	0·90	1·00								
27	LfWB	0·60	0·57	0·59	0·56	0·58	0·53	0·52	0·57	0·57	0·56	0·58	0·70	0·71	0·71	0·69	0·69	0·70	0·69	0·71	0·69	0·79	0·79	0·70	0·76	0·88	0·74	1·00							
28	PPWB	0·52	0·51	0·50	0·51	0·52	0·50	0·49	0·51	0·51	0·50	0·60	0·58	0·64	0·64	0·65	0·57	0·58	0·56	0·58	0·57	0·65	0·63	0·63	0·63	0·69	0·65	0·70	1·00						
29	AP	0·58	0·55	0·57	0·55	0·54	0·54	0·54	0·55	0·55	0·54	0·45	0·60	0·60	0·59	0·57	0·57	0·60	0·60	0·58	0·58	0·57	0·57	0·61	0·61	0·64	0·58	0·62	0·54	1·00					
30	ACLR(Ita)	0·62	0·58	0·60	0·58	0·58	0·59	0·63	0·58	0·58	0·67	0·49	0·61	0·62	0·60	0·55	0·59	0·61	0·60	0·62	0·59	0·59	0·57	0·63	0·63	0·63	0·59	0·62	0·55	0·93	1·00				
31	PD	0·61	0·62	0·56	0·61	0·57	0·57	0·65	0·58	0·58	0·57	0·48	0·60	0·63	0·61	0·56	0·60	0·63	0·61	0·63	0·60	0·60	0·60	0·64	0·64	0·67	0·61	0·65	0·55	0·97	0·94	1·00			
32	RYD	0·63	0·58	0·55	0·60	0·58	0·57	0·56	0·58	0·58	0·59	0·66	0·71	0·74	0·72	0·69	0·71	0·73	0·71	0·75	0·71	0·67	0·67	0·65	0·65	0·76	0·77	0·74	0·65	0·64	0·65	0·67	1·00		
33	SCWL	0·65	0·60	0·57	0·62	0·60	0·57	0·58	0·60	0·60	0·59	0·68	0·75	0·78	0·74	0·73	0·75	0·77	0·75	0·79	0·78	0·68	0·69	0·67	0·67	0·78	0·75	0·77	0·63	0·66	0·65	0·69	0·94	1·00	
34	BGWL	0·58	0·55	0·55	0·56	0·54	0·54	0·58	0·57	0·57	0·56	0·56	0·69	0·72	0·66	0·67	0·69	0·71	0·67	0·73	0·71	0·69	0·71	0·74	0·74	0·80	0·73	0·72	0·63	0·70	0·71	0·73	0·88	0·84	1·00

practice, several of the most useful restriction enzymes, such as *Mse*I, *Alu*I, *Rsa*I, *Hha*I, *Hpa*II and *Taq*I, should be included for preliminary classification of phytoplasmas.

Delineation of phytoplasma groups and sub-groups

The comprehensive classification scheme combined with parallel phylogenetic analyses has formed a basis

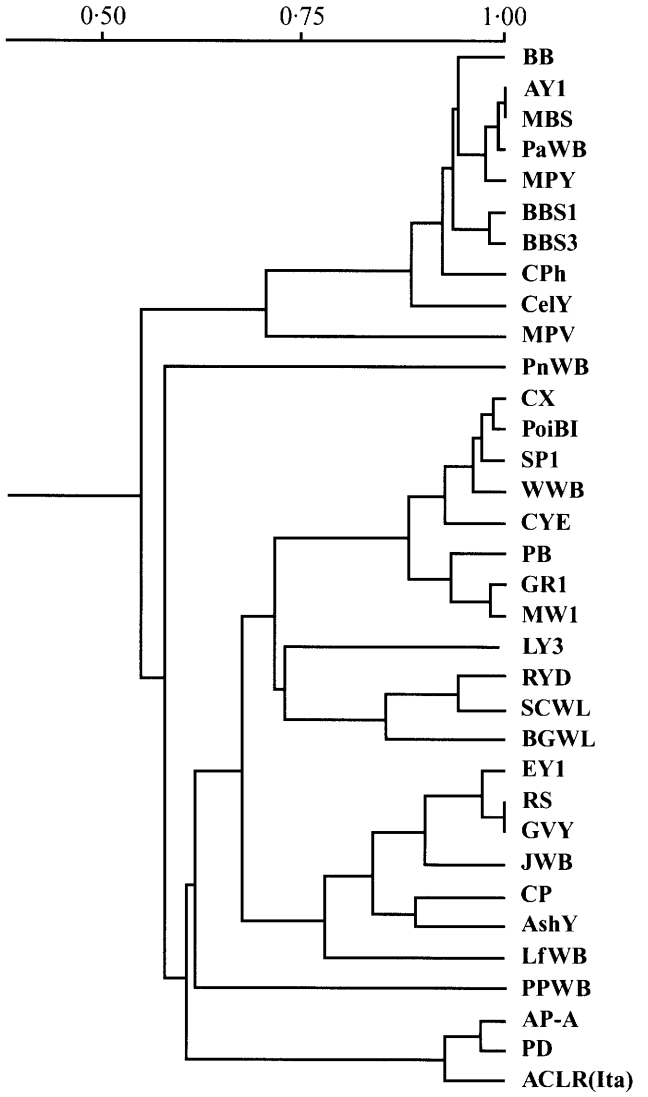

Fig. 3. Dendrogram obtained by cluster analysis of similarity coefficients derived from RFLP analysis of 16S rDNA of the 34 representative phytoplasmas. The scale refers to the similarity index. Phytoplasma strain descriptions are as in Table 1.

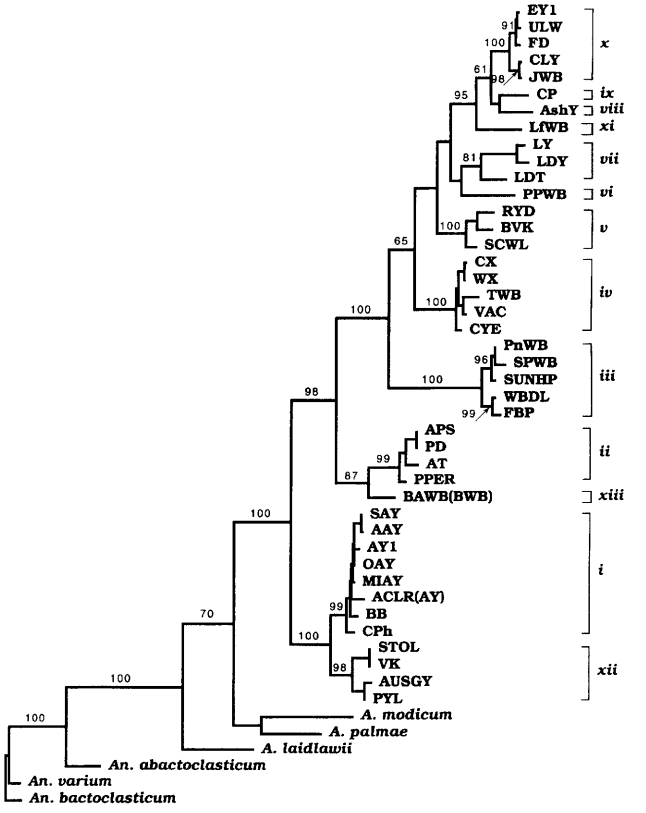

Fig. 4. Phylogenetic tree constructed by parsimony analysis of full 16S rRNA sequences from 42 phytoplasmas, three *Acholeplasma* (*A.*) spp. and three *Anaeroplasma* (*An.*) spp., employing *Anaeroplasma abactoclasticum* as the outgroup. Branch lengths are proportional to the number of inferred character state transformations. Phylogenetic subclades identified are shown on the right. Bootstrap values (measures of support for the inferred subclades) are shown on branches. AAY, American aster yellow collected from Florida by R. E. McCoy. APS, Apple proliferation phytoplasma was collected from Spain (121). Other abbreviations are defined in Table 1.

for establishing a formal phytoplasma taxonomy. Each 16Sr group, which corresponded to a subclade, based on cladistic phylogenetic analysis of 16S rRNA gene sequences was proposed to represent at least one species (49). At the 10th International Congress of the International Organization for Mycoplasmology, the trivial name 'phytoplasma' was officially adopted as the *Candidatus* genus name to replace 'mycoplasma-like organism' and it has been proposed that within the putative genus phytoplasma, each phylogenetic subclade represents a *Candidatus* species. Thus far, two phytoplasma *Candidatus* species, '*Candidatus* Phytoplasma aurantifolia' (associated with the witches'-broom disease of lime) (145) and '*Candidatus* Phytoplasma australiense' (associated with Australian grapevine yellows) (28) have been proposed.

The sub-groups within a given 16Sr group were differentiated based on restriction sites. A new sub-group was assigned if the phytoplasma strain had one or more restriction sites different from those in all the existing members of the given group. While two 16S rRNA gene operons have been reported in phytoplasma (83, 87, 88, 117), in some cases (this work and R. Jomantiene & R. E. Davis, unpublished) differences between phytoplasmas have been detected in only one of the two 16S rRNA genes (e.g. strains CPh, WWB and SP1; see Fig. 1b–d). Sub-group designations for strains WWB and SP1 were based on combined patterns from both operons and will be considered to be only tentative until more substantial evidence indicates that the two operons in these strains are different. Most of the sub-groups identified were consistent with genomic subclusters previously determined based on dot- and Southern-hybridization assays of total genomic DNA (45, 78, 81). However, some previously identified subclusters based on partial DNA homology were not readily differentiated by

RFLP analysis of the highly conserved 16S rRNA gene sequence (81). Therefore, combined RFLP analyses of 16S rRNA and/or ribosomal protein operon gene sequences (50) were applied for a finer sub-group differentiation. In general, sub-group delineation based on RFLP analysis of the 16S rRNA gene was consistent with that based on ribosomal protein gene sequence. Some members within a given 16Sr sub-group can be further differentiated, based on ribosomal protein gene sequences. For example, among members of 16SrI-B, phytoplasma strains HyPH1, IOB and MBS represent additional sub-groups based on combined RFLP analyses of 16S rRNA and ribosomal protein gene sequences (Fig. 2) where ribosomal protein RFLP patterns are identified by comparison only to the members of the 16Sr sub-group. Several members of 16Sr sub-groups III-A, B and V-A also represented additional sub-groups based on analyses of 16S rRNA and ribosomal protein gene sequences (Table 1). Other less-conserved gene sequences (e.g. 23S–16S rRNA intergenic spacer region) or monoclonal antibodies can also be useful for finer sub-group differentiation (63, 66, 98).

While there was consensus in designation of a temporary taxonomic unit, '*Candidatus* Phytoplasma' species, for each of the major phytoplasma groups (phylogenetic subclades or corresponding 16Sr groups), no consensus has been reached for assigning an appropriate taxonomic rank to each of the sub-groups recognized. It is evident that the majority of the designated sub-groups within a given 16Sr group represent distinct phytoplasma subclusters, which have unique ecological niches in nature. For example, apple proliferation phytoplasma (sub-group 16Sr X-A), pear decline phytoplasma (sub-group 16SrX-C), and plum leptonecrosis and European stone fruit phytoplasmas (sub-group 16SrX-B) were associated with their preferential hosts (plants and/or insect vectors) in nature; mutual cross-infections among various hosts by these phytoplasmas have not been reported. Likewise, paulownia witches'-broom phytoplasma (sub-group 16SrI-D), blueberry stunt phytoplasma (sub-group 16SrI-E), maize bushy stunt phytoplasma [sub-group 16SrI-B(rp-L)], pecan bunch phytoplasma (sub-group 16SrIII-C), spiraea stunt phytoplasma (sub-group 16SrIII-E), walnut witches'-broom phytoplasma (sub-group 16SrIII-G), cherry lethal yellows phytoplasma [sub-group 16SrV-B(rp-B)] and jujube witches'-broom phytoplasma [sub-group 16SrV-B(rp-C)] all have their own ecological niches (specific or relatively narrow host range). Many sub-groups are geographically isolated. For example, sub-group 16SrI-A phytoplasmas have only been reported in North America. Sub-group 16SrI-D phytoplasma is present only in eastern Asia (143). A taxonomic rank of at least subspecies level was previously proposed for affiliating each sub-group that was defined on the basis of RFLP analysis of 16S rRNA and/or ribosomal protein gene sequences (50). It is necessary and practical to identify the range of genomic heterogeneity among members of each proposed *Candidatus* species. However, for some sub-groups, for example III-A(rp-A) and III-A(rp-B), which share similar ecological niches, assignment of differential taxonomic ranks may require additional genomic information.

Rationale for proposed taxonomic ranks of RFLP groups and sub-groups

The extensive knowledge accumulated in the last decade on the molecular biology and phylogeny of bacteria has changed the traditional concept of *Mollicutes* taxonomy (4, 5, 10, 38, 58, 102, 111, 129, 136–141). Polyphasic taxonomy, which aims to integrate different kinds of information (phenotypic, genotypic and phylogenetic) on micro-organisms in their classification, has become a consensus approach to modern bacterial systematics (38, 58, 131). It is generally accepted that bacterial classification should reflect the phylogenetic relationship, deduced by analysis of 16S or 23S rRNA sequences. Because of the deficiencies in the traditional phenotypically oriented system, the International Committee on Systematic Bacteriology (ICSB) Subcommittee on the taxonomy of *Mollicutes* has agreed to, and adopted the policy of, basing bacterial taxonomy on phylogeny (38, 58). For uncultured phytoplasmas, basing taxonomy on phylogeny is inevitable because the phenotypic criteria are not attainable. A decision was also made that the complete sequence of the bacterial genome would be the basis for assignment of the basic taxonomic unit, the species. According to the ICSB's recommended criteria, the phylogenetic definition of a species would include strains with at least 70% DNA homology, while a subspecies would include strains with 70–85% DNA homology. For uncultured phytoplasmas, species differentiation based on DNA–DNA homology has not been attempted because pure phytoplasma DNA is difficult to obtain. However, based on their review of data in the literature, Stackebrandt & Goebel (129) recently noted that organisms sharing less than 97% 16S rRNA sequence homology 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–DNA hybridization with 16S rRNA sequence homology in the description of new species, provided that rRNA sequences are available and the sequences are accurately determined.

16S rRNA sequence homologies are 88–94% between two distinct phytoplasma 16S rRNA groups, and 95–98% between two sub-groups within a given group (49, and this study). Based on the criteria proposed by Stackebrandt & Goebel (129), each phytoplasma 16S rRNA group and some 16Sr sub-group phytoplasma strains can be assigned as a taxon at the species level. Because of the highly conserved nature of 16S rRNA gene sequences, there is no defined threshhold of sequence homology for assigning a species. Therefore, the taxonomic rank of the phytoplasma sub-groups

which share 97% or more 16S rRNA sequence homologies are uncertain. For example, in a comparative study of the relationship between 16S rRNA sequence homology and DNA–DNA homologies among species in the genus *Bacillus*, Fox *et al.* (38) noted that although 16S rRNA sequence identity can be used effectively to establish relationships between genera and well-resolved species, 16S rRNA sequence identity alone may not be sufficient to guarantee species identity. Two *Bacillus* species with 23–50% DNA–DNA homology shared 99·8% 16S rRNA sequence similarity. In contrast, they did not find any instances of strains that were well resolved by 16S rRNA sequence analysis which could not also be differentiated on the basis of DNA–DNA hybridization.

The inability to obtain pure cultures of phytoplasmas makes conventional DNA–DNA homology studies difficult. DNA–DNA homology data do not exist to verify the taxonomic ranks assigned to each phytoplasma 16Sr group or sub-group in this study. Our approach, using 16S rRNA gene sequence to differentiate major phytoplasma groups and using the 16S rRNA gene supplemented with less conserved sequences (e.g. ribosomal protein gene clusters) to differentiate sub-groups within each group, should give a classification of phytoplasma strains that reflects the true genomic variation. Based on partial DNA homology data gathered in the last decade, the proposed designation of '*Candidatus* Phytoplasma' species or subspecies on the basis of phylogenetic relatedness determined by RFLP analyses of 16S rRNA (and ribosomal protein) gene sequences seems appropriate. The sub-groups identified by combined RFLP analyses of these conserved gene sequences were consistent with the subclusters identified by relative DNA homology studies of total genomic DNA with a number of cloned phytoplasma DNA probes (45, 50, 78, 81). Hence, small variations in conserved 16S rRNA or ribosomal protein genes, or in 16S–23S intergenic spacer region sequences were phylogenetically significant, since they represent much greater variations in total genomic DNA sequences. This notion was verified in other culturable prokaryotic systems (73), in which the dissimilarities of 16S rRNA gene sequences ranged from 2–10% among established species and 1–3% among subspecies, and the difference in DNA homology between species or subspecies fell within the ranges proposed by the ICSB. While a temporary nomenclature of '*Candidatus* Phytoplasma' species has been accepted by ICSB for assigning each 16SrRNA group (equivalent to a phylogenetic subclade), we propose that a new convention be adopted to refer to the distinct sub-groups encompassed by a *Candidatus* species. For example, maize bushy stunt phytoplasma [16SrI-B(rp-L)] represents one of the several sub-groups identified within aster yellows group 16SrI. The maize bushy stunt sub-group would be referred to as '*Candidatus* Phytoplasma asteri' [16SrI-B(rp-L)] to distinguish from other sub-groups in the aster yellows group (16SrI).

Concluding remarks

Biological properties such as symptomatology, range of susceptible plant hosts and relationships to insect vectors had been the major criteria for diagnosing the phytoplasmal diseases and the associated phytoplasmal strains before molecular-based methods became available (18, 35, 39, 77, 101, 123, 124, 132). In practice, they remain important tools for preliminary identification of putative phytoplasmal diseases. The identities of the putative causal agents can now be accurately identified and defined on the basis of phylogenetic criteria. It is clear that a given disease of the same plant host based on similar symptomatology can be associated with two or more distinct phytoplasmas in different geographical regions, and that a given type of phytoplasma (e.g. 16SrI-B) can potentially inflict various diseases in different plant hosts. The traditional one disease–one phytoplasma concept is changing. Although, in many cases, characteristic biological properties are found to be linked with each of the putative taxonomic units (phytoplasma 16Sr groups or sub-groups) based on phylogenetic criteria, these biological properties can only be used as secondary criteria to define a given presumed causal agent (listed in Table 1).

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