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Ribosomal protein gene-based phylogeny for finer differentiation and classification of phytoplasmas

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Extensive phylogenetic analyses were performed based on sequences of the 16S rRNA gene and two ribosomal protein (rp) genes, *rplV* (*rpl22*) and *rpsC* (*rps3*), from 46 phytoplasma strains representing 12 phytoplasma 16Sr groups, 16 other mollicutes and 28 Gram-positive walled bacteria. The phylogenetic tree inferred from rp genes had a similar overall topology to that inferred from the 16S rRNA gene. However, the rp gene-based tree gave a more defined phylogenetic interrelationship among mollicutes and Gram-positive walled bacteria. Both phylogenies indicated that mollicutes formed a monophyletic group. Phytoplasmas clustered with *Acholeplasma* species and formed one clade paraphyletic with a clade consisting of the remaining mollicutes. The closest relatives of mollicutes were low-G+C-content Gram-positive bacteria. Comparative phylogenetic analyses using the 16S rRNA gene and rp genes were performed to evaluate their efficacy in resolving distinct phytoplasma strains. A phylogenetic tree was constructed based on analysis of rp gene sequences from 87 phytoplasma strains belonging to 12 16Sr phytoplasma groups. The phylogenetic relationships among phytoplasmas were generally in agreement with those obtained on the basis of the 16S rRNA gene in the present and previous works. However, the rp gene-based phylogeny allowed for finer resolution of distinct lineages within the phytoplasma 16Sr groups. RFLP analysis of rp gene sequences permitted finer differentiation of phytoplasma strains in a given 16Sr group. In this study, we also designed several semi-universal and 16Sr group-specific rp gene-based primers that allow for the amplification of 11 16Sr group phytoplasmas.

INTRODUCTION

The highly conserved 16S rRNA gene is the most widely used gene for phylogenetic studies of prokaryotes. Phylogenetic relationships established on the basis of this gene provide a basis for modern prokaryote taxonomy. The 16S rRNA gene sequence has been used as the primary phylogenetic parameter for differentiation and classification of phytoplasmas in the recently emerging phytoplasma taxonomy (Lee *et al.*, 1993b, 1998; Schneider *et al.*, 1995; Seemüller *et al.*, 1994, 1998). Based on extensive RFLP or phylogenetic analysis of 16S rRNA gene sequences from a wide array of phytoplasma strains, 18 RFLP groups and more than 20 distinct phylogenetic groups have been identified to date (Arocha *et al.*, 2005; Lee *et al.*, 2000, 2006b; Seemüller *et al.*, 1998). Each RFLP or phylogenetic group was proposed to represent at least one phytoplasma species (Gundersen *et al.*, 1994; Seemüller *et al.*, 1998). A consensus for naming novel phytoplasmas was reached and recommended by the IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group (2004) that 'a '*Candidatus* (*Ca.*) Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence

Abbreviations: NJ, neighbour-joining; rp, ribosomal protein.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are given in Fig. 1.

Figures presenting the position of the degenerate primers and the analysis of putative restriction sites in ribosomal protein operon sequences are available as supplementary material with the online version of this paper.

(>1200 bp)', 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'. So far, 26 members of '*Ca.* Phytoplasma' have been proposed (Arocha *et al.*, 2005; IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004; Lee *et al.*, 2006b; Schneider *et al.*, 2005; Valiunas *et al.*, 2006). Because of the highly conserved nature of the 16S rRNA gene, many biologically or ecologically distinct phytoplasma strains, each of which may warrant designation of a new taxon but may fail to meet the requirement of sharing <97.5 % sequence similarity with existing '*Ca.* Phytoplasma', cannot be readily differentiated and classified. Additional unique biological properties such as insect vectors and plant hosts as well as other molecular criteria need to be included for speciation. For finer differentiation of phytoplasmas, additional phylogenetic markers such as ribosomal protein (rp) genes, *secY*, *tuf* and 23S rRNA genes and the 16S–23S rRNA intergenic spacer region sequences have been used as supplementary tools. The rp and *secY* genes are the most variable among the five markers and have proved to be useful for finer differentiation of phytoplasma strains. Gundersen *et al.* (1996), Lee *et al.* (1998, 2004a, b, 2006a) and Martini *et al.* (2002) noted that finer subgroup delineation within the aster yellows (16SrI), X-disease (16SrIII) and elm yellows (16SrV) groups could be achieved by combining RFLP analyses of 16S rRNA and rp or *secY* gene sequences. Analysis of rp or *secY* gene sequences delineated biologically and/or ecologically distinct strains that often cannot be readily resolved by the 16S rRNA gene alone.

Because of the current inability to culture phytoplasmas and the resulting lack of information about their phenotypic properties, it is inevitable that the present phytoplasma taxonomy is based on phylogeny. Phylogeny based on the 16S rRNA gene is inadequate for resolving taxonomic rank at the species level. To overcome the limitations of the 16S rRNA gene and to resolve phylogenetic relationships that are more consistent with the actual organism identity, multiple genes with varying degrees of sequence conservation have been employed to introduce additional phylogenetic parameters.

Previous studies indicated that rp genes used as phylogenetic markers have finer resolving power for the differentiation of distinct phytoplasma strains in groups 16SrI and 16SrV (Lee *et al.*, 2004a, b; Martini *et al.*, 2002). The extensive phylogenetic interrelationships among strains in the majority of phytoplasma groups based on rp or *secY* genes have not been established. In the present study, comparative phylogenetic analyses were performed and phylogenetic trees constructed based on sequence analyses of the 16S rRNA gene and two rp genes, *rplV* (*rpl22*) and *rpsC* (*rps3*), from representative phytoplasma strains, other mollicutes and Gram-positive bacteria. The efficacy of rp genes for finer differentiation of phytoplasma strains will be evaluated.

METHODS

Phytoplasma strains and nucleic acid preparation. Representative phytoplasma strains listed in Table 1 were used in this study. Total nucleic acid or DNA was extracted using leaf midribs or other tissues from periwinkle or original hosts that were infected by representative phytoplasma strains belonging to different phytoplasma groups according to the method described by Lee *et al.* (1993a) and Prince *et al.* (1993). These representative phytoplasma strains have been previously characterized and identified by RFLP or sequence analysis of the 16S rRNA gene (Abou-Jawdah *et al.*, 2002; Bertaccini *et al.*, 2000; Jacobs *et al.*, 2003; Lee *et al.*, 1998; Marcone *et al.*, 1997a, b, c; Schneider *et al.*, 1997; unpublished data).

PCR, cloning and sequencing of partial phytoplasma rp operons. The rp gene operon was amplified using the primer pair rpL2F/rp(I)R1A (5′-ATGAACCCGAACGATCACCC-3′/5′-GTTCT-TTTTGGCATTAACAT-3′) from some of the phytoplasma strains listed in Table 1 belonging to different phytoplasma groups (LNS1, LNS2, AP15, AT, BLL, LUM, CPS, VR, PWB, CP, AshY1, EY1, FDD, NJAY, MPV, LY, PDX, PPWB, FBP, GrapeA, PnWB, LfWB and RYD) and *Acholeplasma palmae* J233^T. The forward primer was designed on the basis of the rp gene sequences of an aster yellows phytoplasma (GenBank accession no. M74770) covering the 3′ end of *rplW* (*rpl23*), the *rplB* (*rpl2*), *rpsS* (*rps19*), *rplV* (*rpl22*), *rpsC* (*rps3*) genes and the 5′ end of *rplP* (*rpl16*). The reverse primer, rp(I)R1A, described previously by Lee *et al.* (2003), was modified from the primer rpR1 (located in the 3′ end of *rplP* gene) (Lim & Sears, 1992). For PCR amplification, 38 cycles were conducted in an automated thermal cycler (MJ Research DNA Thermal Cycler PTC-200) with AmpliTaq Gold polymerase (Applied Biosystems). PCR was performed in mixtures containing 1 μl DNA preparation, 200 μM each dNTP and 0.4 μM each primer. The following conditions were used: denaturation at 94 °C for 1 min (11 min for the first cycle to activate the polymerase), annealing for 2 min at 50 °C and primer extension for 3 min (7 min in the final cycle) at 72 °C. A negative control devoid of DNA template in the reaction mixture was included in all the PCR assays. PCR products (5 μl) were electrophoresed through a 1 % agarose gel, stained with ethidium bromide and visualized with a UV transilluminator. The PCR with primer pair rpL2F/rp(I)R1A yielded amplicons of the expected size (about 1.7 kb) covering the 3′ end of *rplB* gene and the *rpsS*, *rplV* and *rpsC* genes from NJAY, PDX, LUM, VR, AshY and *A. palmae* J233^T. The amplicons were purified using the Qiaquick PCR Purification kit (Qiagen) and cloned into *Escherichia coli* TOP10 by using the TOPO TA cloning kit (Invitrogen) according to the manufacturers' instructions, and inserts were sequenced with an automated DNA sequencer (ABI Prism model 3730) at the Center for Biosystems Research (University of Maryland, College Park, MD, USA) using SP6 and T7 promoter primers. The sequences were aligned by using CLUSTAL version 5 and Lasergene software (DNASTAR).

Primer design. To design degenerate rp gene-based primers, the partial rp operon sequences (about 1.7 kb) amplified with primer pair rpL2F/rp(I)R1A from NJAY, PDX, LUM, VR and AshY1 phytoplasma strains and *A. palmae* J233^T were aligned and some conserved regions common to phytoplasma strains were identified.

Three degenerate forward primers were designed based on the sequence near the 5′ end: rpL2F2 (5′-CTCATGGYGGW-GGWGAAGG-3′); rpL2F3 (5′-WCCTTGGGGYAAAAAAGCTC-3′); and rpF1C (5′-ATGGTDGGDCAYAARTTAGG-3′) (W=A+T; Y=C+T; D=A+G+T; and R=A+G). The positions of the forward primers on the alignment are shown in Supplementary Fig. S1 in IJSEM Online.

The primer rpF1C was designed within a conserved portion overlapping the position where primer rpF1 was previously designed

Table 1. Designations, associated diseases, origins and phytoplasma 16Sr and rp group-subgroup affiliations of representative phytoplasma strains

Strain	Associated disease	Geographical origin	RFLP subgroup classification	
			16Sr	rp
'*Ca.* P. asteris' OAY	Oenothera virescence	Michigan, USA	16SrI-B	rpI-B
'*Ca.* P. asteris' AV2192	Aster yellows	Germany	16SrI-L	rpI-B
'*Ca.* P. asteris' AVUT	Aster yellows	Germany	16SrI-M	rpI-B
'*Ca.* P. asteris' PaWB	Paulonia witches'-broom	Taiwan	16SrI-D	rpI-D
'*Ca.* P. asteris' MBS	Maize bushy stunt	Mexico	16SrI-B	rpI-L
'*Ca.* P. asteris' IOWB	*Ipomoea obscura* witches'-broom	Taiwan	16SrI-N	rpI-F
'*Ca.* P. asteris' GD1	Grey dogwood stunt	New York, USA	16SrI-A	rpI-M
'*Ca.* P. asteris' BB	Tomato big bud	Arkansas, USA	16SrI-A	rpI-A
'*Ca.* P. asteris' BBS3	Blueberry stunt	Michigan, USA	16SrI-E	rpI-E
'*Ca.* P. asteris' Strawb2	Strawberry multiplier	Florida, USA	16SrI-K	rpI-J
'*Ca.* P. asteris' KVE	Clover phyllody	France	16SrI-C	rpI-C
'*Ca.* P. asteris' ACLR.AY	Apricot chlorotic leaf roll	Spain	16SrI-F	rpI-N
'*Ca.* P. asteris' CVB	Leafhopper-borne	Germany	16SrI-F	rpI-N
'*Ca.* P. asteris' NJAY	New Jersey aster yellows	USA	16SrI-A	rpI-A
'*Ca.* P. australiense' AGY	Australian grapevine yellows	Australia	16SrXII-B	
'*Ca.* P. australiense' PYCL	Pumpkin yellowing	Australia	16SrXII-B	
'*Ca.* P. australiense' CBWB	*Gomphocarpus physocarpus* witches'-broom	Australia	16SrXII-B	
'*Ca.* P. australiense' CBRYL	Gomphocarpus yellowing	Australia	16SrXII-B	
'*Ca.* P. australiense' SLY2	Strawberry lethal yellows	Australia	16SrXII-B	
'*Ca.* P. australiense' SLY	Strawberry lethal yellows	Australia	16SrXII-B	
'*Ca.* P. australiense' PYL	Phormium yellow leaf	Australia	16SrXII-B	
'*Ca.* P. australiense' PpDB	Papaya dieback	Australia	16SrXII-B	
PTV (TomStol)	Tomato stolbur	Italy	16SrXII-A	
Stol	Pepper stolbur	Serbia	16SrXII-A	
MPV	Mexican periwinkle virescence	Mexico	16SrXIII-A	
Strawb1	Strawberry green petal	Florida, USA	16SrXIII-B	
CbY1	Chinaberry yellows	Bolivia	16SrXIII	
'*Ca.* P. americanum' APPTW10.NE	American potato purple top wilt	Nebraska, USA	16SrXVIII-B	
'*Ca.* P. americanum' APPTW9.NE	American potato purple top wilt	Nebraska, USA	16SrXVIII-A	
'*Ca.* P. mali' AP15	Apple proliferation	Italy	16SrX-A	
'*Ca.* P. mali' AT	Apple proliferation	Germany	16SrX-A	
'*Ca.* P. mali' APSb	Apple proliferation	Italy	16SrX-A	
'*Ca.* P. prunorum' LNS1	European stone fruit yellows	Italy	16SrX-B	
'*Ca.* P. prunorum' LNS2	European stone fruit yellows	Italy	16SrX-B	
'*Ca.* P. prunorum' ESFY.G2	European stone fruit yellows	Germany	16SrX-B	
'*Ca.* P. pyri' PD1	Pear decline	Germany	16SrX-C	
AlfWB	Alfalfa witches'-broom	Oman	16SrII-D	
'*Ca.* P. australasia' GrapeA	Australian grapevine yellows	Australia	16SrII-D	
'*Ca.* P. australasia' TBB	Tomato big bud	Australia	16SrII-D	
'*Ca.* P. australasia' PpM	Papaya mosaic	Australia	16SrII-D	
SUNHP	Sunn hemp witches'-broom	Thailand	16SrII-A	
PnWB	Peanut witches'-broom	Taiwan	16SrII-A	
SPWB	Sweet potato witches'-broom	Taiwan	16SrII-A	
SEPN	Sesame phyllody	Thailand	16SrII-A	
SEPT	Sesame phyllody	Thailand	16SrII-A	
CLP	Cleome phyllody	Thailand	16SrII-A	
IAWB	Italian alfalfa witches'-broom	Italy	16SrII-E	
PEP	*Picris echioides* phyllody	Italy	16SrII-E	
CrP	Crotalaria phyllody	Thailand	16SrII-C	
FBP	Faba bean phyllody	Sudan	16SrII-C	
SOYP	Soybean phyllody	Thailand	16SrII-C	
'*Ca.* P. aurantifolia' LWB	Lime witches'-broom	Oman	16SrII-B	
CoP	Cotton phyllody	Burkina Faso	16SrII-C	

Table 1. cont.

Strain	Associated disease	Geographical origin	RFLP subgroup classification	
			16Sr	rp
CX	Peach X-disease	Canada	16SrIII-A	rpIII-A
WX	Peach X-disease	California, USA	16SrIII-A	rpIII-B
PDX	Pear decline X-disease	California, USA	16SrIII-A	
CYE.L	Clover yellow edge	Lithuania	16SrIII-B	
WWB	Walnut witches'-broom	Georgia, USA	16SrIII-G	rpIII-B
JR (=PoiBI)	Poinsettia branch inducing	USA	16SrIII-H	
SP1	Spirea stunt	New York, USA	16SrIII-E	rpIII-F
GRI	Goldenrod yellows	New York, USA	16SrIII-D	rpIII-E
VAC	Vaccinium witches'-broom	Germany	16SrIII-F	
MWI	Milkweed yellows	New York, USA	16SrIII-F	rpIII-D
PBT	Pecan bunch	Georgia, USA	16SrIII-C	rpIII-G
PPWB	Pigeon pea witches'-broom	Florida, USA	16SrIX-A	
PPWBfl	Pigeon pea witches'-broom	Florida, USA	16SrIX-A	
PPWBja	Pigeon pea witches'-broom	Jamaica	16SrIX-A	
PPWBpr	Pigeon pea witches'-broom	Puerto Rico	16SrIX-A	
RLLfl	Rhynchosia little leaf	Florida, USA	16SrIX	
GLLhon	Gliricidia little leaf	Honduras	16SrIX	
KAP	*Knautia arvensis* phyllody	Italy	16SrIX	
PEY	*Picris echioides* yellows	Italy	16SrIX	
'*Ca.* P. phoenicium' AlmWB.A112	Almond witches'-broom	Lebanon	16SrIX-B	
'*Ca.* P. phoenicium' AlmWB1	Almond witches'-broom	Lebanon	16SrIX-B	
CocLY.Hon	Coconut lethal yellowing	Honduras	16SrIV	
LYTF.FL	Palm lethal yellowing	Florida	16SrIV	
LYF.C5	Coconut lethal yellowing	Florida	16SrIV-A	
LY	Palm lethal yellowing	Florida	16SrIV-A	
LYmex	Coconut lethal yellowing	Mexico	16SrIV	
LYja	Coconut lethal yellowing	Jamaica	16SrIV	
LYJ.C8	Coconut lethal yellowing	Jamaica	16SrIV-A	
LDY	Yucatan coconut lethal decline	Mexico	16SrIV-B	
CLDO	Coconut decline	Honduras	16SrIV	
CPD	Coyol palm decline	Honduras	16SrIV	
CPY	*Carludovica palmata* leaf yellowing	Mexico	16SrIV-D	
'*Ca.* P. trifolii' CP	Clover phyllody	Canada	16SrVI-A	
'*Ca.* P. trifolii' PWB	Potato witches'-broom	Canada	16SrVI-A	
LUM	Lucerne virescence	France	16SrVI	
BLL	Brinjal little leaf	India	16SrVI	
EYIL	Illinois elm yellows	Illinois, USA	16SrVI-C	
VR	Vinca virescence	California, USA	16SrVI-A	
CPS	Catharanthus phyllody	Sudan	16SrVI	
'*Ca.* P. fraxini' AshY1	Ash yellows	New York, USA	16SrVII-A	
'*Ca.* P. fraxini' AshY12	Ash yellows	New Jersey, USA	16SrVII-A	
'*Ca.* P. fraxini' AshY5	Ash yellows	Minnesota, USA	16SrVII-A	
ALY882	Alder yellows	Germany	16SrV-C	rpV-K
FD70	Flavescence dorée	France	16SrV-C	rpV-F
FDD	Flavescence dorée	Italy	16SrV-D	rpV-E
FDC	Flavescence dorée	Italy	16SrV-C	rpV-D
ALY	Alder yellows	Italy	16SrV-C	rpV-H
SpaWB229	Spartium witches'-broom	Italy	16SrV-C	rpV-L
RuS	Rubus stunt	Italy	16SrV-E	rpV-I
HD1	Hemp dogbane	New York, USA	16SrV-C	rpV-J
'*Ca.* P. ulmi' EY1	Elm yellows	New York, USA	16SrV-A	rpV-A
CLY5	Cherry lethal yellows	China	16SrV-B	rpV-B
PYIn	Peach yellows	India	16SrV-B	rpV-M
'*Ca.* P. ziziphi' JWB	Jujube witches'-broom	China	16SrV-B	rpV-C

Table 1. cont.

Strain	Associated disease	Geographical origin	RFLP subgroup classification	
			16Sr	rp
LfWB	Loofah witches'-broom	Taiwan	16SrVIII-A	
RYD (In15)	Rice yellow dwarf	India	16SrXI-A	
SCWL	Sugar cane white leaf	Thailand	16SrXI-B	
SCGS (In16)	Sugar cane grassy shoot	India	16SrXI-B	

by Lim & Sears (1992). Degenerate primers were then used to amplify putative rp gene sequences from phytoplasma groups of which rp gene sequences are not yet known.

Primer pairs rpL2F2/rp(I)R1A, rpL2F3/rp(I)R1A and rpF1C/rp(I)R1A were initially tested on several representative phytoplasma strains of phytoplasma groups listed in Table 1 (LNS2, AP15, AT, PD1, FBP, SUNHP, PnWB, TBB, GrapeA, IAWB, PEP, RYD, SCWL, SCGS, LfWB, MBS, VR, CP, CX, PPWB, KAP, LY, RuS, EY1, AshY1, PTV and MPV). They were also tested in nested PCR assays [direct PCR with primers rpL2F2/rp(I)R1A or rpL2F3/rp(I)R1A followed by nested PCR using primer pair rpF1C/rp(I)R1A] when there was no amplicon or when the amplicons were not of the expected size in the first PCR. The PCR conditions were the same as described above.

16Sr group-specific primers were designed based on a comprehensive alignment of phytoplasma rp gene sequences and their specificity was evaluated against 28 strains belonging to 12 groups (Table 2). For evaluation of the specificity of rp gene-based 16Sr group-specific

Table 2. Semi-universal and 16Sr group-specific rp-based primers designed in this and previous studies

Primer pair	Sequence (5′–3′)	Expected size of PCR product (bp)	Specificity	Reference
rpF1/rpR1	ggacataagttaggtgaattt (*rpsV*) acgatatttagttctttttgg (*rplP*)	1245–1389*	16SrI, III, IV, V, VII, VIII, IX, XIII	Lim & Sears (1992)
rp(I)F1A/rp(I)R1A	ttttcccctacacgtactta (*rplP*) gttctttttggcattaacat (*rpsC*/*rplP*)	1200	16SrI	Lee *et al.* (2004a)
rpL2F3/rp(I)R1A	wccttggggyaaaaaagctc (*rplB*) gttctttttggcattaacat (*rpsC*/*rplP*)	1600	16SrI, III, IV, V, VI, VII, IX, X, XII, XIII, XVIII	This study
rpF1C/rp(I)R1A	atggtdggdcayaarttagg (*rpsV*) gttctttttggcattaacat (*rpsC*/*rplP*)	1212–1386*	16SrI, II, III, IV, V, VI, VII, IX, X, XII, XIII, XVIII	This study
rp(II)F1/rp(II)R1	gctcttactcgtaaayatgtagt (*rpsS*) ttacttgattttctggttttga (*rpsC*)	1200	16SrII	This study
rp(III)F1/rp(III)R1	ttagagaaggcattaaac (*rpsS*) ctctttccccatctaggacg (*rpsC*)	1200	16SrIII	This study
rp(V)F1/rpR1	tcgcggtcatgcaaaaggcg (*rpsS*) acgatatttagttctttttgg (*rplP*)	1200	16SrV	Lee *et al.* (1998) Lim & Sears (1992)
rp(V)F1A/rp(V)R1A	aggcgataaaaaagtttcaaaa (*rpsS*) ggcattaacataatatattatg (*rplP*)	1200	16SrV	Lee *et al.* (2004b)
rp(VI)F2/rp(VI)R2	ggttgttgatttaattcgtggtc (*rplV*) ccagatattcgtctagtatcagaa (*rpsC*)	1000	16SrVI	This study
rp(VIII)F2/rp(VIII)R2	agttgtcgatttaattcgtggca (*rplV*) cagcagatatttgtctagtatctgcg (*rpsC*)	1000	16SrVII, VIII	This study
rp(IX)F2/rp(IX)R2	gcacaagctattttaatgtttacaccc (*rplV*) caaagggactaaacctaaag (*rpsC*)	800	16SrIX	This study
rpStolF/rpStolR†	cgtacaaaataatcgggaga (*rplB*) cgaaacaaaaggtttacgag (*rpsC*)	1372	16SrXII-A	This study
rpStolF2/rpStolR†	aaacttggtcacgtagttcc (*rplP*) cgaaacaaaaggtttacgag (*rpsC*)	1253	16SrXII-A	This study

*Product size is group-dependent.

†PCR conditions with primer pairs rpStolF/rpStolR and rpStolF2/rpStolR were initial denaturation at 94 °C for 2 min, 40 cycles of 1 min at 94 °C, 45 s at 53 °C and 90 s at 72 °C and a final extension at 72 °C for 8 min.

primer pairs, PCR was performed as described previously with an annealing temperature of 50 or 55 °C.

PCR amplification of rp gene operons from representative phytoplasmas. Primer pair rpF1C/rp(I)R1A was used to amplify rp genes from 54 representative phytoplasma strains listed in Table 1 and *A. palmae* J233^T. The PCR conditions were the same as described above. The amplicons (about 1.2 kb, containing the *rplV–rpsC* gene sequence) were purified, cloned and sequenced as described above.

PCR amplification of the 16S rRNA gene from representative phytoplasmas. Primer pair P1/P7 was used to amplify the 16S rRNA gene, spacer region and 5′ end of the 23S rRNA gene from 19 representative phytoplasma strains listed in Table 1. The PCR conditions were the same as described above with an annealing temperature of 55 °C. The PCR products from primers P1/P7 (about 1.8 kb, containing the 16S rRNA gene sequence) were purified, cloned and sequenced as described above.

Phylogenetic analysis. To determine the phylogenetic relationships among phytoplasmas, other mollicutes and closely related walled bacteria, a comprehensive analysis was performed based on nucleotide and deduced amino acid sequences of rp genes (*rplV* and *rpsC*) and on the 16S rRNA gene from representative mollicutes: 46 phytoplasmas (42 based on 16S rRNA), two acholeplasmas, 10 mycoplasmas, one mesoplasma, one ureaplasma, two spiroplasmas and 28 Gram-positive walled bacterial species belonging to the *Bacillales*, '*Clostridia*', '*Lactobacillales*' and *Actinobacteria*. The rp nucleotide and deduced amino acid sequences for mollicutes and other Gram-positive walled bacteria used in this study were obtained mostly from complete genome sequences available in GenBank (Figs 1 and 2). Rp nucleotide and deduced amino acid sequences were aligned as described above. Each output alignment was converted to NBRF/Pir format and was trimmed using GBLOCKS (version 0.91b) to eliminate poorly aligned positions (Castresana, 2000). The trimmed alignment was converted back to MEGALIGN. Cladistic analyses were performed with PAUP, version 4.0 (Swofford, 1998), on a Power Mac G4. Uninformative characters were excluded from analyses. A phylogenetic tree was constructed by a heuristic search [or the neighbour-joining (NJ) algorithm] via random stepwise addition implementing the tree bisection and reconnection branch-swapping algorithm to find the optimal tree(s) (Gundersen *et al.*, 1994). Among the taxa analysed, a high-G+C-content Gram-positive bacterium, *Streptomyces coelicolor* A3(2), was most distantly related to the phytoplasmas. This strain was designated the outgroup to root the tree. The analysis was replicated 1000 times. Bootstrapping was performed to estimate the stability and support for the inferred clades.

Phylogenetic interrelationships among 87 phytoplasma strains (Table 1) were determined based on rp genes (with nucleotide sequences) and among 78 phytoplasma strains (Table 1) based on the 16S rRNA gene. Sequence alignment and cladistic analyses were performed as described above. Two *Acholeplasma* strains and *Bacillus subtilis* 168 were included in the analyses. *B. subtilis* 168 was selected as the outgroup to root the tree. Sequence similarities were calculated by using the MEGALIGN program (Lasergene software; DNASTAR).

Rp and 16S rRNA gene sequences of phytoplasma strains used in phylogenetic analyses were either sequenced in this work or obtained from GenBank.

Restriction maps. To evaluate the efficacy of rp genes for finer differentiation of phytoplasma strains in clover proliferation group (16SrVI) and apple proliferation group (16SrX), putative restriction maps were constructed by using the MapDraw program (Lasergene software; DNASTAR).

RESULTS AND DISCUSSION

Efficacy of degenerate primer pairs for amplification of the phytoplasma rp operon

Primer pairs rpL2F3/rp(I)R1A and rpF1C/rp(I)R1A both amplified a fragment from genomic DNA of representative phytoplasma strains belonging to the following groups: AY (16SrI), X-disease (16SrIII), EY (16SrV), CP (16SrVI), AshY (16SrVII), PPWB (16SrIX), AP (16SrX), Stolbur (16SrXII), MPV (16SrXIII) and APPTW (16SrXVIII) (Table 1). Representative strains of the LY group (16SrIV) were amplified in a semi-nested PCR using primer pair rpL2F3/rp(I)R1A in a first PCR followed by primer pair rpF1C/rp(I)R1A. Rp genes from representative strains of the PnWB group (16SrII) were amplified using the primer pair rpF1C/rp(I)R1A. They were amplified by the primer pair rpL2F2/rp(I)R1A as well, but in many cases the amplification resulted in multiple bands. Members of phytoplasma groups 16SrVIII and 16SrXI didn't yield amplicons of the expected size, probably because of a lack of primer specificity for these phytoplasma groups or degradation of the DNA templates.

PCR-amplified partial rp gene operons from diverse phytoplasmas

PCR using primer pair rpF1C/rp(I)R1A yielded DNA amplicons (partial rp gene operons) from many phytoplasma strains listed in Table 1. The amplicons amplified from members of phytoplasma group 16SrII ranged from 1284 to 1309 bp. The amplicons amplified from members of phytoplasma group 16SrVI were homogeneous in size (1266 or 1267 bp). Members of phytoplasma group 16SrVII yielded an amplicon of 1272 bp. Coconut lethal yellows (LY) phytoplasma strains, representative of the lethal yellows phytoplasma group (16SrIV), produced an amplicon of 1247 bp. Phytoplasma strains belonging to the pigeon pea witches'-broom (PPWB) phytoplasma group (16SrIX) yielded an amplicon of 1262 bp in strains PEY, PPWB and KAP and an amplicon of 1239 bp in strain AlmWB. Members of the X-disease phytoplasma group (16SrIII) yielded an amplicon of 1386 or 1387 bp from all strains except strain MW1, which yielded an amplicon of 1346 bp. Phytoplasma strains AT, PD1 and LNS2 belonging to the AP phytoplasma group (16SrX) yielded an amplicon of 1243 bp, while strains AP15 and APSb yielded amplicons that were respectively 12 and 48 bp longer. Phytoplasmas NJAY and MBS (both 16SrI), MPV (16SrXIII), PTV (TomStol) (16SrXII) and APPTW (16SrXVIII) yielded amplicons of 1260, 1263, 1222 and 1212 bp, respectively.

Congruence between the rp gene-based phylogenetic tree and the 16S rRNA gene-based tree

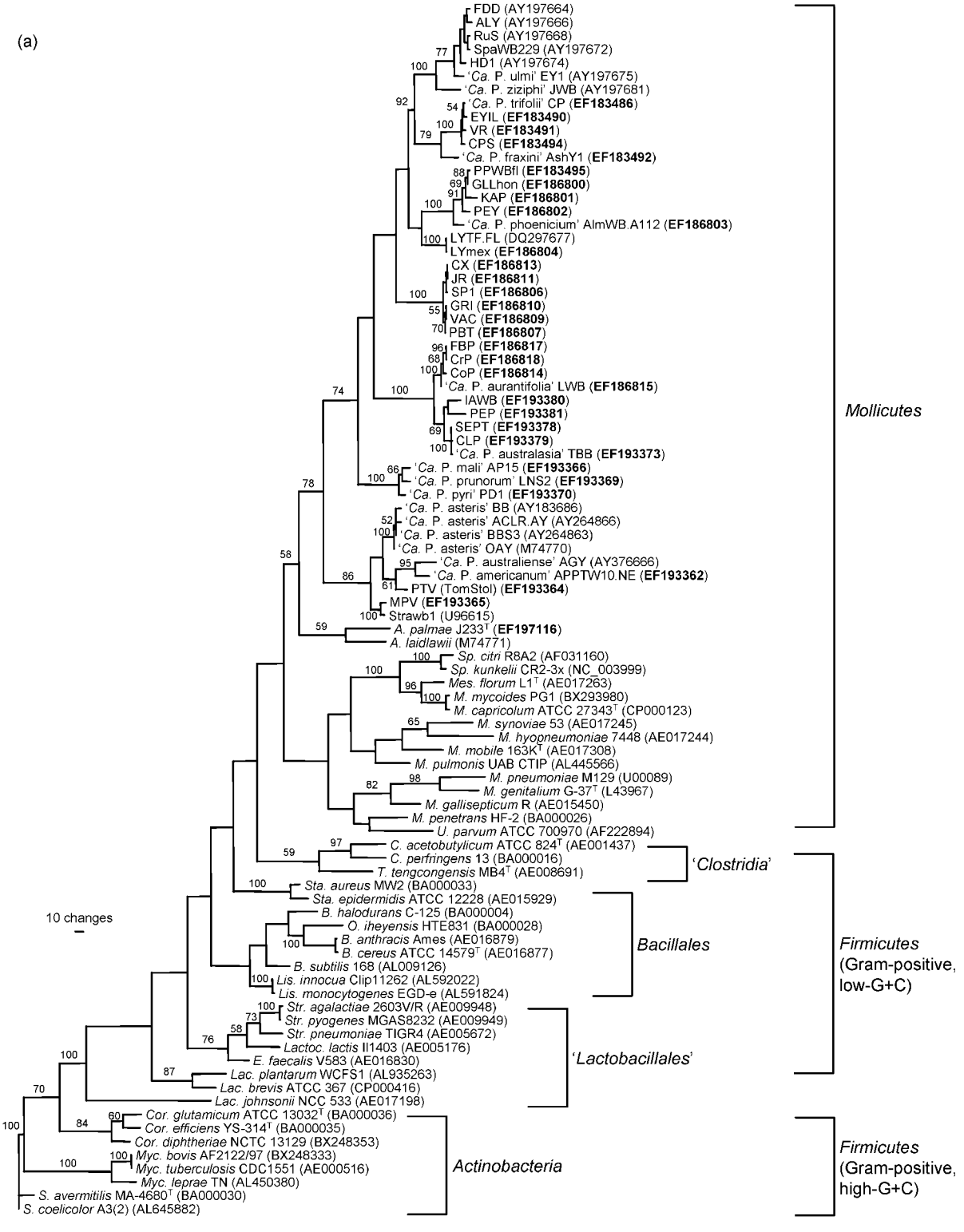
Comparative cladistic analyses were performed by using *rplV–rpsC* and 16S rRNA gene sequences from phytoplasma

strains across 12 distinct phytoplasma 16Sr groups (Lee *et al.*, 1998, 2006b), other representative mollicutes and Gram-positive walled bacteria. The analyses resulted in 32 equally parsimonious trees based on rp genes and 126 trees based on the 16S rRNA gene; representative trees were selected (Fig. 1). The phylogenetic tree inferred from rp genes (Fig. 1a) had an overall topology similar to that inferred from the 16S rRNA gene (Fig. 1b). The two trees showed similar major branching orders, beginning with the outgroup *S. coelicolor* A3(2) followed by the high-G+C-content Gram-positive bacteria, low-G+C-content Gram-positive bacteria and mollicutes. The mollicutes clade contained two distinct subclades for both the rp and 16S rRNA gene analyses. Plant-pathogenic phytoplasmas clustered with *Acholeplasma* strains, forming a subclade. Plant-pathogenic spiroplasmas clustered with animal- and human-pathogenic mycoplasmas, forming another subclade. The closest relatives of the mollicutes were low-G+C-content Gram-positive bacteria belonging to the '*Lactobacillales*', *Bacillales* and '*Clostridia*'. The results are generally in agreement with those reported in previous phylogenetic studies based on the 16S rRNA gene (Jung *et al.*, 2002; Lee *et al.*, 1998; Seemüller *et al.*, 1998). However, the detailed branching orders in the 16S rRNA gene- and rp gene-based trees were not entirely parallel, suggesting unequal rates of change of the 16S rRNA and rp genes over the course of their evolution. The rp genes have probably undergone more rapid change than the 16S rRNA gene. As a result, the rp gene-based tree delineated more defined phylogenetic interrelationships among taxa, especially in the phytoplasma groups, that cannot be readily resolved by the highly conserved 16S rRNA gene-based phylogeny.

The rp gene-based phylogeny using nucleotide sequences indicated that the members of the '*Clostridia*' among the low-G+C-content Gram-positive bacteria are the closest relatives of the mollicutes, followed by members of the *Bacillales* and '*Lactobacillales*' in all 32 of the most parsimonious trees, although the stability of the branching order in this region was supported only by low (<50 %) bootstrap values, which indicated some uncertainty for the inferred character stability among these taxa. The results are in agreement with results reported in a previous study (Zhao *et al.*, 2005). A phylogenetic tree based on deduced amino acid sequences of rp genes for these same taxa resulted in a similar branching order (data not shown).

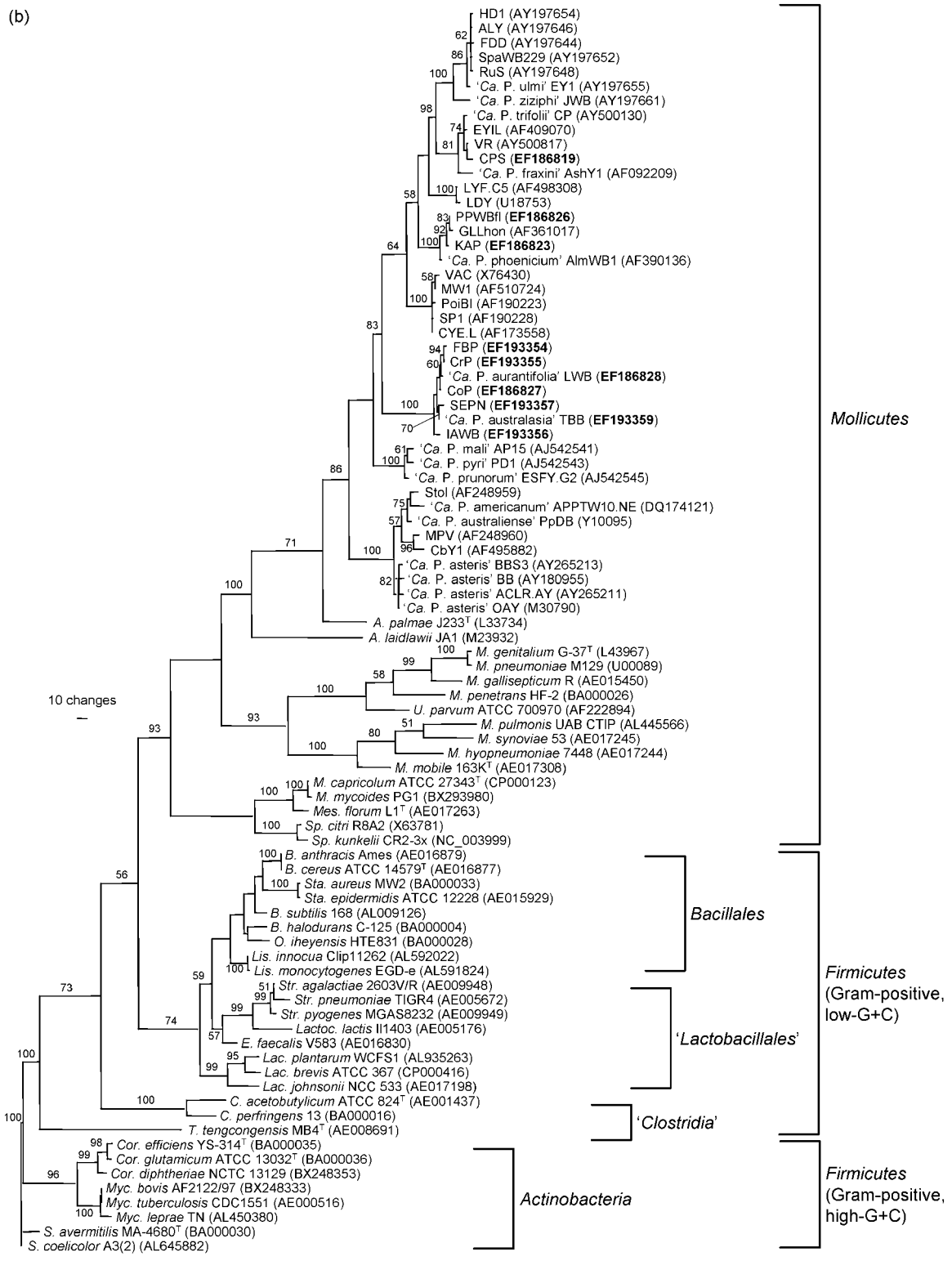
Phylogenetic analyses using the NJ algorithm resulted in a tree with a topology similar to that of the heuristic tree described above when the 16S rRNA gene was used for the analysis, but generated a tree with a different topology (among basal mollicute taxa) when rp genes were used for the analysis (Fig. 2a, b). In the rp NJ tree (Fig. 2a), phytoplasmas together with the two *Acholeplasma* strains formed a monophyletic group that is paraphyletic to the clade consisting of low-G+C-content Gram-positive bacteria in the *Bacillales* and '*Lactobacillales*'. The other mollicutes (spiroplasmas and animal- and human-pathogenic mycoplasmas) together with the low-G+C-content Gram-positive bacteria of the '*Clostridia*' formed a separate monophyletic group. The discrepancies were unexpected and resulted from very few nucleotide substitutions, as shown by the low bootstrap values in several major branches. However, overall, the nucleotide sequence similarity data were consistent with the phylogenetic relationships inferred by the branching order (data not shown). These results implied that phytoplasmas and acholeplasmas may have a small number of rp characteristics more similar to Gram-positive walled bacteria than to other mollicutes such as mycoplasmas and spiroplasmas. Recent findings (Bai *et al.*, 2006; Oshima *et al.*, 2004) based on characterization of phytoplasma genomes support these conclusions. Sequences of phytoplasma genes often showed the best matches with Gram-positive *Bacillus* strains in BLAST and FASTX searches. Miyata *et al.* (2002a, b) reported that the gene organization of some phytoplasma operons such as S10-*spc* and *str* resembled that of *B. subtilis* but not those of mycoplasmas. The different codon usage in members of the class *Mollicutes* offers more evidence in favour of a distinct evolution of phytoplasmas and acholeplasmas from the other mollicutes. *Mycoplasma*, *Ureaplasma*, *Spiroplasma* and *Mesoplasma* species use UGA as a tryptophan codon instead of a stop codon, a feature found in vertebrate, ascidian, echinoderm, *Drosophila*, yeast, mould and protozoan mitochondria (Nei & Kumar, 2000), whereas phytoplasmas and acholeplasmas use the conventional UGG codon for tryptophan and UGA as a stop codon (Razin *et al.*, 1998).

Fig. 1. Phylogenetic trees constructed by parsimony (PAUP version 4.0b) analyses of sequences of rp genes *rplV* (*rpl22*) and *rpsC* (*rps3*) (a) and 16S rRNA genes (b) from 62 and 58 members, respectively, of the class *Mollicutes*, including 46 (a) and 42 (b) phytoplasma strains, and representatives of Gram-positive, high-G+C- and low-G+C-content walled bacteria, employing *Streptomyces coelicolor* A3(2) as the outgroup. Sequences were aligned with CLUSTAL version 5. The trees were obtained through a heuristic search algorithm. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values are shown on branches. Bars, 10 inferred character state changes. Phytoplasma strain abbreviations are defined in Table 1. Bacterial genus abbreviations: *A.*, *Acholeplasma*; *B.*, *Bacillus*; *C.*, *Clostridium*; *Cor.*, *Corynebacterium*; *E.*, *Enterococcus*; *Lis.*, *Listeria*; *Lac.*, *Lactobacillus*; *Lactoc.*, *Lactococcus*; *M.*, *Mycoplasma*; *Mes.*, *Mesoplasma*; *Myc.*, *Mycobacterium*; *O.*, *Oceanobacillus*; *S.*, *Streptomyces*; *Sp.*, *Spiroplasma*; *Sta.*, *Staphylococcus*; *Str.*, *Streptococcus*; *T.*, *Thermoanaerobacter*; *U.*, *Ureaplasma*. GenBank accession numbers of sequences obtained in this study are indicated in bold. The sequence of *Spiroplasma kunkelii* CR2-3x was accessed at http://www.genome.ou.edu/spiro.html



Phylogenetic trees of phytoplasmas based on nucleotide sequences of *rplV* and *rpsC* genes and 16S rRNA gene sequences

Phylogenetic analysis using *rplV–rpsC* nucleotide sequences from 87 phytoplasma strains representing 14 distinct phytoplasma groups (or 12 16Sr groups according to Lee *et al.*, 1998) or more than 40 subgroups, *A. palmae* J233^T, *Acholeplasma laidlawii* JA1 and *B. subtilis* 168 (as an outgroup) resulted in 288 equally parsimonious trees. One of the most parsimonious trees is presented in Fig. 3(a). The phylogeny inferred by *rplV–rpsC* sequences was nearly



congruent with that inferred by 16S rRNA gene analysis (Fig. 3b), indicating similar interrelatedness among phytoplasma taxa. This is in agreement with a previous report in which a phylogenetic study was conducted based on the *rplV* sequences of 11 phytoplasma strains representative of eight phytoplasma subclades (Gundersen *et al.*, 1994).

As shown in previous phylogenetic studies based on the 16S rRNA gene (Jung *et al.*, 2002; Lee *et al.*, 1998, 2000; Seemüller *et al.*, 1998), phytoplasmas formed a large monophyletic group closely related to *A. palmae* and *A. laidlawii*. In this study, both rp- and 16S rRNA gene-based trees revealed that *A. palmae* and *A. laidlawii* constituted a

monophyletic group, paraphyletic to the phytoplasma group. On the basis of the *rplV–rpsC* sequences analysed, a total of 19 distinct phytoplasma monophyletic groups or taxa, which we designated subclades (using lower-case roman numerals), could be recognized based on both tree branch lengths and sequence similarities. Ten rp subclades (*i*, *ii*, *iii*, *iv*, *vi*, *vii*, *viii*, *ix*, *x* and *xii*) corresponded with 16S rRNA gene subclades identified by Gundersen *et al.* (1994), Lee *et al.* (1998) and Montano *et al.* (2001). In these studies, a total of 14 distinct 16Sr subclades were recognized.

The rp gene-based phylogeny clearly resolved more distinct subclades than the 16Sr RNA gene-based phylogeny (Gundersen *et al.*, 1994). For example, the EY group (16SrV) was resolved into two distinct rp subclades (*x* and *xx*), the LY group (16SrIV) was resolved into two rp subclades (*vii* and *xxiii*), the PPWB group (16SrIX) was resolved into two rp subclades (*vi* and *xix*), the PnWB group (16SrII) was resolved into four rp subclades (*iii*, *xviii*, *xvii* and *xvi*) and the stolbur (16SrXII) and American potato purple top wilt (16SrXVIII) groups were resolved into three subclades (*xii*, *xxi* and *xxii*). Each distinct subclade shared no more than 96 % rp sequence similarity to any other subclade. The stability of the subclades was supported by high bootstrap values. We propose that each rp subclade represents at least one phytoplasma species.

Genetic variations of phytoplasmas assessed by comparative sequence analyses of two different conserved genes

The genetic variations among members of a given 16Sr phytoplasma group were determined based on sequences of the rp and 16S rRNA genes obtained both in this work and from GenBank. Overall, the rp genes exhibited greater sequence variation than the 16S rRNA gene, confirming previous results (Gundersen *et al.*, 1994, 1996). The average nucleic acid sequence similarity of rp genes among members of two different phytoplasma groups ranged from 50.4 % (among members of groups 16SrXII-A and 16SrII) to 83.5 % (among members of groups 16SrV and 16SrVI), compared with 85.1 % (groups 16SrIX and 16SrX) to 96.9 % (groups 16SrVI and 16SrVII) similarity for the 16S rRNA gene sequences. Members of phytoplasma groups 16SrX, I, XII, XIII and XVIII are the phytoplasma strains most closely related to *Acholeplasma* strains, sharing an average rp gene sequence similarity of 63 % with *A. palmae* J233^{T} and 55.8 % with *A. laidlawii* JA1. Members of the AY (16SrI) and EY (16SrV) phytoplasma groups exhibited similar degrees of genetic variability in these two genes, showing average sequence similarities ranging from 98.4 to 99.2 % and 98.6 to 99.5 %, respectively, based on the 16S rRNA gene, and from 96.8 to 98.2 % and 96.5 to 98.1 %, respectively, based on the rp gene sequences. Members of the CP (16SrVI), PPWB (16SrIX) and AP (16SrX) phytoplasma groups exhibited different degrees of genetic variability in these two genes. The average sequence similarity among members of the AP phytoplasma group varied from 98.7 to 99.4 % based on the 16S rRNA gene and from 93.4 to 96.5 % based on the rp genes. Based on the 16S rRNA gene, the average sequence similarities ranged from 98.3 to 99.4 % among members of the PPWB phytoplasma group and from 98.4 to 99.2 % among members of the CP phytoplasma group. However, more heterogeneity was evident in the PPWB phytoplasma group than in the CP group when rp genes were analysed. The average sequence similarities ranged from 87.8 to 96.8 % among members of the PPWB phytoplasma group and from 97.8 to 98.8 % among members of the CP phytoplasma group. The lowest average sequence similarities were shown by members of the PnWB (16SrII) phytoplasma group in both genes, ranging from 97.4 to 98.8 % based on the 16S rRNA gene and from 86 to 93.1 % based on the rp genes.

The greater sequence variation makes the rp genes a better molecular tool for phytoplasma classification. More-variable rp gene sequences are ideal for use in RFLP analysis for finer differentiation of ecologically or biologically distinct strains within a given 16Sr phytoplasma group. Genetically distinct phytoplasma strains that cannot be differentiated on the basis of the highly conserved 16S rRNA gene are able to be differentiated on the basis of the less-conserved rp genes. While closely related strains shared similar high similarities (>99 %) on the basis of both 16S rRNA and rp gene sequences, distinct strains, including those unresolved by 16S rRNA gene sequence, between two 16Sr subgroups or within a subgroup manifested their variability when analysed by *rplV* and *rpsC* gene sequences. For example, based on 16S rRNA gene sequences, the beet leafhopper-transmitted agent (BLTVA) phytoplasma (e.g. strain VR), which causes potato purple top wilt in Oregon and Washington states (Lee *et al.*, 2004c), and the potato witches'-broom phytoplasma (e.g. strain PWB), which causes potato witches'-broom in North Dakota and Canada (Lee *et al.*, 1998), are classified with strains CP and PWB as members of subgroup 16SrVI-A, sharing about 99.2 % similarity. Strain VR shared only 97.6 % similarity with CP and PWB based on rp gene sequences. Following phylogenetic analysis based on the rp genes, strain VR appeared to represent a separate lineage distinct from strains CP and PWB. Based on RFLP analysis of rp gene sequences, strains VR and PWB (along with strain CP) were classified into two rp subgroups, consistent with their differing ecological niches and biological properties. In nature, they are carried by different insect vectors and cause different symptoms in infected plants. Maize bushy stunt (MBS) phytoplasma was classified along with other aster yellows phytoplasma strains (e.g. OAY) as members of subgroup 16SrI-B; this provides another example of a strain with distinct ecology that cannot be differentiated based on the 16S rRNA gene but is readily separated from other members of subgroup 16SrI-B based on RFLP analysis of rp gene sequences. In the apple proliferation group (16SrX), three members have been proposed to represent three distinct species, provisionally designated

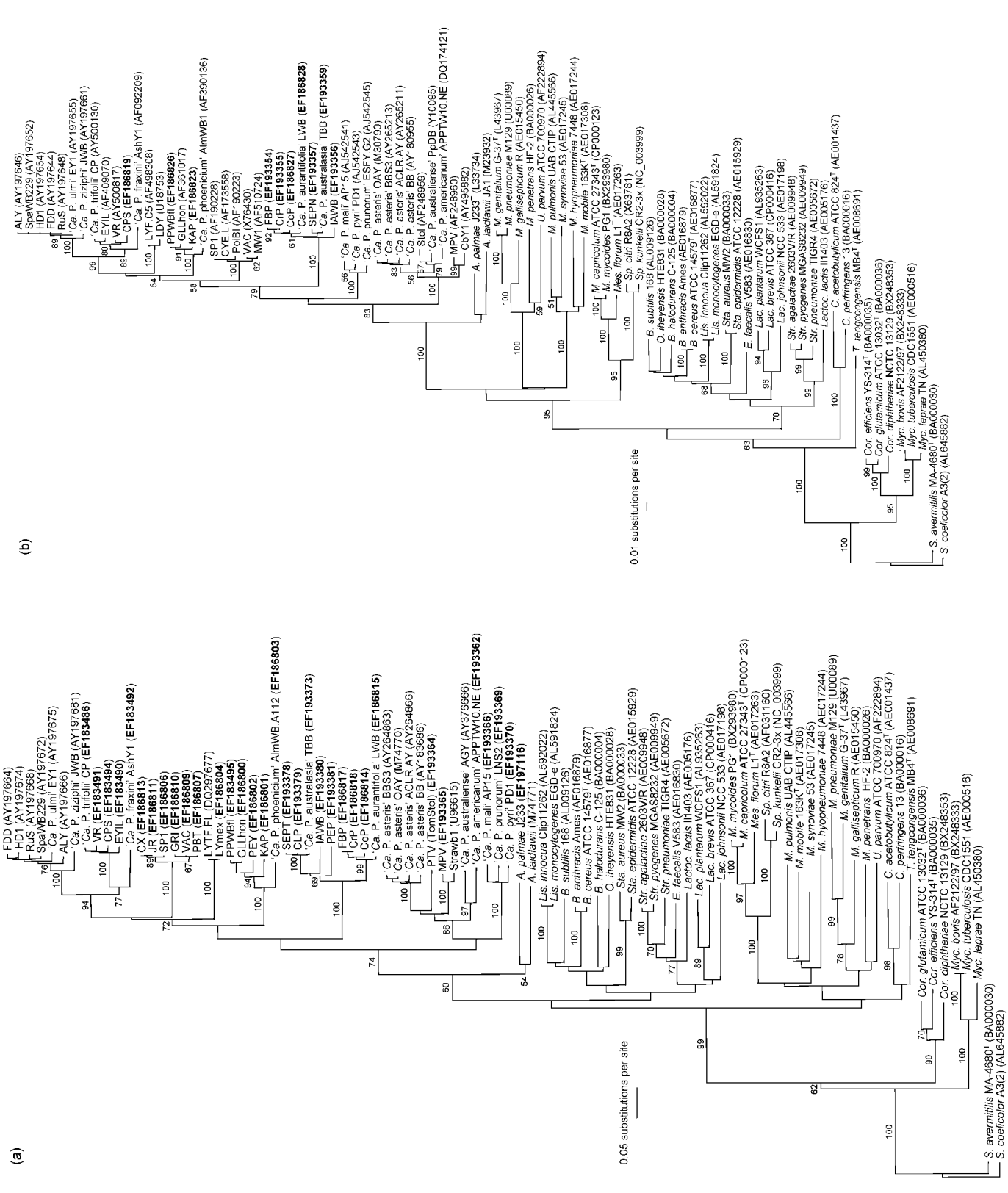


Fig. 2. Phylogenetic trees constructed by parsimony analyses of sequences of rp genes *rplV* (*rpl22*) and *rpsC* (*rps3*) (a) and 16S rRNA genes (b) from members of the class *Mollicutes*, including 46 (a) and 42 (b) phytoplasma strains. Sequences were aligned with CLUSTAL version 5. The trees were obtained through the neighbour-joining algorithm. Bars, 0.05 (a) and 0.01 (b) substitutions per site. Phytoplasma strain abbreviations are defined in Table 1. Bacterial genus abbreviations and other details are as in Fig. 1.

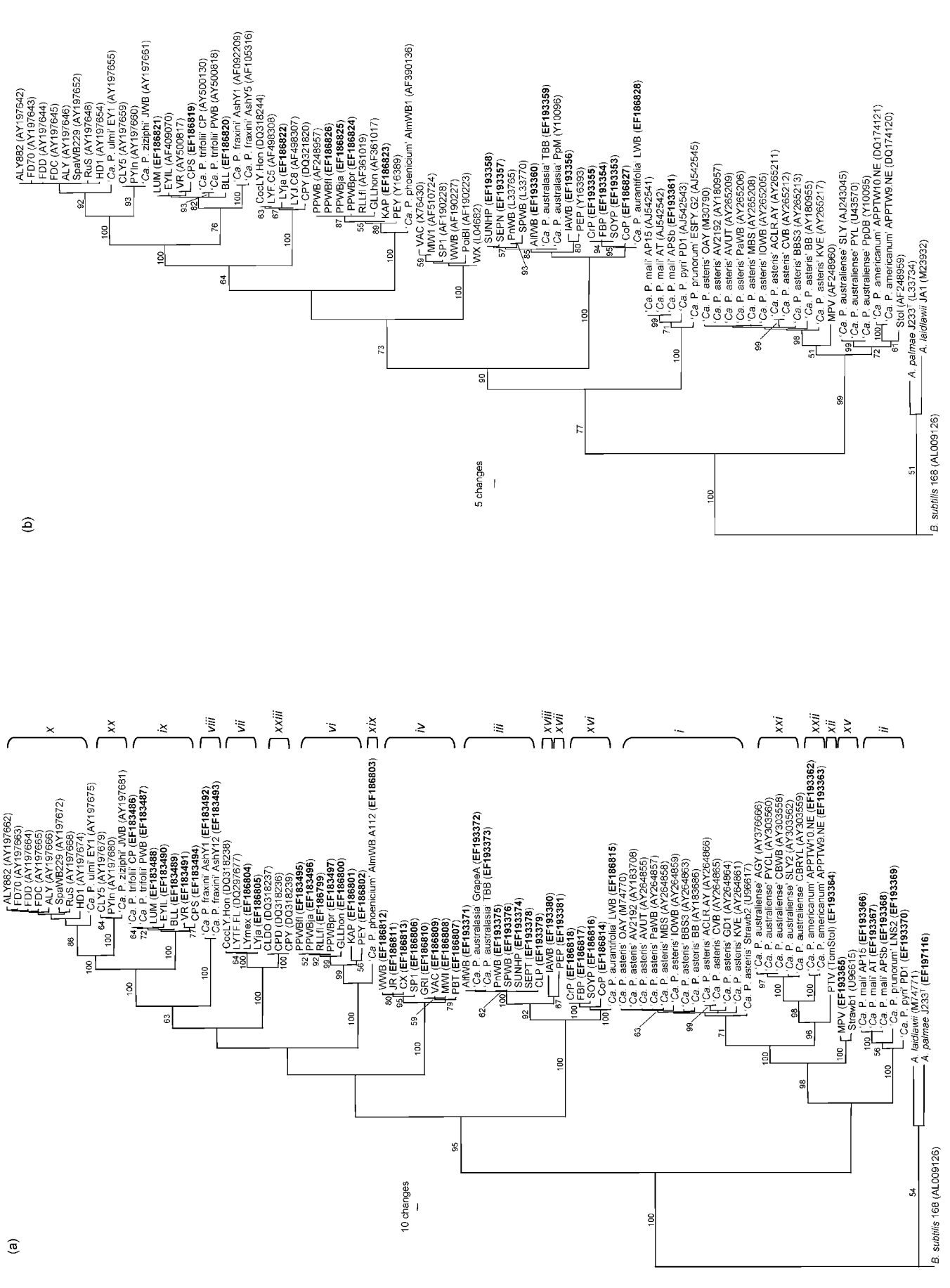


Fig. 3. Phylogenetic trees constructed by parsimony analyses of partial rp operon [covering *rplV* (*rpl22*) and *rpsC* (*rps3*) genes] (a) and 16S rRNA gene (b) sequences from representative phytoplasma strains of different phytoplasma groups. Sequences were aligned with CLUSTAL version 5. *B. subtilis* 168 was employed as the outgroup. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values are shown on branches. Bars, 10 (a) and 5 (b) inferred character state changes. Roman numerals shown to the right in (a) represent the phylogenetic subclades identified. Subclades *i–iv* and *vi–xii* were designated to correlate with 16S rRNA subclades identified previously. Subclades *xvi–xxiii* were identified in this study. Phytoplasma strain abbreviations are defined in Table 1. GenBank accession numbers of sequences obtained in this study are indicated in bold.

'*Candidatus* Phytoplasma mali', '*Ca.* Phytoplasma prunorum' and '*Ca.* Phytoplasma pyri', based primarily on their unique biological properties (vector and host specificities). These three strains have an average sequence similarity >98.7 % based on the 16S rRNA gene and are barely differentiated based on RFLP analysis of 16S rRNA gene sequences. In contrast, they have an average sequence similarity <96.5 % based on *rplV*–rp*sC* and can be readily differentiated based on rp gene sequences.

Greater sequence variation in the rp genes has facilitated the design of phytoplasma 16Sr group-specific primers. In this study, we have designed several semi-universal and group-specific rp primers (Table 2) for amplification of the region extending across *rpsS–rplV–rpsC*. The amplicons can be used for RFLP analysis or for specific identification of 16Sr groups or subgroups.

RFLP analysis of rp gene sequences for finer differentiation among strains

The efficacy of rp gene sequences for finer differentiation among phytoplasma strains in a given 16Sr group was evaluated by RFLP analysis. Two 16Sr groups, 16SrVI and 16SrX, were selected for analysis. Putative rp gene-based RFLP patterns were generated by using the MapDraw program (Lasergene software; DNASTAR). Based on putative RFLP patterns with four restriction enzymes (*Alu*I, *Dra*I, *Taq*I and *Tsp509*I), representative strains CP (subgroup 16SrVI-A), PWB (16SrVI-A), VR (16SrVI-A), EYIL (16SrVI-C), LUM (16SrVI), BLL (16SrVI) and CPS (16SrVI) in group 16SrVI were differentiated into six distinct rp subgroups (Supplementary Fig. S2 in IJSEM Online). Strain VR along with strains CP and PWB have been classified into the same subgroup, 16SrVI-A. Based on rp gene sequences, strain VR represented a separate subgroup of rp(VI), distinct from strains CP and PWB, which formed another subgroup of rp(VI). Based on putative RFLP patterns with two enzymes (*Alu*I and *Dra*I), strains AP15 (subgroup 16SrX-A), AT (16SrX-A), LNS2 (16SrX-B) and PD1 (16SrX-C) were readily differentiated into three distinct rp subgroups (Supplementary Fig. S3 in IJSEM Online).

Conclusions

Ribosomal proteins and rRNA are essential components of the ribosome, a ubiquitous cellular organelle. Like the rRNA genes (16S rRNA and 23S rRNA), ribosomal proteins are conserved among bacterial species. Ribosomal protein genes are among a core of genes in bacteria that share a common history of evolution and carry a strong phylogenetic signal (Daubin *et al.*, 2002; Wolf *et al.*, 2001). These genes are more variable than the 16S rRNA gene and serve as a good phylogenetic parameter useful for differentiation and classification of bacterial strains at taxonomic ranks below the genus level.

In the present study, the rp gene-based phylogeny revealed more insights into the phylogenetic relationships among phytoplasma strains. With more variability than the 16S rRNA gene, the rp gene sequences provide more phylogenetic markers useful for differentiation of genetically closely related but distinct ecological strains that are not readily separated on the basis of the highly conserved 16S rRNA gene. So far, 16S rRNA gene-based RFLP analysis has been applied extensively for differentiation and classification of phytoplasmas. It proves to be very suitable for classification of phytoplasma strains into major 16Sr RFLP groups. However, finer differentiation into subgroups cannot be achieved precisely based on the rather limited number of variable markers present in the 16S rRNA gene. It was proposed that each group represents at least one phytoplasma species. However, there are not sufficient phylogenetic markers to determine the taxonomic rank for each subgroup. The present study and our previous studies indicate that more-variable genes, such as rp or *secY*, can serve as additional phylogenetic tools for finer differentiation of subgroups within a given 16Sr group.

REFERENCES

Abou-Jawdah, Y., Karakashian, A., Sobh, H., Martini, M. & Lee, I.-M. (2002). An epidemic of almond witches'-broom in Lebanon: classification and phylogenetic relationships of the associated phytoplasma. *Plant Dis* **86**, 477–484.

Arocha, Y., López, M., Piñol, B., Fernández, M., Picornell, B., Almeida, R., Palenzuela, I., Wilson, M. R. & Jones, P. (2005). '*Candidatus* Phytoplasma graminis' and '*Candidatus* Phytoplasma caricae', two novel phytoplasmas associated with diseases of sugarcane, weeds and papaya in Cuba. *Int J Syst Evol Microbiol* **55**, 2451–2463.

Bai, X., Zhang, J., Ewing, A., Miller, S. A., Radek, A. J., Shevchenko, D. V., Tsukerman, K., Walunas, T., Lapidus, A. & other authors (2006). Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. *J Bacteriol* **188**, 3682–3696.

Bertaccini, A., Carraro, L., Davies, D., Laimer da Camara Machado, M., Martini, M., Paltrinieri, S. & Seemüller, E. (2000). Micropropagation of a collection of phytoplasma strains in periwinkle

and other host plants. In *Proceedings of the 13th International Congress of the International Organization for Mycoplasmology*, Fukuoka, Japan, 14–19 July 2000, p. 101.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* **17**, 540–552.

Daubin, V., Gouy, M. & Perriere, G. (2002). A phylogenomic approach to bacterial phylogeny: evidence of a core of genes sharing a common history. *Genome Res* **12**, 1080–1090.

Gundersen, D. E., Lee, I.-M., Rehner, S. A., Davis, R. E. & Kingsbury, D. T. (1994). Phylogeny of mycoplasmalike organisms (phytoplasmas): a basis for their classification. *J Bacteriol* **176**, 5244–5254.

Gundersen, D. E., Lee, I.-M., Schaff, D. A., Harrison, N. A., Chang, C. J., Davis, R. E. & Kinsbury, D. T. (1996). Genomic diversity and differentiation among phytoplasma strains in 16S rRNA group I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). *Int J Syst Bacteriol* **46**, 64–75.

IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group (2004). '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *Int J Syst Evol Microbiol* **54**, 1243–1255.

Jacobs, K. A., Lee, I.-M., Griffiths, H. M., Miller, F. D., Jr & Bottner, K. D. (2003). A new member of the clover proliferation phytoplasmas group (16SrVI) associated with elm yellows in Illinois. *Plant Dis* **87**, 241–246.

Jung, H. Y., Sawayanagi, T., Kakizawa, S., Nishigawa, H., Miyata, S., Oshima, K., Ugaki, M., Lee, J. T., Hibi, T. & Namba, S. (2002). '*Candidatus* Phytoplasma castaneae', a novel phytoplasma taxon associated with chestnut witches' broom disease. *Int J Syst Evol Microbiol* **52**, 1543–1549.

Lee, I.-M., Davis, R. E., Sinclair, W. A., DeWitt, N. D. & Conti, M. (1993a). Genetic relatedness of mycoplasmalike organisms detected in *Ulmus* spp. in the United States and Italy by means of DNA probes and polymerase chain reactions. *Phytopathology* **83**, 829–833.

Lee, I.-M., Hammond, R. W., Davis, R. E. & Gundersen, D. E. (1993b). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology* **83**, 834–842.

Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E. & Bartoszyk, I. M. (1998). Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int J Syst Bacteriol* **48**, 1153–1169.

Lee, I.-M., Davis, R. E. & Gundersen-Rindal, D. E. (2000). Phytoplasma: phytopathogenic mollicutes. *Annu Rev Microbiol* **54**, 221–255.

Lee, I.-M., Martini, M., Bottner, K. D., Dane, R. A., Black, M. C. & Troxclair, N. (2003). Ecological implications from a molecular analysis of phytoplasmas involved in an aster yellows epidemic in various crops in Texas. *Phytopathology* **93**, 1368–1377.

Lee, I.-M., Martini, M., Marcone, C. & Zhu, S. F. (2004a). Classification of phytoplasma strains in the elm yellows group (16SrV) and proposition of '*Candidatus* Phytoplasma ulmi' for the phytoplasma associated with elm yellows. *Int J Syst Evol Microbiol* **54**, 337–347.

Lee, I.-M., Gundersen, D. E., Davis, R. E., Bottner, K. D., Marcone, C. & Seemüller, E. (2004b). '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. *Int J Syst Evol Microbiol* **54**, 1037–1048.

Lee, I.-M., Bottner, K. D., Munyaneza, J. E., Secor, G. A. & Gudmestad, N. C. (2004c). Clover proliferation group (16SrVI) subgroup A (16SrVI-A) phytoplasma is a probable causal agent of potato purple top disease in Washington and Oregon. *Plant Dis* **88**, 429.

Lee, I.-M., Zhao, Y. & Bottner, K. D. (2006a). SecY gene sequence analysis for finer differentiation of diverse strains in the aster yellows phytoplasma group. *Mol Cell Probes* **20**, 87–91.

Lee, I.-M., Bottner, K. D., Secor, G. & Rivera-Varas, V. (2006b). '*Candidatus* Phytoplasma americanum', a phytoplasma associated with a potato purple top wilt disease complex. *Int J Syst Evol Microbiol* **56**, 1593–1597.

Lim, P. O. & Sears, B. B. (1992). Evolutionary relationships of a plant-pathogenic mycoplasmalike organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. *J Bacteriol* **174**, 2606–2611.

Marcone, C., Ragozzino, A. & Seemüller, E. (1997a). Detection and identification of phytoplasmas in yellows-diseased weeds in Italy. *Plant Pathol* **46**, 530–537.

Marcone, C., Ragozzino, A. & Seemüller, E. (1997b). Identification and characterization of the phytoplasma associated with elm yellows in southern Italy and its relatedness to other phytoplasmas of the elm yellows group. *Eur J For Pathol* **27**, 45–54.

Marcone, C., Ragozzino, A. & Seemüller, E. (1997c). Detection and identification of phytoplasmas infecting vegetable, ornamental and forage crops in southern Italy. *J Plant Pathol* **79**, 211–217.

Martini, M., Botti, S., Marcone, C., Marzachì, C., Casati, P., Bianco, P. A., Benedetti, R. & Bertaccini, A. (2002). Genetic variability among Flavescence dorée phytoplasmas from different origins in Italy and France. *Mol Cell Probes* **16**, 197–208.

Miyata, S., Furuki, K., Sawayanagi, T., Oshima, K., Kuboyama, T., Tsuchizaki, T., Ugaki, M. & Namba, S. (2002a). Gene arrangement and sequence of *str* operon of phytoplasma resemble those of *Bacillus* more than those of *Mycoplasma*. *J Gen Plant Pathol* **68**, 62–67.

Miyata, S., Furuki, K., Oshima, K., Sawayanagi, T., Nishigawa, H., Kakizawa, S., Jung, H., Ugaki, M. & Namba, S. (2002b). Complete nucleotide sequence of the S10-*spc* operon of phytoplasma: gene organization and genetic code resemble those of *Bacillus subtilis*. *DNA Cell Biol* **21**, 527–534.

Montano, H. G., Davis, R. E., Dally, E. L., Hogenhout, S., Pimentel, P. & Brioso, P. S. T. (2001). '*Candidatus* Phytoplasma brasiliense', a new phytoplasma taxon associated with hibiscus witches'-broom disease. *Int J Syst Evol Microbiol* **51**, 1109–1118.

Nei, M. & Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.

Oshima, K., Kakizawa, S., Nishigawa, H., Jung, H., Wei, W., Suzuki, S., Arashida, R., Nakata, D., Miyata, S. & other authors (2004). Reductive evolution suggested from the complete genome sequence of a plant-pathogenic phytoplasma. *Nat Genet* **36**, 27–29.

Prince, J. P., Davis, R. E., Wolf, T. K., Lee, I.-M., Mogen, B. D., Dally, E. L., Bertaccini, A., Credi, R. & Barba, M. (1993). Molecular detection of diverse mycoplasmalike organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. *Phytopathology* **83**, 1130–1137.

Razin, S., Yogev, D. & Naot, Y. (1998). Molecular biology and pathology of mycoplasmas. *Microbiol Mol Biol Rev* **62**, 1094–1156.

Schneider, B., Seemüller, E., Smart, C. D. & Kirkpatrick, B. C. (1995). Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In *Molecular and Diagnostic Procedures in Mycoplasmology*, vol. 1, pp. 369–380. Edited by R. Razin & J. G. Tully. San Diego: Academic Press.

Schneider, B., Marcone, C., Kampmann, M., Ragozzino, A., Lederer, W., Cousin, M. T. & Seemüller, E. (1997). Characterization and classification of phytoplasmas from wild and cultivated plants by RFLP and sequence analysis of ribosomal DNA. *Eur J Plant Pathol* **103**, 675–686.

Schneider, B., Torres, E., Martín, M. P., Schröder, M., Behnke, H.-D. & Seemüller, E. (2005). '*Candidatus* Phytoplasma pini', a novel taxon from *Pinus silvestris* and *Pinus halepensis*. *Int J Syst Evol Microbiol* **55**, 303–307.

Seemüller, E., Schneider, B., Mäurer, R., Ahrens, U., Daire, X., Kison, H., Lorenz, K. H., Firrao, G., Avinent, L. & other authors (1994). Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. *Int J Syst Bacteriol* **44**, 440–446.

Seemüller, E., Marcone, C., Lauer, U., Ragozzino, A. & Göschl, M. (1998). Current status of molecular classification of the phytoplasmas. *J Plant Pathol* **80**, 3–26.

Swofford, D. L. (1998). PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4. Sunderland, MA: Sinauer Associates.

Valiunas, D., Staniulis, J. & Davis, R. E. (2006). '*Candidatus* Phytoplasma fragariae', a novel phytoplasma taxon discovered in yellows diseased strawberry, *Fragaria* × *ananassa*. *Int J Syst Evol Microbiol* **56**, 277–281.

Wolf, Y. I., Rogozin, I. B., Grishin, N. V., Tatusov, R. L. & Koonin, E. V. (2001). Genome trees constructed using five different approaches suggest new major bacterial clades. *BMC Evol Biol* **1**, 8.

Zhao, Y., Davis, R. E. & Lee, I.-M. (2005). Phylogenetic positions of '*Candidatus* Phytoplasma asteris' and *Spiroplasma kunkelii* as inferred from multiple sets of concatenated core housekeeping proteins. *Int J Syst Evol Microbiol* **55**, 2131–2141.