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Genotyping Points to Divergent Evolution of '*Candidatus* Phytoplasma asteris' Strains Causing North American Grapevine Yellows and Strains Causing Aster Yellows

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Abstract

Grapevine yellows diseases occur in cultivated grapevine (*Vitis vinifera* L.) on several continents, where the diseases are known by different names depending upon the identities of the causal phytoplasmas. In this study, phytoplasma strains associated with grapevine yellows disease (North American grapevine yellows [NAGY]) in vineyards of Pennsylvania were characterized as belonging to 16S ribosomal RNA (rRNA) gene restriction fragment length polymorphism group 16SrI (aster yellows phytoplasma group), subgroup 16SrI-B (I-B), and variant subgroup I-B*. The strains (NAGYI strains) were subjected to genotyping based on analyses of 16S rRNA and *secY* genes, and to in silico three-dimensional modeling of the SecY protein. Although the NAGYI strains are closely related to aster yellows (AY) phytoplasma strains and are classified like AY strains in subgroup I-B or in variant subgroup I-B*, the results from genotyping and protein modeling may signal ongoing evolutionary divergence of NAGYI strains from related strains in subgroup 16SrI-B.

Grapevine yellows is a term that is applied to diseases of cultivated grapevine (*Vitis vinifera* L.) that are caused by phytoplasmas: plant-pathogenic bacteria that lack a cell wall, reside in phloem tissue of infected plants, and are transmitted from plant to plant by phloem-feeding insects. Phytoplasmas are characterized by genomes that have undergone significant reduction during evolutionary adaptation to an obligately parasitic life style, and by the presence of chromosomally integrated prophage-based pathogenicity islands termed sequence-variable mosaics (Davis et al. 2007; Jomantiene and Davis 2006; Wei et al. 2008a; Zhao et al. 2014). In spite of decades of repeated attempts to isolate phytoplasmas in axenic culture, no independently confirmed report of culture has appeared in the published literature (Davis et al. 2015; Zhao et al. 2014). Thus, phenotypic properties remain elusive, hampering advances in the classification and taxonomy of phytoplasmas.

Currently, phytoplasmas are classified in a series of groups and subordinate subgroups on the basis of collective restriction fragment length polymorphism (RFLP) patterns of a specific segment (F2n/R2 segment) of the 16S ribosomal RNA (rRNA) gene. Each 16Sr group is believed to represent at least one phytoplasma species but, because phytoplasmas cannot be isolated in pure culture and, therefore, are not eligible for formal description as species within class *Mollicutes* (Marcone 2014; Zhao et al. 2014), the convention of '*Candidatus* Phytoplasma' taxon has been adopted (IRPCM 2004). Each described '*Ca.* Phytoplasma' taxon presumably represents a distinct biological species; however, it is widely recognized that a taxon such as the group 16SrI '*Ca.* Phytoplasma asteris' probably embraces multiple biologically distinct species (Wei et al. 2007; Zhao et al. 2015). Although subgroups may distinguish distinct species in some cases, it is generally agreed that the 16S rRNA gene is insufficiently informative to distinguish biologically distinct species-level lineages that are closely related to one another. Therefore, genes that are more variable in nucleotide sequence, including the *secY* gene, have been employed for finer differentiation of related phytoplasma strains (Davis et al. 2015; Jomantiene et al. 1998; Lee et al. 2010; Li et al. 2014; Martini et al. 2007; Šeruga Musić et al. 2014). This approach has bearing on the study of phytoplasma lineages causing grapevine yellows in North America.

Grapevine yellows diseases occur on several continents, where they are known by different names, depending upon the identities of the associated, or causal, phytoplasmas. For example, the grapevine yellows disease known as bois noir occurs in Europe and is caused by '*Ca.* Phytoplasma solani' (Quaglino et al. 2013); flavescence dorée (FD), also found in Europe, is attributed to infection by a phytoplasma for which the name '*Ca*. Phytoplasma vitis' has been informally proposed (IRPCM 2004); and Australian grapevine yellows is caused by '*Ca*. Phytoplasma australiense' (Davis et al. 1997). Grapevine yellows has also been associated with infection of *V. vinifera* by phytoplasma strains classified in groups 16SrI ('*Ca*. P. asteris'-related strains) and 16SrIII ('*Ca*. Phytoplasma pruni'-related strains) in North America (Davis et al. 1998, 2015; Olivier et al. 2009, 2014; Prince et al. 1993). Characteristic symptoms of the grapevine yellows diseases include shriveling of grape clusters, downward rolling of the margins of leaves, yellowing and necrosis of leaf veins, yellowing of leaf lamina in white grape varieties, leaf reddening in red grape varieties, uneven lignification of canes, dieback of canes, and plant death. Although they are caused by varied phytoplasma taxa, the different grapevine yellows diseases cannot be distinguished from one another on the basis of disease symptoms. Therefore, identification of the associated phytoplasma is a necessary prelude to correct diagnosis of a grapevine yellows disease.

In 1977, plants of cultivated *V. vinifera* exhibiting symptoms typical of FD were observed in New York State; subsequent reports indicated that symptomatic grapevine plants were infected by a phytoplasma serologically related to FD phytoplasma (Chen et al. 1993; Maixner et al. 1993; Pearson et al. 1985; Uyemoto et al. 1977). Grapevine yellows was observed and reported in Virginia in the early 1990s commensurate with the expansion of *V. vinifera* plantings in that state; phytoplasma strains related to aster yellows (AY) phytoplasma (group 16SrI, '*Ca*. P. asteris') and strains related to peach X-disease phytoplasma (group 16SrIII, '*Ca*. P. pruni') were associated with the disease in different individual plants, often in the same vineyard (Davis et al. 1998; Prince et al. 1993; Wolf et al. 1994). The

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North American grapevine yellows accession numbers KF990037, KF990038, KX236145 to KX236154, and KX364387 to KX364395.

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FD phytoplasma was not found. A resurgence of grapevine yellows in the United States prompted a more recent study of vineyards in Virginia, Maryland, Ohio, Missouri, New York State, and Pennsylvania, where '*Ca*. P. pruni'-related strains associated with the disease were found to represent two different phytoplasmal sequevars, both of which were genotypically distinct from strains previously shown to be associated with X-disease of peach (Davis et al. 2013, 2015).

Following our recent study of group 16SrIII phytoplasma strains associated with North American grapevine yellows (NAGY) disease (NAGYIII strains) (Davis et al. 2015), we resumed genetic characterization of NAGY group 16SrI phytoplasma strains (NAGYI strains). Previous work had shown that NAGYI strains are related to strains of '*Ca*. P. asteris' that cause AY disease (Davis et al. 1998; Olivier et al. 2009, 2014). However, we reasoned that NAGYI strains may differ genotypically from at least some strains causing AY disease in herbaceous plants, because NAGYI strains have been reported in *V. vinifera* in areas where AY disease is not known to be prevalent (Davis et al. 1998), and AY disease occurs in regions where grapevine yellows has not been reported (Gundersen et al. 1996; Kuske and Kirkpatrick 1990; Lee et al. 2004a; Severin 1934). Our findings prompt the hypothesis that the evolutionary trajectory of NAGYI strains is diverging from strains causing AY disease in herbaceous plant hosts.

Table 1. 16S ribosomal RNA (rRNA) and *secY* gene sequences determined in this work from phytoplasma strains infecting plants of *Vitis vinifera* L. growing in vineyards of Pennsylvania

Vineyard, *V. vinifera* cultivar[a]	Strain	Subgroup	GenBank accession number	
			rRNA[b]	*secY*
Fr				
Pinot gris	BG1	16SrI-B*	KX364387	…
Chardonnay	BG2	16SrI-B*	KX364388	…
Merlot	BG3	16SrI-B*	KX364389	…
Syrah	BG4	16SrI-B*	KX236150†	KX236151
	…	…	KX236149‡	…
Cabernet Sauvignon	BG5	16SrI-B*	KX364390	…
Pinot noir	BG6	16SrI-B*	KX364391	…
Cabernet franc	BG9	16SrI-B*	KX364392	…
Ha				
Merlot	BG10	16SrI-B*	KF990037†	KX236152
	…	…	KF990038‡	…
Pinot gris	BG12	16SrI-B*	KX364393	…
Pinot noir	BG13	16SrI-B*	KX364394	…
Cabernet franc	BG15	16SrI-B*	KX364395	…
Sq				
Chardonnay	TW224	16SrI-B	KX236148†	KX236153
	…	…	KX236147‡	…
Chardonnay	TW308	16SrI-B	KX236146†	KX236154
	…	…	KX236145‡	…

[a] Samples were collected in 2011 from vineyards Fr and Ha and in 2003 from vineyard Sq.

[b] Symbols † and ‡ indicate rRNA operons *rrnA* and *rrnB*, respectively. Polymerase chain reaction amplicons from strains BG4, BG10, TW224, and TW308 were cloned to separate and sequence the interoperon heterogeneous rRNA gene segments. The remaining rRNA gene sequences show positions of double peaks that signal interoperon single-nucleotide polymorphisms in the rRNA genes.

Materials and Methods

Plant samples and DNA amplification. Symptomatic leaves were collected from 13 grapevine-yellows-diseased plants of *V. vinifera* in vineyards of Pennsylvania (Table 1). Petioles and veins were excised from the leaves and stored frozen at −20°C; DNA for use in polymerase chain reactions (PCR) was extracted from the excised leaf veins and petioles, as previously described (Davis et al. 2015). Amplification of rRNA gene sequences was carried out in direct high-fidelity PCR (hf-PCR) primed by primer pair P1/16S-SR, or in seminested PCR primed by P1/16S-SR followed by P1A/16S-SR, catalyzed by AccuPrime *Taq* DNA Polymerase High Fidelity (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA), as previously described (Davis et al. 2015). Amplification of genomic regions containing a partial (3′ end) *rpl15 rp* gene, a complete *secY* gene, and a partial (5′ end) methionine aminopeptidase (*map*) gene was carried out in hf-PCR primed by L15F1/MapR1 and catalyzed by LA Taq DNA polymerase (Takara Bio USA, Madison, WI) followed by nested PCR with primer pair AYsecYF1/AYsecYR1 under conditions previously described (Lee et al. 2006, 2010). Nucleotide sequences of the oligonucleotides used as primers in PCR are given in Table 2. All PCR assays included a negative control reaction mix devoid of DNA template. PCR products were analyzed and purified from gels, and both DNA strands were sequenced, as previously described (Davis et al. 2015). 16S ribosomal DNA (rDNA) amplicons derived from sequence heterogeneous phytoplasmal rRNA operons were separated by cloning PCR products, using the TOPO TA Cloning Kit (Life Technologies), and the cloned segments were separately sequenced. The assembled nucleotide sequences were deposited in the GenBank database (Table 1). Nucleotide sequences from other phytoplasmas used in analyses were obtained from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) (Table 3).

Phytoplasma classification. Classification of phytoplasmas into 16S rRNA gene (rDNA) RFLP groups and subgroups, and taxonomic placement of strains into '*Ca.* Phytoplasma' species, were accomplished by subjecting 16S rRNA gene sequences to nucleotide sequence comparisons and virtual restriction RFLP analyses using *i*PhyClassifier (Zhao et al. 2009) at website https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi. Nucleotide and amino acid sequence alignments, determinations of nucleotide sequence identities, and single nucleotide polymorphism identifications were done using the Lasergene software MegAlign program (DNASTAR, Madison, WI). In this article, we have adopted the convention proposed previously for distinguishing NAGY phytoplasma strains according to their 16S rDNA RFLP group affiliation (Davis et al. 2015), whereby NAGY strains classified in group 16SrI (AY phytoplasma group) are referred to as NAGYI strains.

Three-dimensional protein structure predictions. For protein structure predictions, the amino acid sequences of proteins were deduced from the respective gene nucleotide sequences using the Lasergene EditSeq program (DNASTAR), and three-dimensional (3D) structures were modeled using SWISS-MODEL (https://swissmodel.expasy.org/), with SecY of *Bacillus subtilis* as template (Swiss Model 3dl8H). Predicted 3D structures were visualized using Swiss

Table 2. Nucleotide sequences of polymerase chain reaction primers used in this study

Target	Primer	Nucleotide sequence	Reference
rRNA operon	P1	5′-AAGAGTTTGATCCTGGCTCAGGATT-3′	Deng and Hiruki 1991
	P1A	5′-AACGCTGGCGGCGCGCCTAATAC-3′	Lee et al. 2004b
	16S-SR	5′-GGTCTGTCAAAACTGAAGATG-3′	”
secY region[a]	L15F1	5′-CCTGGTAGTGGYAMTGGWAAAAC-3′	Lee et al. 2010
	MapR1	5′-ATTARRAATATARGGYTCTTCRTG-3′	”
	AYsecYF1	5′-CAGCCATTTTAGCAGTTGGTGG-3′	Lee et al. 2006
	AYsecYR1	5′-CAGAAGCTTGAGTGCCTTTACC-3′	”

[a] This DNA region included the 3′ end of the L15 ribosomal protein gene (*rpl15*), the complete *secY* gene, and the 5′ end of the methionine aminopeptidase gene (*map*).

PDB-Viewer (Guex and Peitsch 1997), and orientations of the proteins embedded in the phytoplasma cell membrane were interpreted based on SecY structure reference *Escherichia coli*.

Phylogenetic analyses. The minimum evolution algorithm was implemented in MEGA6 through close-neighbor-interchange search using the neighbor-joining method (Tamura et al. 2013) for phylogenetic analyses of phytoplasma strains. A bootstrap test with 1,000 replicates was used to evaluate reliability of the analysis.

Results

Symptoms characteristic of NAGY disease were observed in plants of diverse cultivars growing in three vineyards of Pennsylvania (Table 1). The symptoms consisted of shriveling of grape

Table 3. Subgroup 16SrI-B phytoplasma strains and GenBank accession numbers of nucleotide and protein amino acid sequences used in this work[a]

Strain	Associated disease	Source host	Geographic region	GenBank accession number	
				rRNA[b]	SecY
AY1	Maryland aster yellows	*Catharanthus roseus* (Madagascar periwinkle)	Maryland, United States	AF322644†	AAV73936
				AF322645‡	…
HyPH	Hydrangea phyllody	*Hydrangea macrophylla* (French hydrangea)	Italy	AY265207	AAV73932
PRIVC	Primrose virescence	*Primula* sp. (primrose)	Germany	AY265210	AAV73935
MBS	Maize bushy stunt	*Zea mays* (corn, maize)	Mexico	AF487779	ADO33828
AV2192	Aster yellows	*Callistephus chinensis* (China aster)	Germany	AY180957	AAV73926

[a] GenBank at the National Center for Biotechnology.
[b] rRNA = ribosomal RNA and symbols † and. ‡ indicate *rrnA* and *rrnB*, respectively.

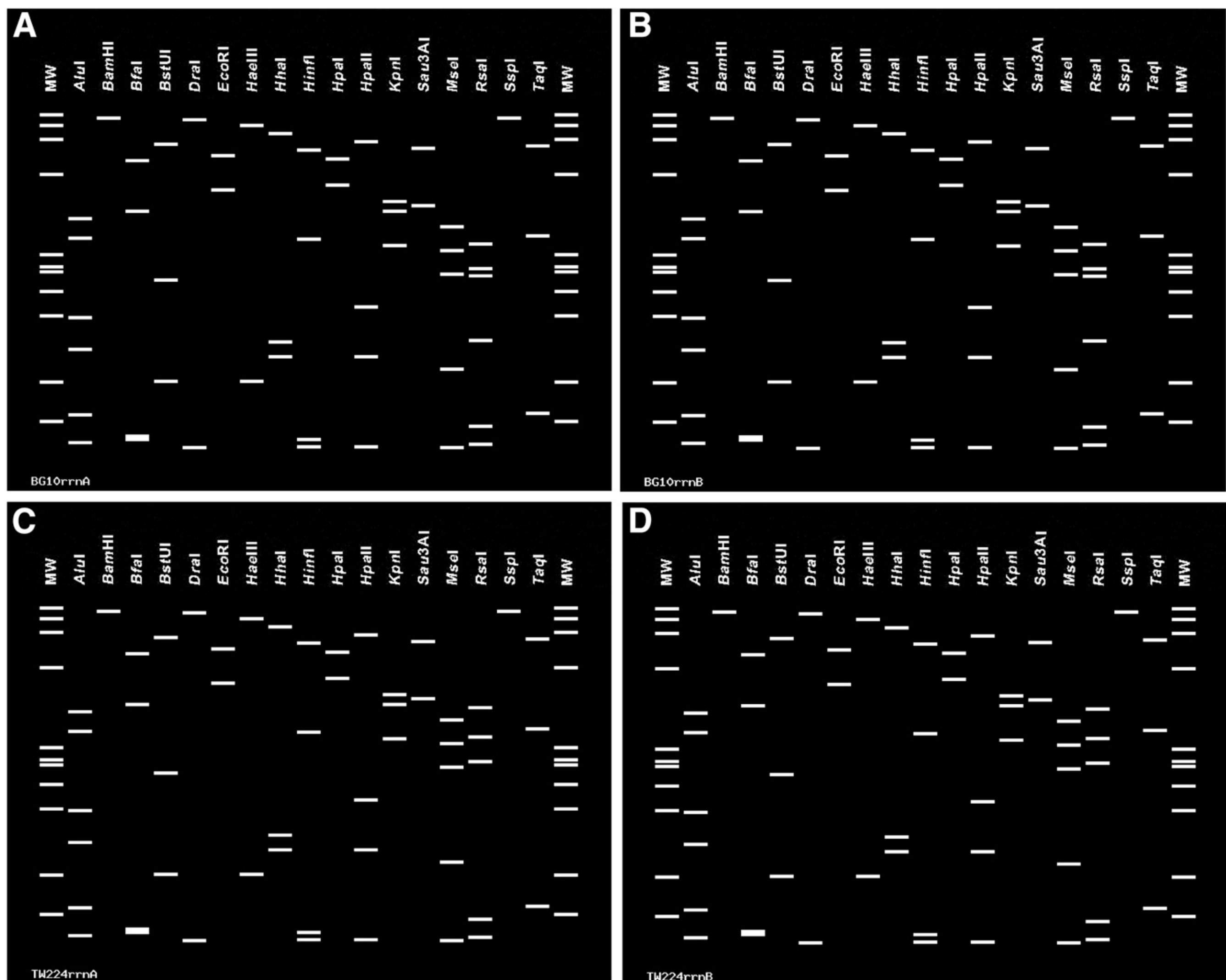


Fig. 1. Virtual restriction fragment length polymorphism (RFLP) patterns derived from computer-simulated restriction endonuclease digestions of 1.2-kbp segments of 16S ribosomal RNA (rRNA) gene (16S ribosomal DNA [rDNA]) sequences from group 16SrI phytoplasma strains associated with North American grapevine yellows (NAGY) in Pennsylvania. **A,** *rrnA* (GenBank number KF990037) and **B,** *rrnB* (GenBank number KF990038), RFLP patterns of 16S rDNA from strain BG10, a variant (16SrI-B*) of subgroup 16SrI-B. **C,** *rrnA* (GenBank number KX236148) and **D,** *rrnB* (GenBank number KX236147), RFLP patterns of 16S rDNA from strain TW224, a member of subgroup 16SrI-B. In silico digestion based on the recognition sites of 17 restriction endonuclease enzymes (left to right): *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. The in silico RFLP patterns were derived using the online *i*PhyClassifier interactive tool. MW = ΦX174 DNA-*Hae*III digest.

clusters, downward rolling of the margins of leaves, and necrosis or yellowing of leaf veins. These symptoms were indistinguishable from those recently attributed to infection of cultivated grapevines in the United States by group 16SrIII (‘*Ca.* P. pruni’-related) phytoplasma strains (NAGYIII strains) (Davis et al. 2015).

16S rRNA gene sequences, interoperon heterogeneity, and classification. DNA segments of the size expected (1.5 kbp) for phytoplasmal rRNA genes (rDNA) were amplified in PCR primed by phytoplasma-universal oligonucleotide primers, indicating possible phytoplasmal infection in the 13 symptomatic grapevines that were sampled. Results from nucleotide sequencing and in silico RFLP analysis confirmed that all 13 amplicons were rDNA derived from group 16SrI strains (NAGYI strains) related to subgroup 16SrI-B (Table 1). The nucleotide sequences of strains TW224 and TW308 were identical to each other. The nucleotide sequences of all 11 BG strains were identical to each other, and both rRNA operons of each BG strain contained a recognition site, for the restriction endonuclease *Rsa*I, that was absent from the two rRNA operons of both TW strains. Virtual RFLP patterns of the 16S rRNA gene sequences indicated that strains TW224 and TW308 belong to subgroup 16SrI-B, whereas the BG strains represent a variant, subgroup 16SrI-B*. The variant subgroup classification takes into account the presence of the unique *Rsa*I site in the 16S rDNA of the BG strains, in accordance with a convention introduced earlier (Wei et al. 2008b) and recently applied to NAGYIII phytoplasma strains (Davis et al. 2015).

The base-call nucleotide sequencing chromatogram for every NAGYI strain contained double peaks. The chromatograms for every BG strain exhibited double peaks at three specific base positions, whereas the TW224 and TW308 chromatograms exhibited double peaks at four specific base positions. None of the double-peak base positions within the F2n/R2 segment was located in a recognition site for any of the 17 restriction endonucleases used for group-subgroup classification. Because double peaks can suggest possible rRNA interoperon sequence heterogeneity, as in previous work (Davis et al. 2013), we cloned and separately sequenced both operons from each of four representative NAGYI strains (namely, BG4, BG10, TW224, and TW308) (Table 1); group and subgroup classification was confirmed by in silico RFLP analysis of the cloned sequences (Fig. 1).

Table 4. SecY amino acid residues distinguishing North American grapevine yellows group 16SrI (NAGYI) phytoplasma strains from related phytoplasma strains of subgroup 16SrI-B

	Amino acid residue at indicated position in the SecY protein of strain										
Strain[a]	**3**	**43**	**110**	**117**	**192**	**193**	**229**	**267**	**278**	**289**	**310**
BG4	H	F	F	V	K	N	N	S	V	G	F
BG10	H	F	F	V	K	N	N	S	V	G	F
TW224	H	F	F	A	K	I	N	S	V	G	F
TW308	H	F	F	A	K	I	N	S	V	G	F
AV2192	R	S	F	A	N	N	N	A	L	E	F
AY1	R	S	F	A	N	N	N	A	L	E	F
HyPH	R	S	F	A	N	N	N	A	L	E	F
PRIVC	R	S	F	A	N	N	N	A	L	E	F
MBS	R	S	L	A	K	N	D	S	V	E	L

[a] NAGYI strains = BG4, BG10, TW224, and TW308.

Deduced amino acid sequences and modeling of SecY proteins. The *secY* genomic regions were amplified from strains BG4, BG10, TW224, and TW308 and were sequenced (Table 1), and the *secY* genes were translated in silico. The deduced amino acid sequences of the SecY proteins encoded by the NAGYI strains were of two types that differed from one another by two residues as follows: position 117 (valine [V] in strains BG4 and BG10 → alanine [A] in strains TW224 and TW308) and position 193 (asparagine [N] → isoleucine [I]) (Table 4). The SecY-deduced amino acid sequences encoded by the BG and the TW strains differed from the SecY proteins encoded by ‘*Ca.* P. asteris’ subgroup 16SrI-B strains associated with aster-yellows-type diseases in herbaceous plants (Table 4).

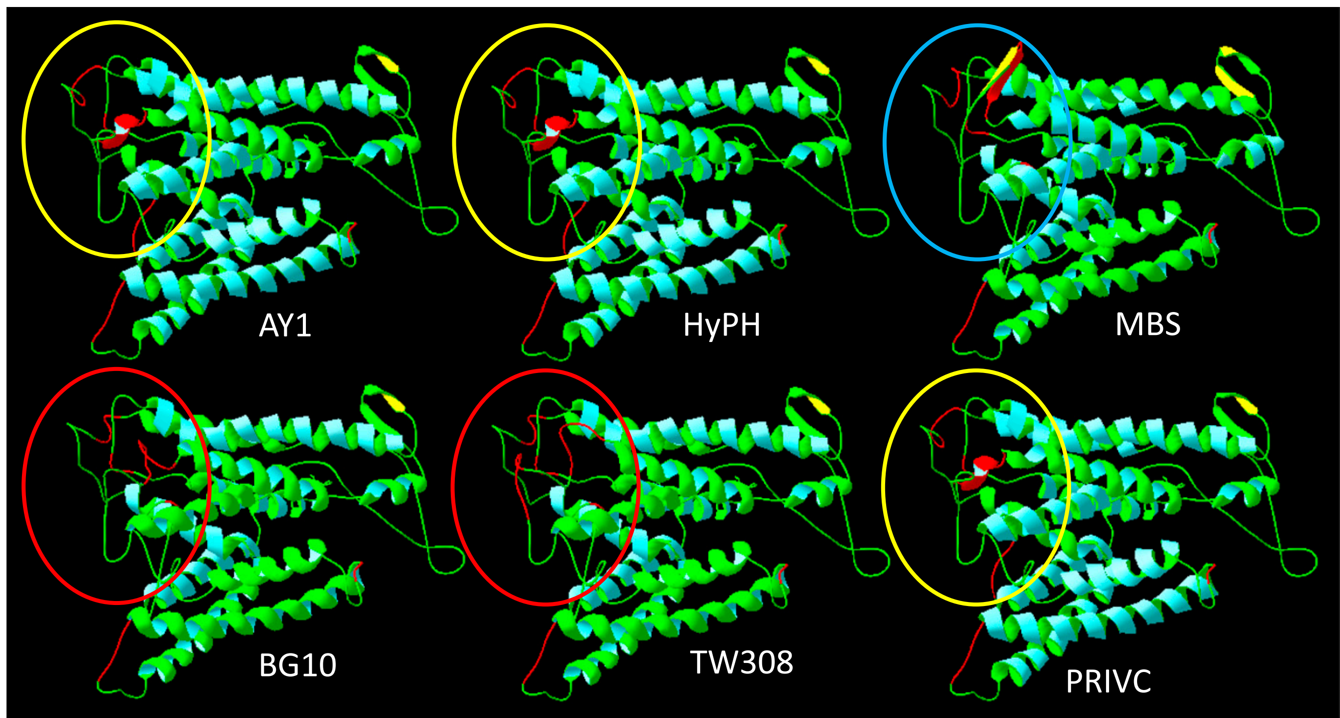


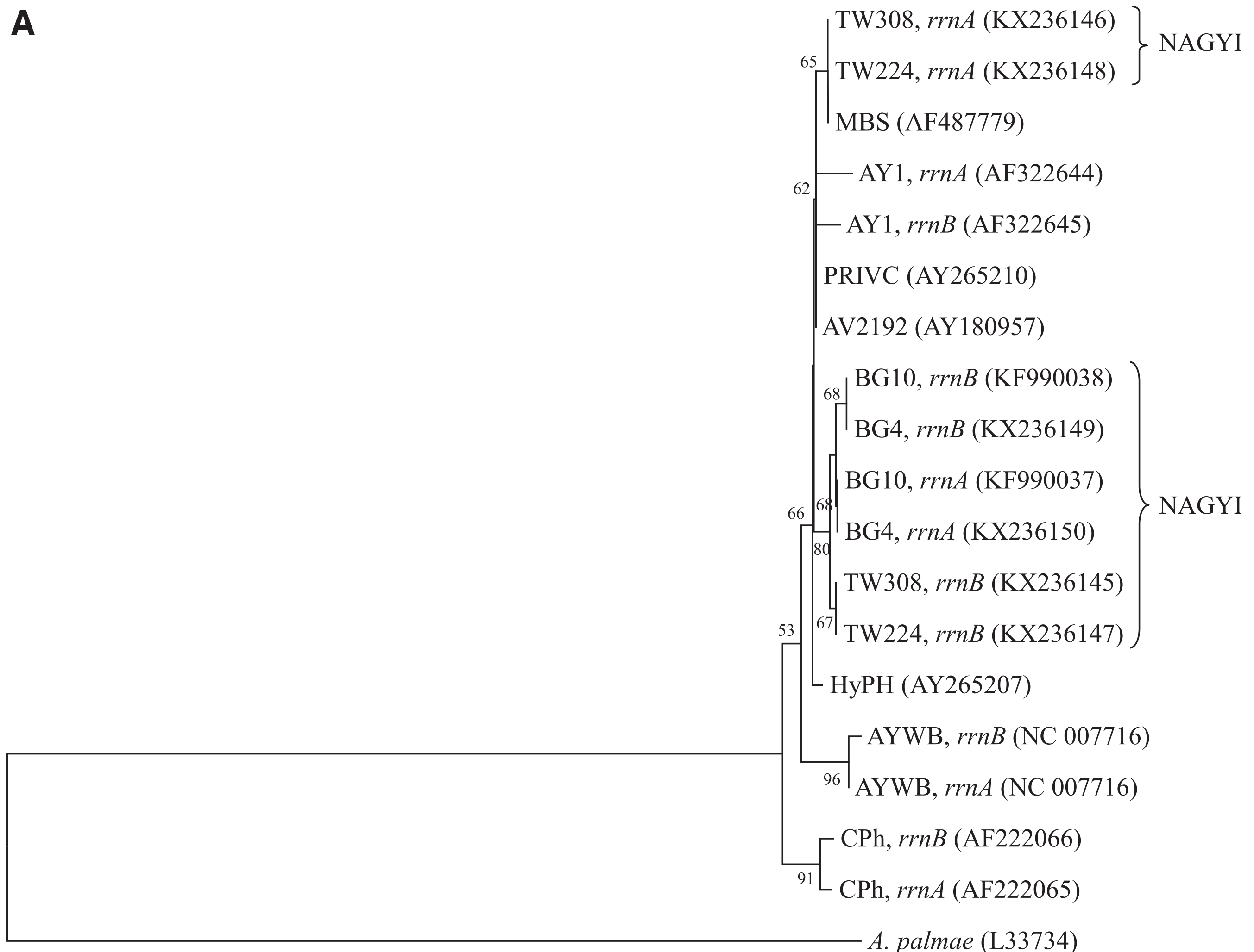
Fig. 2. Predicted three-dimensional structures of SecY proteins encoded by North American grapevine yellows group 16SrI phytoplasma strains BG10 (protein id AOI27800) and TW224 (protein id AOI27801), and by subgroup 16SrI-B strains AY1 (protein id AAV73936), HyPH (AAV73932), PRIVC (AAV73935), and MBS (ADO33828). The amino acid sequence and predicted structure of SecY from subgroup 16SrI-B strain AV2192 (not shown) were identical to those of SecY proteins from strains AY1, HyPH, and PRIVC. Differing regions putatively exposed at or present near the outer surface of the phytoplasma membrane are circled.

For example, the SecY proteins of the BG and TW strains each differed nonidentically by seven residues from the SecY proteins of the AY strains AY1 (Maryland AY phytoplasma, protein id AAV73936), AV2192 (AY phytoplasma, protein id AAV73926), PRIVC (primrose virescence phytoplasma, protein id AAV73935), HyPH (Hydrangea phyllody phytoplasma, protein id AAV73932) and of maize bushy stunt (MBS) phytoplasma (protein id ADO33828) (Table 4).

Because previous work illustrated that differences in SecY amino acid sequences may distinguish among diverse phytoplasma lineages (Davis et al. 2015; Wei et al. 2011), we constructed 3D models of the SecY proteins encoded by the NAGYI phytoplasmas and compared the predicted structures with those of the SecY proteins encoded by closely related subgroup 16SrI-B phytoplasma strains from herbaceous hosts. In particular, the SecY proteins of strains AY1, HyPH, PRIVC, and AV2192 were mutually identical in predicted 3D structure, whereas the structure of the strain MBS SecY protein was unique. The SecY proteins of NAGYI strains BG10 and TW308 differed slightly from one another but differed markedly from the SecY proteins of related strains AY1, HyPH, PRIVC, AV2192 (not shown), and MBS (Fig. 2). Interestingly, the differences in amino acid residues altered the 3D conformation of the folded proteins in regions putatively located at or near the outer surface of the phytoplasma membrane.

Phylogenetic relationships of NAGYI phytoplasma strains. Relationships of strains BG4, BG10, TW224, and TW308 with other phytoplasma strains classified in group 16SrI are shown in a phylogenetic tree based on analysis of 16S rRNA genes. Both *rrnA* and *rrnB* from strains BG4 and BG10, and operon *rrnB* from strains TW224 and TW308, formed a branch divergent from other group 16SrI strains (Fig. 3A). The *rrnA* operon sequences from strains TW224 and TW308 occurred on a branch bearing the divergent lineage represented by strain MBS.

Relationships inferred from phylogenetic analysis of SecY proteins were consistent with the concept that the NAGYI phytoplasma strains are closely related to one another but may be evolutionarily diverging from subgroup 16SrI-B phytoplasma strains AY1, HyPH, PRIVC, AV2192, and MBS, as well as from subgroup 16SrI-A and



(*Continued*)

Fig. 3. Neighbor-joining phylogenetic trees constructed on the basis of **A,** 16S ribosomal RNA (rRNA) gene sequences and **B,** amino acid sequences of SecY proteins from North American grapevine yellows group 16SrI (NAGYI) phytoplasma strains (BG4, BG10, TW224, and TW308), related strains associated with aster yellows (AY; strains AY1, HyPH, PRIVC, and AV2192) or maize bushy stunt disease (strain MBS) and classified in subgroup 16SrI-B, and phytoplasma strains representing subgroups 16SrI-A (strain AYWB, aster yellows witches'-broom) and 16SrI-C (strain CPh, clover phyllody), and from *Acholeplasma palmae* strain J233. Numbers at nodes are bootstrap confidence levels. GenBank accession numbers for the 16S rRNA gene sequences and for the SecY proteins are in parentheses.

subgroup 16SrI-C lineages represented by strains AYWB and CPh, respectively (Fig. 3B).

Discussion

In a previous article, we presented evidence of NAGY in several states in the United States and provided molecular markers distinguishing two novel sequevars of the group 16SrIII NAGY phytoplasma strains (NAGYIII strains related to '*Ca*. P. pruni') (Davis et al. 2015). The present article extends and complements that work and earlier reports of NAGY phytoplasma strains in Virginia (Davis et al. 1998; Prince et al. 1993). Here, we characterize recently found NAGYI strains (strains related to '*Ca*. P. asteris') that are members or variants of group 16SrI (AY phytoplasma group) subgroup 16SrI-B that can be distinguished from related phytoplasma strains associated with AY disease in herbaceous plants.

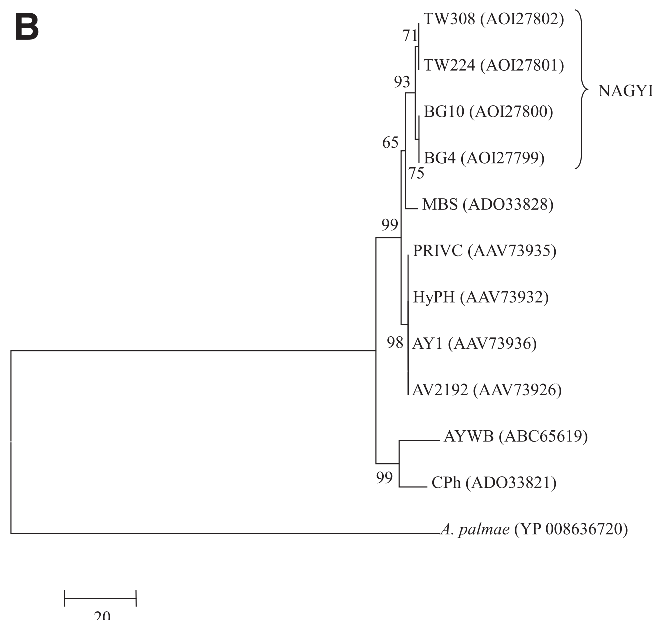
Interestingly, group 16SrI phytoplasmas related to those causing AY disease in herbaceous plants have been reported in diseased *V. vinifera* in several regions of the world. In addition to North America, grapevine yellows-associated phytoplasmas belonging to subgroup 16SrI-B have been reported in South America, Europe, and Africa (Alma et al. 1996; Engelbrecht et al. 2010; Ertunc et al. 2015; Gajardo et al. 2009; M'hirsi et al. 2004; Olivier et al. 2009, 2014) (this study). The design and implementation of effective strategies to manage these diseases will rest, in part, upon knowledge of non-*V. vinifera* plant hosts and insect vectors of these phytoplasmas. Fortunately, genotyping and structural analysis of SecY proteins, as in the present study, provide molecular markers for determining which non-*V. vinifera* plants harbor NAGYI phytoplasma strains.

Classification of NAGYI phytoplasma strains in subgroup 16SrI-B or 16SrI-B* in this article might be interpreted to indicate that NAGYI disease can be attributed to grapevine infection by strains that are also responsible for AY disease in herbaceous plants, but such an interpretation may not be completely accurate. The genotyping and in silico modeling of SecY proteins indicated that, although closely related to AY phytoplasma, the NAGYI strains in this study can be distinguished from subgroup 16SrI-B phytoplasma strains such as AY1, HyPh, and PRIVC that cause AY diseases in herbaceous plants, as well as from subgroup 16SrI-B MBS phytoplasma (this study).

The structural differences, predicted to be located in surface-exposed regions of the membrane-embedded SecY protein, conceivably signal divergent evolution of the phytoplasma lineages. Just as the 3D structure of the SecY proteins distinguished NAGYI strains from AY phytoplasma subgroup 16SrI-B strains, it also distinguished MBS phytoplasma from the AY strains (this study). Thus, the evolution of NAGYI strains in the present study may be analogous to the evolutionary trajectory of MBS phytoplasma, in that both lineages may be diverging from strains causing AY in herbaceous plants. Notably, MBS phytoplasma is restricted in nature to particular plant hosts (corn [maize], *Zea mays* L., and teosinte, *Z. mays* L. *mexicana* (Schrad.) Iltis) and to maize-feeding leafhopper vectors, including *Dalbulus maidis* (DeLong and Wolcott), *D. elimatus* (Ball), and *Graminella nigrifrons* (Forbes) (Nault 1980). Conversely, phytoplasma strains that cause AY disease in other plants have not been reported in maize or teosinte, and *D. maidis* has not been reported as a vector of AY phytoplasma strains. Thus, what appear to be small differences in 16S rRNA gene or *secY* gene sequences may, in some cases, signal important differences in evolutionary biology. Conceivably, some structural features distinguishing the NAGYI SecY proteins in this study are a consequence of evolutionary adaptation to specific plant or insect hosts. With this reasoning, a question arises as to whether some NAGYI strains have an unknown biological cycle or insect vector–plant host niche that differs from that of closely related but genotypically distinct subgroup 16SrI-B phytoplasma strains causing disease in herbaceous plants.

Although our data distinguished NAGYI strains from AY phytoplasma strains, the findings do not eliminate the possibility that AY phytoplasma strains from herbaceous plants also can cause grapevine yellows disease in North America. Notably, AY phytoplasma strains belonging to subgroups 16SrI-A (AY witches' broom subgroup), 16SrI-B (AY phytoplasma subgroup), and 16SrI-C (clover phyllody subgroup) have been reported in NAGY-diseased *V. vinifera* in Canada (Olivier et al. 2014), and a subgroup 16SrI-A strain was reported from NAGY-diseased *V. vinifera* in Virginia (Davis et al. 1998). It is not known whether these particular strains from *V. vinifera* are associated with AY in herbaceous plants, and the *secY* sequences from these earlier NAGY strains were unavailable for the current study's nucleotide sequence analysis and 3D structure comparisons of the encoded SecY proteins. Although additional data will be needed to determine the full geographic extent of NAGY and to identify the insect vectors of the varied NAGY phytoplasma genotypes, a growing body of knowledge continues to emphasize that grapevine yellows diseases are caused by a wide range of phylogenetically diverse phytoplasma genotypes.

Fig. 3. (*Continued from previous page*)



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