Phylogenetic analysis and delineation of phytoplasmas based on *secY* gene sequences

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The *secY* gene sequence is more variable than that of the 16S rRNA gene. Comparative phylogenetic analyses with 16S rRNA and *secY* gene sequences from 80 and 83 phytoplasma strains, respectively, were performed to assess the efficacy of these sequences for delineating phytoplasma strains within each 16Sr group. The phylogenetic interrelatedness among phytoplasma taxa inferred by *secY* gene-based phylogeny was nearly congruent with that inferred by 16S rRNA gene-based phylogeny. Phylogenetic analysis based on the *secY* gene permitted finer differentiation of phytoplasma strains, however. The *secY* gene-based phylogeny not only readily resolved 16Sr subgroups within a given 16Sr group, but also delineated distinct lineages irresolvable by 16S rRNA gene-based phylogeny. Such high resolving power makes the *secY* gene a more useful genetic marker than the 16S rRNA gene for finer differentiation of closely related phytoplasma strains based on RFLP analysis with selected restriction enzymes. Such strains were readily identified by collective *secY* RFLP patterns. The genetic interrelationships among these strains were determined by pattern similarity coefficients, which coincided with delineations by phylogenetic analysis. This study also revealed two heterogeneous *spc* operons present in the phytoplasma clade. This latter finding may have significant implications for phytoplasma evolution.

INTRODUCTION

Phytoplasmas, formerly termed mycoplasma-like organisms (MLOs), are minute, cell-wall-less prokaryotes that are associated with diseases in several hundred plant species (Lee *et al.*, 2000; Bertaccini, 2007; Hogenhout *et al.*, 2008). Extensive phylogenetic studies have placed phytoplasmas in the class *Mollicutes*. Because it has proved impossible to obtain pure cultures of any phytoplasma and the paucity of accessible phenotypic criteria, it is difficult to classify phytoplasmas according to the criteria applied to taxonomy of the *Mollicutes* (Weisburg *et al.*, 1989; Murray & Schleifer, 1994; Vandamme *et al.*, 1996; Razin *et al.*, 1998), and phytoplasma taxonomy is inevitably based heavily on molecular characteristics and phylogeny. The 16S rRNA gene sequence has been used as the primary parameter for differentiation and classification of phytoplasmas (Lee *et al.*, 1993b, 1998a; Schneider *et al.*, 1995; Seemüller *et al.*, 1994, 1998). So far, a total of 19 distinct groups based on actual RFLP analysis of PCR-amplified 16S rRNA gene sequences, termed 16S rRNA groups (16Sr groups), or 30 groups based on *in silico* RFLP analysis have been identified (Lee *et al.*, 1998a, 2000; Wei *et al.*, 2007). Eighty-nine 16Sr subgroups were further classified by *in*

silico analysis. The latter RFLP-based grouping is highly consistent with phylogenetic relationships based on analysis of 16S rRNA gene sequences. It was proposed that each 16S rRNA gene RFLP group represents at least one phytoplasma species (Gundersen *et al.*, 1994). At present, species designation is based primarily on dissimilarity of 16S rRNA gene sequences among phytoplasmas. An arbitrary threshold of 2.5% dissimilarity was applied as a guideline for erecting a novel species (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004). Because of the highly conserved nature of the 16S rRNA gene, this guideline may exclude many ecologically or biologically distinct phytoplasma strains, some of which may warrant designation as novel taxa.

Classification of distinct strains below the species level has been based primarily on RFLP analysis of 16S rRNA gene sequences. Over the last decade, epidemiological studies have revealed that diverse phytoplasma strains, which are very closely related based on analysis of 16S rRNA gene sequences, are involved in similar diseases associated with different cultivars of a given plant species grown in the same or different geographical regions (Martini *et al.*, 2002; Lee *et al.*, 2003, 2004c, 2006b). It is not uncommon that closely related phytoplasma strains have unique ecological niches encompassing both plant host range and insect vectors (Lee *et al.*, 1998b, 2000).

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

Table 1. Phytoplasma strains used in this study

'Ca. P.', 'Candidatus Phytoplasma'.

Strain	Associated disease(s)	Geographical origin	16S rRNA gene RFLP classification	GenBank accession number(s)	
				16S rRNA gene	secY
BB	Tomato big bud	Arkansas, USA	16SrI-A	AY180955	AY803178
NJAY	New Jersey aster yellows	New Jersey, USA	16SrI-A	–	GU004345*
GD1	Grey dogwood stunt	New York, USA	16SrI-A	DQ112021	AY803171
CHRYM	Chrysanthemum yellows	Germany	16SrI-A	AY265214	AY803170
HYDP	Hydrangea phyllody	Belgium	16SrI-A	AY265215	AY803181
PVM	Plantago virescence	Germany	16SrI-A	AY265216	AY803185
'Ca. P. asteris' AYWB	Aster yellow witches'-broom	Ohio, USA	16SrI-A	NC_007716	NC_007716
AY (AY1)	Maryland aster yellows	Maryland, USA	16SrI-B	AF322644	AY803177
CY	Chrysanthemum yellow	Italy	16SrI-B	EF634457	DQ787851
HyPH	Hydrangea phyllody	Italy	16SrI-B	AY265207	AY803173
PRIVC	Primrose virescence	Germany	16SrI-B	AY265210	AY803176
OAY	Oenothera virescence	Michigan, USA	16SrI-B	M30790	–
AV2192	Aster yellows	Germany	16SrI-B	AY180957	AY803167
MBS	Maize bushy stunt	Mexico	16SrI-B	AY265208	AY803175; GU004341*
CPh-a	Clover phyllody	Canada	16SrI-C	AF222065	AY803179; GU004339*
KVG	Clover phyllody	Germany	16SrI-C	AY265218	AY803183
KVE	Clover phyllody	France	16SrI-C	AY265217	AY803174
PaWB	Paulownia witches'-broom	Taiwan	16SrI-D	AY265206	AY803184; GU004337*
BBS3	Blueberry stunt	Michigan, USA	16SrI-E	AY265213	AY803169; GU004357*
ACLR-AY	Apricot chlorotic leaf roll	Spain	16SrI-F	AY265211	AY803166
CVB	Leafhopper borne	Germany	16SrI-F	AY265212	AY803172
STRAWB2	Strawberry multiplier	Florida, USA	16SrI-K	U96616	AY803180
AVUT	Aster yellows	Germany	16SrI-M	AY265209	AY803168
IOWB	<i>Ipomoea obscura</i> witches'-broom	Taiwan	16SrI-N	AY265205	AY803182
PnWB	Peanut witches'-broom	Taiwan	16SrII-A	L33765	GU004331*
SPWB	Sweet potato witches'-broom	Taiwan	16SrII-A	L33770	GU004321*
SEPN	Sesame phyllody	Thailand	16SrII-A	EF193357	GU004362*
SEPT	Sesame phyllody	Thailand	16SrII-A	GU004373†	GU004322*
CaWBYN16	Cactus witches'-broom YN16	China	16SrII-C	EU099561	GU004323*
SOYP	Soybean phyllody	Thailand	16SrII-C	EF193353	GU004324*
CrP	Crotalaria phyllody	Unknown	16SrII-C	EF193355	GU004349*
CoP	Cotton phyllody	Unknown	16SrII-C	EF186827	GU004350*
PEP	<i>Picris echioides</i> phyllody	Italy	16SrII-E	Y16393	GU004348*
TBB	Australian tomato big bud	Australia	16SrII-D	Y08173	GU004347*
CX	Peach X-disease	Canada	16SrIII-A	L33733	GU004327*
WX	X-disease	Canada	16SrIII-A	L04682	GU004354*
CYE	Clover yellow edge	Lithuania	16SrIII-B	AF173558	GU004332*
Vac	Vaccinium witches'-broom	Germany	16SrIII-F	X76430	GU004360*
PBT	Pecan bunchy top	Georgia	16SrIII-C	GU004371†	GU004361*
GR1	Goldenrod yellows	New York, USA	16SrIII-D	GU004372†	GU004364*
SP1	Spirea stunt	New York, USA	16SrIII-E	AF190228	GU004326*
MW1	Milkweed yellows	New York, USA	16SrIII-F	AF510724	GU004340*
AKpot7	Potato purple top-AK	Alaska, USA	16SrIII-F	GU004370†	GU004358*
WWB	Walnut witches'-broom	Georgia, USA	16SrIII-G	AF190227	GU004325*
JR1Ph (PoiBI)	Poinsettia branch inducing	USA	16SrIII-H	AF190223	GU004328*
MT117	Potato purple top-MT	Montana, USA	16SrIII-M	FJ226074	GU004333*
AKpot6	Potato purple top-AK	Alaska, USA	16SrIII-N	GU004365†	GU004359*
LYF (LYF-C5)	Coconut lethal yellows	Florida, USA	16SrIV-A	AF498308	GU004320*

Table 1. cont.

Strain	Associated disease(s)	Geographical origin	16S rRNA gene RFLP classification	GenBank accession number(s)	
				16S rRNA gene	<i>secY</i>
' <i>Ca. P. ulmi</i> ' EY1	Elm yellows	New York, USA	16SrV-A	AY197655	AY197690; GU004330*
' <i>Ca. P. ulmi</i> ' EYEu (EY-626)	Elm yellows	Italy	16SrV-A	AY197657	AY197691
CLY5	Cherry lethal yellows	China	16SrV-B	AY197659	AY197693
PY-In	Peach yellows	India	16SrV-B	AY197660	AY197694
' <i>Ca. P. ziziphi</i> ' JWB	Jujube witches'-broom	China	16SrV-B	AY197661	AY197695
ALY882	Alder yellows	Germany	16SrV-C	AY197642	AY197692
FD70	Flavescence dorée	France	16SrV-C	AY197643	AY197686
FD-C	Flavescence dorée	Italy	16SrV-C	AY197645	AY197688
ALY	Alder yellows	Italy	16SrV-C	AY197646	AY197684
SpaWB229	Spartium witches'-broom	Italy	16SrV-C	AY197652	AY197689
HD1	Hemp dogbane	New York, USA	16SrV-C	AY197654	AY197687
FD-D	Flavescence dorée	Italy	16SrV-D	AY197644	AY197685
RuS	Rubus stunt	Italy	16SrV-E	AY197648	AY197696
' <i>Ca. P. trifolli</i> ' CP	Clover phyllody	Canada	16SrVI-A	AY500130	GU004315*
' <i>Ca. P. trifolli</i> ' PWB	Potato witches'-broom	Canada	16SrVI-A	AY500818	GU004316*
VR	Vinca virescence	California, USA	16SrVI-A	AY500817	GU004317*
LUM	Lucerne virescence	France	16SrVI-A	EF186821	GU004318*
AkPot1	Potato purple top-AK	Alaska, USA	16SrVI-A	GU004366†	GU004351*
AKpot2	Potato purple top-AK	Alaska, USA	16SrVI-A	GU004367†	GU004342*
AKpot4	Potato purple top-AK	Alaska, USA	16SrVI-A	GU004368†	GU004343*
AKpot5	Potato purple top-AK	Alaska, USA	16SrVI-A	GU004369†	GU004344*
DBPh2	Dry bean phyllody	Washington, USA	16SrVI-A	AY496002	GU004352*
DBPh3	Dry bean phyllody	Washington, USA	16SrVI-A	AY496003	GU004353*
BLL	Brinjal little leaf	India	16SrVI-D	EF186820	GU004356*
AshY1	Ash yellows	New York, USA	16SrVII-A	AF092209	GU004329*
LfWB	Loofah witches'-broom	Taiwan	16SrVIII-A	L33764	GU004319*
' <i>Ca. P. mali</i> ' AT	Apple proliferation	Germany	16SrX-A	CU469464	CU469464
' <i>Ca. P. mali</i> ' AP15	Apple proliferation	Italy	16SrX-A	EF193366	GU004335*
' <i>Ca. P. prunorum</i> ' PLN	European stone fruit yellows/plum leptonecrosis	Germany/Italy	16SrX-B	AJ542545	GU004334*
' <i>Ca. P. pyri</i> ' PD1	Pear decline	Italy	16SrX-C	AJ542543	GU004363*
STOL	Pepper stolbur	Serbia	16SrXII-A	AF248959	–
Stolbur PO	Vinca stolbur	France	16SrXII-A	–	FM163376
Rus93	Stolbur-Rus	Russia	16SrXII-A	GU004375†	GU004346*
PTV	Stolbur-It	Italy	16SrXII-A	GU004374†	GU004355*
' <i>Ca. P. australiense</i> ' PYL	Phormium yellow leaf/Australian grapevine yellows	New Zealand/Australia	16SrXII-B	U43570	NC_010544
MPV	Mexican periwinkle virescence	Mexico	16SrXIII-A	AF248960	GU004336*
' <i>Ca. P. americanum</i> ' APPT10-NE	American potato purple top wilt	Nebraska, USA	16SrXVIII-B	DQ174121	GU004338*

*Submitted as partial *spc* operon sequence in this study.

†16S rRNA gene sequence submitted in this study.

Often, such strains cannot be readily differentiated by analysis of 16S rRNA gene sequences, hindering the development of targeted and efficient disease control measures and underscoring the need to seek additional markers that permit finer differentiation of closely related strains. In the last decade, several other conserved genes or specific genomic DNA fragments have been studied in

the search for molecular markers for finer strain differentiation (Lee *et al.*, 2006a; Marcone *et al.*, 2000; Martini *et al.*, 2007).

Earlier studies on differentiation of phytoplasma strains within the aster yellows group (16SrI) (Lee *et al.*, 2006a) and the elm yellows group (16SrV) (Lee *et al.*, 2004a)

indicated that, because of its high resolving power, the *secY* gene, encoding a protein translocase subunit, represents one of the most promising markers for finer differentiation of phytoplasma strains and for delineating biologically and/or ecologically distinct strains that often cannot be readily resolved by analysis of the 16S rRNA gene alone. Phylogenetic interrelationships based on *secY* gene analysis have not been explored for strains constituting the majority of phytoplasma groups. In the present study, comparative phylogenetic analyses were performed and phylogenetic trees constructed based on sequence analyses of 16S rRNA and *secY* genes from representative phytoplasma strains in order to evaluate the efficacy of *secY* gene sequences for differentiation of closely related phytoplasma strains.

METHODS

Phytoplasma strains and nucleic acid preparation. Phytoplasma strains used in this study are listed in Table 1; they are representative of 12 16Sr groups. Many of the phytoplasma strains were available in our inventory (as extracted total nucleic acid preparations). For those that were unavailable, total nucleic acid was extracted using leaf midribs or other tissues from periwinkle or original hosts that were infected by these phytoplasma strains according to the method described by Lee *et al.* (1993a) or Green *et al.* (1999). The strains were

previously characterized and identified by RFLP or sequence analysis of the 16S rRNA gene (Bertaccini, 2007; Jacobs *et al.*, 2003; Lee *et al.*, 1993b, 1998a, 2000; Marcone *et al.*, 1997a, b, c, 2000; Martini *et al.*, 2007; Schneider *et al.*, 1997; Seemüller *et al.*, 1998; Cai *et al.*, 2008).

Primer design. Degenerate primer pair L15F1/MapR1 (Table 2) was initially designed based on conserved regions of the ribosomal protein gene *rpl15* (or *rplO*) and methionine aminopeptidase gene *map* in the *spc* ribosomal protein operon, which contains the *rpl15* gene, the adenylate kinase gene (*adk*), a protein translocase gene (*secY*) and the *map* gene (Suh *et al.*, 1996). Conserved regions were identified by alignment of sequences of the *rpl15* and *map* genes from four phytoplasma and *Acholeplasma* strain genome sequences available in GenBank and the LY phytoplasma sequence (N. A. Harrison, unpublished) using CLUSTAL V from the LaserGene software MEGALIGN program (DNASTAR). Additional primers were designed within the L15F1/MapR1 amplicon to facilitate sequencing of the *spc* operon (Table 2). Subsequently, more specific primers were designed based on alignment of sequences from partial *spc* operons amplified from representative 16Sr group phytoplasmas using the primer pair L15F1/MapR1. Specific primer pairs were designed for amplification of DNA fragments (1700–1850 bp) from particular 16Sr phytoplasma groups (Table 2) for use in computer-simulated RFLP analysis (Wei *et al.*, 2008). Primer positions are indicated in Fig. 1.

PCR amplification, cloning and sequencing of the partial *spc* operon. The *secY* gene was amplified from some of the phytoplasma strains listed in Table 1 belonging to different phytoplasma groups

Table 2. Primers designed in this study for amplification of the partial *spc* operon and DNA fragments containing the complete *secY* gene and partial sequence of adjacent genes for virtual RFLP analysis

Primer(s)	Sequence(s) (5'–3')	16Sr group(s) amplified	Annealing temperature (°C)
Partial <i>spc</i> operon			
L15F1/MapR1	CCTGGTAGTGGYAMTGGWAAAAC/ATTARRAATATARGGYTCTTCRTG	I–VIII, X, XII, XIII, XVIII	50
L15F1A-a/MapR1A-a	TGGWAAAAC TKCBGGWAARGG/AAGMTKYACCRATDCCATG	I, XII, XIII	55
L15F1A-b/MapR1A-b	GGWAAAACYTSHGGYMRVGGHCATAAAGG/CCWATMCCRTGWCCDGWAAAA	II, III, V, VI	64
L15F1A(I)/MapR1A(I)	CTTCTGGTAAAGGACATAAAGG/GTTCTTCGTGCAAAGATGTACC	I	52
L15F1A(II)/MapR1A(II)	CTTGCGGTCGCGGCCATAAAGG/GTTCTTCGTGTAAGATTACC	II	60
L15F1A(III)/MapR1A(III)	CTTCTGGTAAAGGACATAAAGG/GTTCTTCGTGCAATTGCAAACC	III	55
L15F1A(VI)/MapR1A(VI)	CTTCAGGYAARGGTCATAAAGG/GTTTCTTCATCAAGTCTAGTACC	VI	50
L15F-646-a	TGGAARGAMCAAGGACAAAT	I, XII, XIII	53
L15F-696-b	DMAAGAATGGMRWRAACAAGG	II, III, V, VI	53
MapR-703-a	TYWGGTTTTRTCATCTKYTCCTTG	I, XII, XIII	53
MapR-696-b	AGMHACRCCDACMAYAATTA	II, III, V, VI	48
L15F-806-a	SCTTTAATBGCWRCTGCWGG	I, XII, XIII	57
<i>secY</i> gene			
SecYF1a/SecYR1 (XII)	GGACAATTAGCWC GTTCAGG/CAGGAAC TAACTTCCCTTGA	XII	55
SecYF1(II)/SecYR1(II)	CGCGTATAGGTTTGAAGGTG/CCTGCCATTTTCATTATAGCG	II	55
SecYF2(II)/SecYR1(II)	TGAAGGTGGTCAAACCTCT/CCTGCCATTTTCATTATAGCG	II	55
SecYF1(III)/SecYR1(III)	CTAGACCAGGTTTGAAGG/GACCTGCTTTTCTCATTATAGC	III	50
SecYF2(III)/SecYR1(III)	TGAAGGYGGACAAATCCCT/GACCTGCTTTTCTCATTATAGC	III	55
SecYF1(VI)/SecYR1(VI)	CTAGATTAGGATTYGAGGG/GACCRCCAAAACCTTGATAATC	VI	50
SecYF2(VI)/SecYR1(VI)	ATTYGAGGGYGGYCAAACAC/GACCRCCAAAACCTTGATAATC	VI	55
SecYF1(X)/SecYR1(X)	GGTGGTGTTAGACCAGGTTT/GGAATACCTGAACTACTAC	X	50
SecYF2a/SecYR1(XII)	CTCTTCGMCCYGGTTTGAAGG/CAGGAAC TAACTTCCCTTGA	XII	55

Fig. 1. Schematic gene arrangement and positions of primers designed in this study for the amplification of the partial *secY* operon (a) and of DNA fragments containing the complete *secY* gene and partial sequences of adjacent genes of selected 16Sr phytoplasma groups (b). ▲, Position to which each amplicon was trimmed for *in silico* RFLP analysis for phytoplasma groups 16SrII, 16SrIII and 16SrVI.

using the primer pair L15F1/MapR1. The amplicon size is 2.8 kb for groups 16SrI, 16SrXII, 16SrXIII and 16SrXVIII and 2.2 kb for groups 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrVI, 16SrVII and 16SrX. For PCR amplification, 35 cycles were conducted in an automated thermal cycler (MJ Research DNA thermal cycler PTC-200) with TaKaRa LA *Taq* polymerase (Takara Mirus Bio). PCR was performed in mixtures containing 1 µl DNA extract (approx. 10–30 ng), 400 µM each dNTP, 0.8 µM each primer and 2.5 U *Taq* polymerase. The following conditions were used: denaturation at 94 °C for 30 s (1 min for the first cycle), annealing for 1 min at 50 °C and primer extension for 5 min at 68 °C (10 min in the final cycle, at 72 °C). A negative control devoid of DNA template in the reaction mixture was included in all PCRs. Aliquots of the PCR products (3 µl) were electrophoresed through a 1% agarose gel, stained with ethidium bromide and visualized with a UV transilluminator. The amplicons were purified using PCR Kleen spin columns (Bio-Rad) or, for amplicons with multiple bands, the QIAquick gel extraction kit (Qiagen) according to the manufacturers' instructions. The majority of purified products were cloned into *Escherichia coli* TOP10 by using the TOPO TA

cloning kit (Invitrogen) according to the manufacturer's instructions and 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. For purified products with a low concentration, direct sequencing with L15F1/MapR1 primers was performed. Additional walking primers were needed to determine the complete nucleotide sequence. For 16Sr groups I, XII, XIII and XVIII, the walking primers used were L15F-646-a, L15F-806-a and MapR-703-a. For all other 16Sr groups, the walking primers used were L15F-696-b and MapR-696-b (Table 2). Other primers listed in Table 2 were used to amplify additional 16Sr phytoplasma groups for sequencing according to the procedure described above. All sequences were submitted to GenBank; accession numbers are listed in Table 1.

Phylogenetic analysis. *secY* nucleotide and deduced amino acid sequences and 16S rRNA gene sequences from 83 and 80 phytoplasma strains, respectively, representative of 12 distinct phytoplasma 16Sr groups (consisting of more than 40 16Sr subgroups) and from two

— 50 changes

— 5 changes

Bacillus subtilis 168

Acholeplasma strains and *Bacillus subtilis* 168 were aligned using CLUSTAL V from the LaserGene software MEGALIGN program (DNASTAR). Cladistic analyses were performed with PAUP version 4.0 (Swofford, 1998) on a Power Mac G4. Uninformative characters were excluded from analyses. Phylogenetic trees were constructed by a heuristic search (or neighbour-joining algorithm) by random stepwise addition, implementing the tree bisection and reconnection branch-swapping algorithm to find the optimal tree(s) (Gundersen *et al.*, 1994). *B. subtilis* 168 was used as the outgroup to root the trees. The analysis was replicated 1000 times. Bootstrapping was performed to estimate the stability and support for the inferred clades. Full-length *secY* and near full-length (1.43 kb) 16S rRNA gene sequences of phytoplasma strains used for phylogenetic analysis were either sequenced in this work or obtained from the GenBank database.

Strain differentiation by computer-simulated RFLP analysis of *secY* sequences. To demonstrate the efficacy of using the *secY* gene sequence for finer differentiation of phytoplasma strains, DNA fragments ranging from 1700 to 1850 bp containing the entire *secY* gene and partial sequences of adjacent genes (*rpl15* and *map*) were amplified using nested PCR and sequenced from phytoplasma strains in groups 16SrII, 16SrIII and 16SrVI using specific primers listed in Table 2. These sequences were trimmed to about 1.4, 1.34 and 1.37 kb for groups 16SrII, 16SrIII and 16SrVI, respectively, each of which contains the complete *secY* gene and partial *rpl15* and *map* genes or intergenic spacer regions, for *in silico* RFLP analysis (Fig. 1b). Collective RFLP pattern types of full-length phytoplasma *secY* gene sequences were based on analysis by 18 restriction enzymes. *In silico* restriction digestion and pairwise virtual RFLP pattern comparison were performed using a modified Perl program developed previously (Wei *et al.*, 2008), with the inclusion of an additional restriction enzyme, *Tsp509I*. Virtual gel images were generated using the program VGelME. Key enzymes that distinguish different (group/subgroup) pattern types were identified by using the program VGelMS. The programs VGelME and VGelMS were described previously (Zhao *et al.*, 2009).

RESULTS AND DISCUSSION

Amplification of the partial phytoplasma *spc* operon

A partial *spc* ribosomal protein operon from each of 50 phytoplasma strains representing 12 16Sr groups was amplified by PCR and sequenced. Annotated sequences revealed the presence of two distinct *spc* operons among the phytoplasma strains analysed. Strains belonging to groups 16SrI, 16SrXII, 16SrXIII and 16SrXVIII had an *spc* operon with the gene order *rpl15–secY–adk–map*, while strains belonging to groups 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrVI, 16SrVII, 16SrVIII and 16SrX had an operon with the gene order *rpl15–secY–map*, lacking the gene *adk*. The distinctness of the two *spc* operon types separated phytoplasmas into two major subclades, which seem to coincide with the divergence of the two major phylogenetic

groups from an *Acholeplasma*-like ancestor early in the course of evolution, as shown on phylogenetic trees constructed previously and in this study based on 16S rRNA, ribosomal protein and *secY* gene sequences. This finding of two heterogeneous *spc* operons in ‘*Candidatus* Phytoplasma’ may have implications for phytoplasma evolution. Members of the genus *Acholeplasma*, the closest known relatives of phytoplasmas, and strains in the *Clostridium/Lactobacillus* group, believed to contain the common ancestor of the class *Mollicutes*, have an *spc* operon with a gene order identical to that found in phytoplasma strains belonging to groups 16SrI, 16SrXII, 16SrXIII and 16SrXVIII. This implies that phytoplasma strains in groups lacking the *adk* gene may have diverged from the phytoplasma common ancestor, which is most closely related to *Acholeplasma* species, during the course of phytoplasma evolution. Phylogenies based on *secY*, 16S rRNA and ribosomal protein gene sequences also support this suggestion.

Phylogenetic interrelationships and genetic variations of phytoplasmas based on nucleotide sequences of the *secY* gene

The phylogenetic analyses resulted in 77 equally parsimonious trees based on *secY* nucleotide sequences and 82 trees based on deduced SecY amino acid sequences. The trees inferred from the *secY* gene had a topology similar to that based on deduced SecY amino acid sequences (not shown). One representative parsimonious tree inferred from the *secY* gene was selected (Fig. 2). There were two subclades within the phytoplasma clade: one comprised strains in groups 16SrI, 16SrXII, 16SrXIII and 16SrXVIII and the other comprised strains in the remainder of the groups analysed in this study. The latter subclade comprised two major branches: one included strains in group 16SrX and the other contained strains in groups 16SrII, 16SrIII, 16SrIV, 16SrV, 16SrVI, 16SrVII, 16SrVIII and 16SrIX. The interrelatedness among phytoplasma taxa inferred from *secY* gene-based phylogeny was nearly congruent with that inferred from 16S rRNA gene-based phylogenies shown in this study and reported previously (Lee *et al.*, 1998a, 2000; Seemüller *et al.*, 1998; Jung *et al.*, 2002; Martini *et al.*, 2007). Clearly, phylogenetic analysis based on *secY* gene sequences permitted finer differentiation of phytoplasma strains. The *secY* gene-based phylogeny readily resolved subgroups within a given 16Sr group, and also delineated distinct lineages irresolvable by 16S rRNA gene-based phylogeny. For example, members in each of groups 16SrV, 16SrII and 16SrIII clearly represented two distinct genetically divergent lineages, as supported by high bootstrap

Fig. 2. Phylogenetic trees constructed by parsimony analyses of partial 16S rRNA (a) and full *secY* (b) gene sequences from phytoplasma strains representative of 12 distinct phytoplasma 16Sr groups (consisting of more than 40 16Sr subgroups) and from two *Acholeplasma* strains and *Bacillus subtilis*. *B. subtilis* 168 was used as the outgroup to root the trees. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values are shown on the main branches. Bars, 5 (a) and 50 (b) inferred character state changes. 16Sr groups are indicated; subgroup affiliations are indicated in parentheses in (a). Phytoplasma strain abbreviations are defined in Table 1.

values, which were not well resolved by 16S rRNA gene-based phylogenetic analysis.

Genetic variations of phytoplasmas assessed by comparative sequence analyses of *secY* and 16S rRNA genes

The *secY* gene nucleic acid sequence similarity between members of different phytoplasma groups ranged from 53.5 to 77.9 %, compared with 85.1–96.9 % for 16S rRNA gene sequences. Examples are members of the PnWB group (16SrII) analysed in this study, where the sequence similarity ranged from 87.7 to 100 % for the *secY* gene and 97.4 to 99.6 % for the 16S rRNA gene. Among members of the X-disease (16SrIII) group, sequence

similarities ranged from 93.6 to 99.8 % for the *secY* gene and 99.0 to 99.8 % for the 16S rRNA gene, whereas, among members of the clover proliferation (16SrVI) group, the sequence similarities ranged from 94.4 to 99.8 % for the *secY* gene and 98.4 to 99.2 % for the 16S rRNA gene. The greater sequence variability indicates that the *secY* gene is a more informative molecular tool for classification of closely related phytoplasma strains.

Strain differentiation by virtual RFLP analysis of *secY* sequences

The relatively variable *secY* gene sequences should be highly useful in RFLP analysis for finer differentiation of ecologically or biologically distinct strains within a given

Table 3. Summary of profiles produced by virtual RFLP analyses of the *secY* gene from phytoplasma 16Sr groups II, III and VI

Strain	Subgroup	RFLP pattern type											
		<i>AluI</i>	<i>BfaI</i>	<i>DraI</i>	<i>HaeIII</i>	<i>HinfI</i>	<i>HpaII</i>	<i>MseI</i>	<i>RsaI</i>	<i>Sau3AI</i>	<i>SspI</i>	<i>TaqI</i>	<i>Tsp509I</i>
Group 16SrII													
PnWB	A	1	1	1	1	1	1	1	1	1	1	1	1
SPWB	A	1	2	2	1	1	1	1	1	1	1	1	1
SEPT	A	1	1	2	1	1	1	1	1	1	1	1	1
SEPN	A	1	1	2	1	1	1	1	1	1	1	1	1
CacWBYN16	C	2	3	3	2	2	2	2	2	2	2	1	2
SOYP	C	2	3	3	2	2	2	2	2	3	2	1	2
CrP	C	2	3	3	2	2	3	2	2	2	2	1	2
CoP	C	3	3	4	1	2	2	2	2	2	2	1	2
TBB	D	1	1	1	1	1	1	1	1	1	1	1	1
PEP	E	4	4	5	3	3	2	3	3	4	3	2	3
Group 16SrIII													
CX	A	1	1	1	1	1	1	1	1	1	1	1	1
WX	A	1	1	1	1	1	1	2	1	1	1	1	1
CYE	B	2	2	2	1	2	2	3	2	1	2	1	2
Vac	F	3	2	1	2	3	2	4	1	1	2	1	3
PBT	C	4	2	1	1	4	3	5	1	1	2	1	4
GR1	D	3	2	1	2	3	2	4	1	1	2	1	3
SP1	E	5	3	1	1	1	1	6	3	1	1	1	5
MW1	F	6	2	1	1	3	2	4	1	1	2	1	3
AKpot7	F	3	2	1	2	3	2	4	1	1	2	1	6
WWB	G	1	1	1	1	1	1	7	1	1	1	1	7
JR1Ph	H	2	1	1	1	1	1	1	1	1	1	1	1
MT117	M	3	2	1	2	3	2	4	1	1	2	1	3
AKpot6	N	3	2	1	2	3	2	4	1	1	2	1	7
Group 16SrVI													
CP	A	1	1	1	1	1	1	1	1	1	1	1	1
PWB	A	1	1	1	1	1	1	1	1	1	1	2	1
AKpot1	A	1	1	1	1	1	1	1	1	1	1	1	1
AKpot4	A	1	1	1	1	1	1	2	2	1	2	1	1
AKpot5	A	1	1	1	1	1	1	2	2	1	2	1	1
AKpot2	A	1	1	1	1	1	1	2	2	1	2	1	1
LUM	A	2	1	1	1	1	1	3	1	1	1	3	2
DBPh2	A	3	1	1	1	2	1	4	1	1	3	1	3
DBPh3	A	3	1	1	1	2	1	4	1	1	3	1	3
VR	A	3	1	1	1	2	1	4	1	1	3	4	3
BLL	D	2	2	1	1	1	1	2	1	1	1	1	4

16Sr phytoplasma group. To evaluate the efficacy of the *secY* gene for finer differentiation of phytoplasma strains, computer-simulated virtual RFLP patterns were generated using *iPhyClassifier* (Zhao *et al.*, 2009). Table 3 summarizes the collective pattern types of strains analysed in groups 16SrII, 16SrIII and 16SrVI using 12 key restriction enzymes among the 18 used for analysis. Biologically distinct strains within a given 16Sr group and, in most cases, 16Sr subgroup were readily differentiated based on collective patterns. For example, ten members of subgroup 16SrVI-A were differentiated into seven *secY*(VI) genotypes

represented by the following individual strains or strain clusters: CP and AKpot1; AKpot2, AKpot4 and AKpot5; DBPh2 and DBPh3; PWB; LUM; BLL; and VR. Four members of subgroup 16SrII-A (SEPT, SEPN, PnWB and SPWB) were differentiated into three *secY*(II) genotypes, with two strains, SEPT and SEPN, having the same genotype. Four members of subgroup 16SrII-C (CaWBYN16, SOYP, CrP and CoP) were differentiated into four *secY*(II) genotypes. However, in one case, two different 16Sr subgroups were found to have similar *secY* genotypes; AKpot6 and AKpot7, which are classified as

Fig. 3. Computer-simulated virtual RFLP patterns derived from *in silico* digestions of phytoplasma *secY* gene fragments (about 1.4, 1.34 and 1.37 kb, respectively, for groups 16SrII, 16SrIII and 16SrVI) from representative strains of groups 16SrII, 16SrIII and 16SrVI with six key enzymes: *AluI*, *BfaI*, *MseI*, *Sau3AI*, *TaqI* and *Tsp509I*. Lanes MW, *HaeIII* digest of ϕ X174 RFI DNA; fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. Phytoplasma strain abbreviations are defined in Table 1. Subgroup affiliations: PnWB, SPWB, SEPT and SEPN, 16SrII-A; CaWBYN16, SOYP, CrP and CoP, 16SrII-C; TBB, 16SrII-D; PEP, 16SrII-E; CX and WX, 16SrIII-A; CYE, 16SrIII-B; PBT, 16SrIII-C; GR1, 16SrIII-D; SP1, 16SrIII-E; Vac, MW1 and AKpot7, 16SrIII-F; WWB, 16SrIII-G; JRPh1, 16SrIII-H; MT117, 16SrIII-M; AKpot6, 16SrIII-N; CP, PWB, AKpot1, AKpot4, AKpot5, AKpot2, LUM, DBPh2, DBPh3 and VR, 16SrVI-A; BLL, 16SrVI-D.

16SrIII-N and 16SrIII-F, respectively, have a similar *secY*(III) genotype. Fig. 3 shows computer-simulated virtual gel patterns with six key enzymes (*AluI*, *HpaII*, *MseI*, *RsaI*, *TaqI* and *Tsp509I*). Greater sequence variability in the *secY* gene provides additional genetic markers that increase the resolving power substantially for differentiation of subgroups within a given 16Sr group. Using *secY*, it is feasible to separate closely related, but biologically or geographically distinct, strain variants within a given subgroup. The genetic variability among subgroups in a given 16Sr group was reinforced in general while, in some cases, two 16Sr subgroups revealed little or no variability in the *secY* gene. Discrepancies in determining the genetic variability of closely related strains based on the 16S rRNA gene can only be resolved by incorporating multiple genes with varying degrees of genetic variability in analyses. Many 16Sr subgroups appear to have significant biological implications. In many subgroups, the constituent strains are often associated with specific plant hosts and have specific vector interrelationships in nature, while, in some subgroups, the constituent members are genetically diverse and are often associated with multiple plant hosts and insect vectors (Lee *et al.*, 2004b). Identification of biologically distinct strains is essential and highly relevant for epidemiological studies.

Conclusion

A classification system based on the highly conserved 16S rRNA gene sequence is insufficient for fine differentiation of closely related phytoplasma strains, underscoring the urgent need to utilize additional molecular markers that exhibit moderate genetic variability. The *secY* gene, located in the operator-distal part of the *spc* ribosomal protein operon, encodes protein translocase subunit SecY. A single copy of the *secY* gene is present in all known phytoplasma genomes. The *secY* gene is one of the most variable among the phylogenetic markers used so far for differentiation of phytoplasma strains and was found to be more efficient than other gene markers for differentiation and classification of phytoplasmas and especially for resolving closely related strains within the same 16S rRNA gene RFLP group (Hodgetts *et al.*, 2008; Lee *et al.*, 2006a; Martini *et al.*, 2007).

In the present study, *secY* gene-based phylogeny revealed new insights into the phylogenetic relationships among phytoplasma strains. Being more variable than the 16S rRNA gene, *secY* provided more phylogenetically informative markers useful for differentiation of genetically closely related but ecologically distinct strains, not readily separated by analysis of the 16S rRNA gene. The 16S rRNA gene has been the primary phylogenetic marker used for delineation of major phytoplasma groups and candidate species within 'Candidatus Phytoplasma'. However, finer differentiation of strains into subgroups cannot be achieved on the basis of the rather limited variable regions present in the 16S rRNA gene. Including the 16S rRNA

gene in combination with an additional marker that displays moderate genetic variability in phylogenetic analysis overcomes the limitations of the 16S rRNA gene for classification of phytoplasma strains below the species level. The present study and our previous studies (Lee *et al.*, 2006a; Martini *et al.*, 2002, 2007) indicate that phylogenetic analyses based on moderately variable genes, such as *secY* or ribosomal protein genes, increased the resolving power substantially for delineation of 16S rRNA groups and subgroups and for closely related but biologically distinct strains. For example, strain MBS, strains FD-C, ALY and SpaWB and strains DBPh2, DBPh3 and VR can be differentiated readily from other members of groups 16SrIB, 16SrV and 16SrVI, respectively. These strains inhabit mutually distinct ecological niches (Table 1). Emerging multiple-gene-based classification systems should provide molecular criteria for improved delineation of species and strains.

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