Unraveling the Etiology of North American Grapevine Yellows (NAGY): Novel NAGY Phytoplasma Sequevars Related to ‘*Candidatus* Phytoplasma pruni’

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Abstract

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North American grapevine yellows (NAGY) disease has sometimes been attributed to infection of *Vitis vinifera* L. by *Prunus* X-disease phytoplasma (‘*Candidatus* Phytoplasma pruni’) but this attribution may not be fully adequate. In this study, phytoplasma strains related to ‘*Ca. Phytoplasma pruni*’ were found in NAGY-diseased grapevines in Maryland, Pennsylvania, Virginia, Ohio, Missouri, and New York State. Based on restriction fragment length polymorphism analysis of 16S ribosomal RNA gene (16S rDNA) sequences, the strains (termed NAGYIII strains) were classified in group 16SrIII (X-disease group) but they contained a recognition site for the restriction endonuclease *Mse*I that is not present in the 16S rDNA of ‘*Ca. Phytoplasma pruni*’. The 16S rDNA of the strains differed by three or four nucleotides from that of ‘*Ca. Phytoplasma pruni*’, indicating that they belonged to two novel 16S rDNA sequevars, designated NAGYIII α and NAGYIII β . Both sequevars

differed from ‘*Ca. Phytoplasma pruni*’ by a single base in each of three regions corresponding to species-unique (signature) sequences described for ‘*Ca. Phytoplasma pruni*’. Phylogenetic analyses of 16S rRNA genes and SecY proteins, and single-nucleotide polymorphism analyses of *secY* and ribosomal protein genes, further distinguished the two grapevine sequevar lineages from one another and from ‘*Ca. Phytoplasma pruni*’. The NAGYIII α and NAGYIII β sequevars also differed from ‘*Ca. Phytoplasma pruni*’ in regions of the folded SecY protein that are predicted to be near or exposed at the outer surface of the phytoplasma membrane. No evidence indicated that diseased grapevines contained any phytoplasma strain conforming to ‘*Ca. Phytoplasma pruni*’ *sensu stricto*. Because the NAGYIII sequevars have not been reported in X-disease, a question is raised as to whether NAGYIII and *Prunus* X-disease are caused by different phytoplasma genotypes.

Grape has been cited as the “most valuable agricultural crop in the United States” and “the most valuable horticultural crop in the world” (Ayala 2011; Myles et al. 2011), and grapevine yellows (GY) is one of the most damaging diseases known in *Vitis vinifera* L. grapevines. The term “grapevine yellows” is applied individually or collectively to any of several diseases of cultivated grapevine. Typical symptoms include shriveling of grape clusters, uneven lignification or maturation of stems (canes), downward rolling of leaves, yellowing or necrosis of leaf veins, reddening of leaves in red grape varieties, dieback of canes, and, in sensitive cultivars, eventual plant death. Although characterized by very similar or indistinguishable symptom syndromes, the GY diseases in different geographic regions of the world are attributed to plant infection by different species of phytoplasmas (formerly mycoplasma-like organisms) that differ from one another in their spread by specific insect vectors, plant host range, and natural ecology (Daire et al. 1997; Davis et al. 1993, 1997a,b, 1998; Engelbrecht et al. 2010; M’hirsi et al. 2004; Olivier et al. 2009; Prince et al. 1993; Quaglino et al. 2013).

Phytoplasmas are unique plant-pathogenic bacteria that lack a cell wall, inhabit phloem of diseased plants, are transmitted from plant to plant by phloem-feeding insects (mainly leafhoppers) (Davis and Lee 1982; Weintraub and Beanland 2006; Zhao et al. 2014), and possess chromosomally integrated prophage-based genomic islands termed sequence variable mosaics (Davis et al. 2007; Jomantiene and Davis 2006; Wei et al. 2008a). Taxonomically, phytoplasmas are classified in class *Mollicutes* (mycoplasmas), along with species of *Mycoplasma*, *Spiroplasma*, *Acholeplasma*, and other genera (Krieg et al. 2011). Attempts to isolate phytoplasmas in culture have been made by researchers since the early 1970s and, while several claims of axenic culture of phytoplasmas have appeared in the published literature, none has been independently confirmed thus far (Contaldo et al. 2012; Ghosh et al. 1975; Giannotti and Vago 1971; Jacoli 1981; Lin et al. 1970; Lombardo and Pignatelli 1970; Teranaka and Otsuka 1973; Zhao et al. 2014). Thus, phytoplasmas have long remained refractory to many approaches utilized to ascertain phenotypic properties of cultivable bacteria.

Analysis of gene sequences has consequently been adopted for phytoplasma classification and taxonomy (Davis et al. 2013; Lee et al. 1998, 2010; Martini et al. 2007; Quaglino et al. 2013; Zhao et al. 2014). Based on restriction fragment length polymorphism (RFLP) analyses of 16S ribosomal RNA genes (16S rDNA), phytoplasmas have been classified in more than 30 groups, and over 35 ‘*Candidatus* Phytoplasma’ spp. have been described thus far, principally on the basis of comparisons of nucleotide sequence identity of the 16S rRNA gene (Davis et al. 2013; Lee et al. 1998; Nejat et al. 2013; Quaglino et al. 2013). Evolutionary adaptation to survival in widely diverse ecological niches, involving varied insect vectors and plant hosts in different geographic and climatic regions, likely gave rise to divergent radiation of numerous phytoplasma species,

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most of which undoubtedly have not yet been studied. Molecular genetic characterization based on sequence analyses of conserved genes currently remains the most useful approach for distinguishing different phytoplasmas. Because it is difficult, if not impossible, to distinguish the diverse GY diseases from one another based on symptoms alone, genotyping of GY phytoplasma strains forms a critically important basis for understanding etiology and epidemiology of the diseases worldwide, for assessing the impacts of infection by specific GY pathogens, and for devising effective disease management strategies.

In North America, a GY disease was first reported in 1977 in cultivated grapevines in New York State; based on disease symptoms alone, the disease was thought possibly to be *flavescence dorée* (FD), a GY disease occurring in Europe (Pearson et al. 1985; Uyemoto et al. 1977). These and other findings led to suggestions that the FD phytoplasma, via the insect vector *Scaphoideus titanus*, was

introduced into Europe from North America (Chen et al. 1993; Maixner et al. 1993; Pearson et al. 1985; Uyemoto et al. 1977), although the New York GY phytoplasma had not been definitively identified and characterized. In the 1990s, GY was reported in Virginia (Davis et al. 1998; Prince et al. 1993; Wolf et al. 1994). During 2009 to 2011, a resurgence of GY occurred in Virginia (T. K. Wolf, unpublished), prompting the present study of phytoplasmas associated with the disease, now termed North American grapevine yellows (NAGY), in the eastern United States.

In the current study, we investigated the possible presence and explored the etiology of NAGY disease in the states of Maryland, Pennsylvania, Ohio, and Missouri. We also definitively identified and characterized NAGY phytoplasmas infecting cultivated grapevines in New York State, where NAGY-associated strains had not been clearly identified, and in Virginia, where a more detailed characterization of NAGY phytoplasma strains was needed. An objective of

Table 1. Grapevine cultivars exhibiting symptoms of North American grapevine yellows (NAGY) and found in this study to be infected by ‘*Candidatus* Phytoplasma pruni’-related strains (strains classified in group 16SrIII) in vineyards in Maryland, Virginia, Pennsylvania, New York State, Ohio, and Missouri

State, county	Vineyard	Grapevine cultivar	Year ^a	Phytoplasma	
				Strain	Sequovar ^b
Maryland					
Montgomery	A	Chardonnay	2011	MD1	α
		Chardonnay	2011	MD2	α
		Chardonnay	2011	MD3	α
		Chardonnay	2011	MD4	α
		Chardonnay	2011	MD5	α
		Chardonnay	2011	MD6	β
		Chardonnay	2011	MD7	α
		Chardonnay	2012	MD8	α
		Chardonnay	2012	MD9	α
Washington	B	Pinot gris	2011	UMD1	α
		Chardonnay	2011	UMD4	α
		Chardonnay	2011	UMD6	α
		Black Malvasia	2011	UMD7	α
Talbot	C	Chardonnay	2011	ES1	α
		Chardonnay	2011	ES2	α
Pennsylvania					
Lancaster	D	Chardonnay	2003	TW518	β
Lancaster	E	Chardonnay	2011	PA1	β
New York State					
Schuyler	F	Chardonnay	2003	TW285	β
Schuyler	G	Chardonnay	2003	TW295	β
Seneca	H	Chardonnay	2003	TW382	β
Missouri					
Warren	I	Unspecified	2010	MO9	α
Boone	J	Unspecified	2010	MO12	α
Virginia					
Loudoun	K	Chardonnay	1995	WC7A	α
		Chardonnay	1996	WC11-96	β
Rappahannock	L	Chardonnay	1995	FH13A	α
		Chardonnay	1996	FH1-96	α
Fauquier	M	Cabernet Sauvignon	2010	VG1	β
		Cabernet Sauvignon	2010	VG14	α
Rappahannock	N	Cabernet Sauvignon	2010	VG2	α
		Chardonnay	2010	VG3	α
		Chardonnay	2010	VG4	α
		Chardonnay	2010	VG16	α
Rappahannock	O	Viognier	2010	VG10B	α
		Petit Manseng	2010	VG12	α
Rappahannock	P	Malbec	2010	VG20	α
Ohio					
Ashtabula	Q	Chardonnay	2012	OH1	α
		Chardonnay	2012	OH2	α
		Chardonnay	2012	OH7	α
		Chardonnay	2012	OH9	α

^a Year of sample collection.

^b Single-nucleotide polymorphism lineage SNP790T/C of 16S rRNA genes; see text for details.

this work was to gain greater understanding of the etiology of NAGY and provide molecular genetic markers that would clearly distinguish NAGY phytoplasma strains and subsequently be useful in identification of insect vectors in epidemiological studies and in disease management. In pursuit of this objective, we used sensitive molecular diagnostic procedures to detect grapevine-infecting phytoplasmas; carried out multilocus genotyping of NAGY phytoplasma strains based on analyses of 16S rRNA, *secY*, and ribosomal protein (*rp*) gene sequences; and analyzed the three-dimensional (3D)

structure of SecY proteins. A preliminary synoptic report of some of the findings has been published in abstract form (Davis et al. 2012).

Materials and Methods

Plant samples and DNA amplification. Samples of symptomatic leaves were collected from 39 cultivated grapevine plants (*V. vinifera* or hybrid) in Maryland, Missouri, New York State, Ohio, Pennsylvania, and Virginia (Table 1). Veins were excised from symptomatic

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'-AACGCTGGCGCGCCTAATAC-3'	Lee et al. 2004b
	16S-SR	5'-GGTCTGTCAAACTGAAGATG-3'	Lee et al. 2004b
<i>secY</i> region ^a	L15F1A(III)	5'-CTTCTGGTAAAGGACATAAAGG-3'	Lee et al. 2010
	MapR1A(III)	5'-GGTTCTTCGTGCAATTGCAAACC-3'	Lee et al. 2010
	SecYF1(III)	5'-CTAGACCAGGTTTTGAAGG-3'	Lee et al. 2010
	SecYR1(III)	5'-GACCTGCTTTTCTCATTATAGC-3'	Lee et al. 2010
<i>rp</i> genes ^b	rpL2F3	5'-WCCTTGGGGYAAAAAAGCTC-3'	Martini et al. 2007
	rp(I)R1A	5'-GTTCTTTTGGCATTAAACAT-3'	Martini et al. 2007
	rp(III)-FN	5'-GGTGAATTTTCTCCAACCTCG-3'	Davis et al. 2013

^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*).

^b Ribosomal protein (*rp*) gene locus encoding S19 (*rpsS* gene), L22 (*rplV* gene), and S3 (*rpsC* gene).

Table 3. GenBank accession numbers of 16S rRNA, *secY*, and ribosomal protein (*rp*) gene sequences determined in this work

State	Vineyard	Strain	GenBank accession number ^a			
			<i>rrnA</i>	<i>rrnB</i>	<i>secY</i>	<i>rp</i>
Maryland	A	MD1	KF915151	KF915150	ND	ND
		MD2	KF915125 ^b	...	KF853435	KF880830
		MD3	KF915124 ^b	...	ND	ND
		MD4	KF915149	KF915148	KF853436	KF880829
		MD6	KF915147	KF915146	KF853437	KF880828
		MD8	KF915145	KF915144	KF853438	KF880827
		MD9	KF915123 ^b	...	KF853439	KF880826
	B	UMD1	KF915118 ^b	...	KF853448	KF880816
		UMD4	KF915117 ^b	...	KF853449	KF880815
		UMD6	KF915116 ^b	...	ND	ND
		UMD7	KF915115 ^b	...	KF853450	KF880814
	C	ES1	KF915129 ^b	...	KF853459	KF880806
		ES2	KF915128 ^b	...	ND	ND
		...	...	...	...	...
Pennsylvania	D	TW518	KF915119 ^b	...	KF853447	KF880817
	E	PA1	KF915137	KF915136	KF853458	KF880823
New York	F	TW285	KF915135	KF915134	KF853444	KF880820
	G	TW295	KF915133	KF915132	KF853445	KF880819
	H	TW382	KF915131	KF915130	KF853446	KF880818
	...	...	...	...	...	...
Missouri	I	MO9	KF915122 ^b	...	KF853441	KF880825
	J	MO12	KF915121 ^b	...	KF853440	KF880824
	...	...	...	...	...	...
Virginia	K	WC7A	KF915111 ^b	...	KF853457	KF880808
		WC11-96	KF915110 ^b	...	KF853456	KF880807
	L	FH13A	KF915126 ^b	...	KF853434	KF880831
		FH1-96	KF915127 ^b	...	KF853433	KF880832
	M	VG1	KF915114 ^b	...	KF853451	KF880813
		VG14	KF990039	KF990040	KF853452	KF880810
	N	VG2	KF915113 ^b	...	KF853454	KF880812
		VG3	KF915112 ^b	...	KF853455	KF880811
		VG16	KF990041 ^b	...	KF853453	KF880809
	...	...	...	...	...	...
Ohio	Q	OH1	KF915143	KF915142	ND	ND
		OH2	KF915141	KF915140	KF853442	KF880822
		OH7	KF915139	KF915138	ND	ND
		OH9	KF915120 ^b	...	KF853443	KF880821

^a *rrnA* and *rrnB* = ribosomal RNA operons *rrnA* and *rrnB*, respectively. The nucleotide sequence for operon *rrnB* was determined for strains in which evidence for interoperon sequence heterogeneity was observed in 16S rRNA genes. ND = not determined.

^b No evidence for interoperon sequence heterogeneity of 16S rRNA genes was observed for this strain.

leaves and frozen at -20°C until they were used for DNA extraction. DNA for use as template in polymerase chain reactions (PCRs) was extracted from excised veins by a modified method (Green et al. 1999) using DNeasy Plant DNA Extract Kit (Qiagen, Germantown, MD) and stored at -20°C until further use. rRNA gene sequences were amplified in direct high-fidelity (hf)-PCRs primed by primer pair P1/16S-SR, or in seminested PCRs primed by P1/16S-SR followed by P1A/16S-SR, and catalyzed by AccuPrime Taq DNA Polymerase High Fidelity (Life Technologies, Carlsbad, CA) under conditions previously described (Lee et al. 2004a,b). The rp genomic regions containing a partial (3' end) *rpsS*, a complete *rplV*, and a complete *rpsC* gene were amplified in nested hf-PCRs primed by primer pair rpL2F3/rp(I)R1A (Martini et al. 2007) followed by rp(III)-FN/rp(I)R1A (Davis et al. 2013). *SecY* genomic regions containing a partial (3' end) *rpl15* *rp* gene, a complete *secY* gene, and a partial (5' end) methionine aminopeptidase (*map*) gene were amplified in hf-PCRs primed by L15F1A(III)/MapR1A(III) and catalyzed by LA Taq DNA polymerase (Takara Bio USA, Madison, WI) followed by nested PCR with primer pair SecYF1(III)/SecYR1(III) under conditions previously described (Lee et al. 2010). Nucleotide sequences of primers are given in Table 2. A negative control devoid of DNA template in the reaction mix was included in all PCR assays. PCR products were analyzed by 1% agarose gel electrophoresis (10 V/cm).

Fig. 1. Symptomatic North American grapevine yellows (NAGY)-diseased and symptomless grapevine (*Vitis vinifera*) plants. **A**, Symptomless Chardonnay grapevine. **B**, NAGY-diseased Chardonnay; note shriveled fruit cluster. **C**, Symptomatic leaf from NAGY-diseased Chardonnay. **D**, Symptomatic leaf from NAGY-diseased Black Malvasia.

Fig. 2. Analysis of putative restriction sites in 16S rRNA gene (rDNA) sequences from North American grapevine yellows phytoplasma strains belonging to group 16SrIII (North American grapevine yellows [NAGY]III strains) and from '*Candidatus* Phytoplasma pruni'. Maps were generated using the MAPDRAW option of the DNASTAR program and manually aligned for comparison of recognition sites for the restriction endonuclease *MseI*. MD1, MD6, MO9, and WC11-96 = NAGYIII phytoplasma strains. rrmA and rrnB indicate rDNA sequences derived from the two sequence heterogeneous rRNA operons, respectively, in a given phytoplasma genome. No evidence for 16S rRNA interoperon sequence heterogeneity was observed for strains MO9 and WC11-96. Each bar represents the 1,247-bp rDNA segment that is amplified in polymerase chain reaction primed by oligonucleotide pair F2n/R2n and used for group and subgroup classification based on restriction fragment length polymorphism analysis. Vertical lines indicate recognition sites for the restriction endonuclease *MseI*. GenBank numbers are given in parentheses. Arrows indicate *MseI* recognition sites that are present in NAGYIII strains and absent in '*Ca. Phytoplasma pruni*'. Horizontal scale bars indicate base positions.

Phytoplasma-specific DNA bands were extracted from gels using QIAquick Gel Extraction Kit (Qiagen), and amplicons were sequenced by automated DNA sequencing (ABI Prism Model 3730; Applied Biosystems, Foster City, CA) of both strands. GenBank accession numbers of nucleotide sequences determined in this work are given in Table 3. Nucleotide sequences of genes from other phytoplasmas in this study were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/>).

Phytoplasma classification. 16S rRNA gene sequences were subjected to virtual RFLP analyses for group and subgroup classification, and to nucleotide sequence identity comparisons for taxonomic placement, using iPhyClassifier (Zhao et al. 2009) (<http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>). Input data for iPhyClassifier usage consisted of nucleotide sequences, at least 1.2 kb in size, including the annealing sites for PCR primers R16F2n and R16R2 (Gundersen and Lee 1996), the minimum 16S rDNA sequence region required by the program. Nucleotide and amino acid sequence alignments were done using the Lasergene software MegAlign program (DNASTAR, Madison, WI).

Protein structure predictions. The amino acid sequences of SecY proteins were deduced from respective *secY* gene nucleotide sequences using the Lasergene EditSeq program (DNASTAR). The 3D structures of the SecY proteins were predicted using the online modeling program SWISS-MODEL (<http://swissmodel.expasy.org/>) with the *Bacillus subtilis* SecY protein as a template (Swiss Model 3dl8H). Predicted 3D structures were visualized and analyzed using Swiss PDB-Viewer (Guex and Peitsch 1997).

Phylogenetic analyses. Phylogenetic analyses were accomplished by using the software MEGA4 with the minimum evolution algorithm implemented in the software (Tamura et al. 2007). The initial tree was obtained through close-neighbor-interchange search using the neighbor-joining method. The reliability of the analysis was subjected to a bootstrap test with 1,000 replicates.

Results

Geographic occurrence of NAGY and classification of associated phytoplasma strains. GY disease symptoms were observed in *V. vinifera* 'Chardonnay', 'Pinot gris' ('Pinot grigio'), 'Viognier', 'Petit Manseng', 'Cabernet Sauvignon', 'Malbec', and 'Black Malvasia' in the states of Maryland, Missouri, Pennsylvania, Ohio, Virginia, and New York State (Table 1). The findings were new records of the disease for Maryland, Ohio, Pennsylvania, and Missouri. Symptoms observed in white or light-skinned grape cultivars included mild chlorosis of leaf lamina, yellowing and necrosis of leaf veins, necrosis of interveinal tissue, downward curling of leaf margins, incomplete lignification of affected branches, and shriveling of fruit clusters (Fig. 1). In addition, diseased red and dark grape cultivars generally exhibited abnormal reddening of leaf interveinal tissue. Downward rolling of leaf margins was often not observed.

Phytoplasma-characteristic rDNA fragments were amplified in PCRs containing template DNA from all 39 NAGY-symptomatic plants sampled, indicating that all the plants were infected by phytoplasma (Table 1). Based on the results from the virtual RFLP analysis,

we classified the NAGY strains in group 16SrIII and termed them NAGYIII phytoplasma strains. The collective RFLP patterns of 16S rDNA were highly similar to those of '*Candidatus* Phytoplasma pruni', the reference phytoplasma for group 16SrIII, subgroup 16SrIII-A, but

	Phytoplasma strain	16S rRNA gene				<i>secY</i>	rp (S3 protein gene)
		242	790	1003	1093	308, 316, 318	1100, 1113
NAGYIII α	WC7A	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	FH13A	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	FH1-96	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	VG2	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	VG3	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	VG14	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	VG16	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	MO9	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	MO12	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	MD2	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	MD4	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	MD8	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	MD9	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	ES1	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	UMD1	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	UMD4	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	UMD7	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	OH2	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	OH9	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
NAGYIII β	WC11-96	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	VG1	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	PA1	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	MD6	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	TW285	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	TW295	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	TW382	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	TW518	AGTTGGCAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
'Ca. Phytoplasma pruni'	PX11-1	AGTTGGTAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	PX11-2	AGTTGGTAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	PX92-1	AGTTGGTAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	PX92-4	AGTTGGTAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	CX-95	AGTTGGTAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG
	WX95	AGTTGGTAGGG	CGCTGTAA	AGGTTATCA	GTTAATTGC	TAAGATGAAAATTGTAATA	GTATTGATTTATCATGGTGAAG

Fig. 3. Selected single-nucleotide polymorphisms (SNPs) in segments of genes encoding 16S rRNA, and SecY and rpS3 proteins, of North American grapevine yellows (NAGY) phytoplasma strains and '*Candidatus* Phytoplasma pruni' strains. Representative NAGY strains are shown. Bases distinguishing NAGYIII α , NAGYIII β , and 'Ca. Phytoplasma pruni' from one another are boxed. Numbers indicate base positions of SNPs with reference to 'Ca. Phytoplasma pruni' strain PX11CT1^R GenBank sequences JQ044393 (16S rRNA; bases 242, 790, 1,003, and 1,093), JQ268254 (*secY*, bases 308, 316, and 318) and JQ360960 (ribosomal protein [rp] locus, bases 1,100 and 1,113). In the present study, strains (NAGYIII α strains) bearing a T in the 16S rRNA gene SNP790T/C position were found in vineyards in Maryland, Virginia, Ohio, and Missouri; strains (NAGYIII β strains) bearing a C in the SNP790T/C position were found in vineyards in Maryland, Virginia, New York, and Pennsylvania. GenBank accession numbers for 16S rRNA gene sequences from 'Ca. Phytoplasma pruni' strains PX11CT1^R (PX11-1), PX11CT2 (PX11-2), PX92CT1 (PX92-1), PX92CT4 (PX92-4), CX-95, and WX95 are JQ044393, JQ044391, JQ044389, JQ044383, JQ044397, and JQ044396, respectively; those for *secY* genes are JQ268254, JQ268253, JQ268251, JQ268249, and JQ268250, respectively; and those for *rpS3* genes are JQ360960, JQ360959, JQ360958, JQ360957, JQ360955, and JQ360956, respectively.

Fig. 4. Predicted three-dimensional structures of the deduced amino acid sequences of the SecY proteins encoded by lineage North American grapevine yellows (NAGY)III α strain MO9, lineage NAGYIII β strain MD6, and '*Candidatus* Phytoplasma pruni' strain PX11CT1^R. Differing regions putatively exposed at or present near the outer surface of the phytoplasma membrane are boxed.

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Fig. 5. Phylogenetic relationships of North American grapevine yellows (NAGY)III phytoplasma strains and reference strains of '*Candidatus Phytoplasma*' spp. inferred from analysis of **A and B**, 16S rRNA gene sequences and **C and D**, SecY amino acid sequences (5C and 5D). B and D are expanded views of portions of A and C, respectively. Representative NAGY strains are shown. *Achleoplasma palmae* was selected to root the trees. NAGYIIIα and NAGYIIIβ indicate two distinct sequevar lineages of NAGYIII phytoplasma. For analyses of NAGYIII strains exhibiting rRNA interopereon sequence heterogeneity, 16S rRNA gene sequences from *rrnA* were used. GenBank accession numbers for 16S rDNA sequences from NAGYIII phytoplasma strains are given in Table 3. GenBank numbers for 16S rDNA sequences from other phytoplasma strains are given in parentheses. Amino acid sequences of SecY proteins from NAGYIII strains (C and D) are those encoded by respective nucleotide sequences for which GenBank numbers are listed in Table 3; amino acid sequences of SecY proteins from '*Candidatus Phytoplasma pruni*' strains PX92CT4, PX92CT1, PX11CT2, and PX11CT1^R are encoded by GenBank accessions JQ268251 to JQ268254, respectively. GenBank accession numbers for the remaining SecY proteins are given in parentheses. Scale bars indicate the number of base substitutions per site.

the 16S rDNA of all the grapevine-infecting NAGYIII strains possessed a recognition site for *MseI* that was not present in strains of '*Ca. Phytoplasma pruni*' (Fig. 2). In accordance with revised phytoplasma classification (Wei et al. 2008b), this difference of a single restriction endonuclease recognition site places the NAGYIII strains into a new variant subgroup, designated 16SrIII-A*. Because all NAGYIII strains in this study were 16SrIII-A subgroup variants (16SrIII-A*), we reexamined RFLP patterns of 16S rDNA from phytoplasma strain VGYIII (strain WC7A, GenBank accession number AF060875), which was previously reported from NAGY-diseased grapevine in Virginia and classified in subgroup 16SrIII-I (Davis et al. 1998). The 16S rDNA RFLP patterns that we obtained included a differentiating *MseI* restriction site in the 16S rRNA gene, indicating that strain WC7A should also be classified in variant subgroup 16SrIII-A*.

NAGYIII strains represent two novel sequevars. Alignment of the 16S rDNA nucleotide sequences revealed that each strain was represented by one or the other of two distinct 16S rDNA sequences that shared >99% 16S rDNA sequence identity with '*Ca. Phytoplasma pruni*' but differed by three or four nucleotides from '*Ca. Phytoplasma pruni*' (Fig. 3). To distinguish the strains carrying the two different 16S rDNA sequences, we adopted the term "sequevar", previously used to distinguish walled bacteria. Because "a sequevar consists of the strain or strains which have a particular sequence" (Frothingham and Wilson 1993), we recognized among the NAGYIII strains two novel 16S rDNA sequevars, designated sequevars NAGYIII α and NAGYIII β (Table 1; Fig. 3).

A single-nucleotide polymorphism (SNP) in the 16S rRNA genes distinguished the two sequevars. Sequevar NAGYIII α contained a T whereas sequevar NAGYIII β contained a C at the base position equivalent to position 790 in the 16S rRNA gene sequence of '*Ca. Phytoplasma pruni*' reference strain PX11CT1^R (GenBank sequence JQ044393). Sequevar NAGYIII α was found in vineyards in Maryland, Virginia, Ohio, and Missouri; sequevar NAGYIII β was found in vineyards in Maryland, Virginia, New York, and Pennsylvania (Table 1; Fig. 3). In strains exhibiting 16S rDNA interoperon heterogeneity (Table 3), the operon designated as *rrnA* matched the 16S rDNA sequences of all other strains of a given sequevar.

Unique 16S rRNA gene sequence regions of NAGYIII phytoplasma strains. Because both NAGYIII sequevars are closely related to '*Ca. Phytoplasma pruni*', we examined both sequevars for the presence of species-unique 16S rDNA sequences as designated in the original description of '*Ca. Phytoplasma pruni*' (Davis et al. 2013). Although both sequevars shared >99% 16S rRNA gene sequence identity with '*Ca. Phytoplasma pruni*', their 16S rRNA genes differed in regions that corresponded to three '*Ca. Phytoplasma pruni*'-specific sequences. These nucleotide sequences in the 16S rRNA genes of sequevars NAGYIII α and NAGYIII β are 5'-CACATTAGTTAGTTGGCAGGGTAAAGGCCTACC-3', 5'-ATGGAGGTTATCAGGAAAACAGGTGGTGC-3', and 5'-CTTGTGCGTTAATTGCCAGCATGTAAT-3'. The base positions by which these regions differ from the corresponding '*Ca. Phytoplasma pruni*'-specific sequences are underlined, and a recognition site for the restriction endonuclease *MseI* that distinguishes both sequevars from '*Ca. Phytoplasma pruni*' is in italics. Based on these findings, under criteria specified in the original description of '*Ca. Phytoplasma pruni*' (Davis et al. 2013) and consistent with earlier guidelines for recognizing '*Ca. Phytoplasma*' (IRPCM 2004), sequevars NAGYIII α and NAGYIII β should not be termed strains of '*Ca. Phytoplasma pruni*' but should be referred to as '*Ca. Phytoplasma pruni*'-related strains.

***secY* gene SNP lineages among NAGYIII phytoplasmas.** We next found evidence that the two novel sequevars NAGYIII α and NAGYIII β are also distinguishable from each other and from '*Ca. Phytoplasma pruni*' by lineage-specific divergence in the *secY* gene. In nested PCR, primer pair SecYF1(III)/SecYR1(III) primed amplification of a 1.7-kb DNA fragment that included the partial sequence (3' end) of the *L15 rp* gene (*rpl15*), the complete *secY* gene, and partial sequence (5' end) of the *map* gene from sequevars NAGYIII α and NAGYIII β . Nucleotide sequence alignment revealed that the *secY* genes in all NAGYIII α strains were identical; the *secY* genes in all NAGYIII β strains were also identical, with the exception of a single base difference in strain TW518. The NAGYIII α and NAGYIII β strains shared 99.6% *secY* nucleotide sequence identity but both sequevars shared just 98.7% *secY* nucleotide sequence

Fig. 5. Continued from previous page.

identity with '*Ca. Phytoplasma pruni*'. The alignment of the *secY* nucleotide sequences revealed SNPs at four base positions (Fig. 3; data not shown) that further distinguished sequevars NAGYIII α and NAGYIII β . One of the SNPs was located in the *secY* translational start codon.

Results from automated delineation of putative protein coding regions (open reading frames) indicated that the start codon for the *secY* gene from both NAGYIII α and NAGYIII β sequevars was ATG but the deduced amino acid sequences of the encoded sequevar NAGYIII β SecY proteins were two amino acids shorter than the SecY proteins encoded by *secY* genes in sequevar NAGYIII α . We further observed that the ATG start codon initially predicted for sequevar NAGYIII β was preceded by the triplet GTG, beginning 6 bases upstream of the in-frame ATG; moreover, this GTG was preceded by a purine-rich putative ribosome binding site within 7 to 14 bases upstream of the GTG. For example, this sequence region in strain MD6, as in all other strains of sequevar NAGYIII β , is as follows: ATATTGAAGGTGGCATAAGGTGAAAATGGCAAT, where the purine-rich region is underlined and bold, as is the newly annotated GTG start codon, while the in-frame ATG is underlined. The corresponding sequence in the *secY* of strain MO9 and other sequevar NAGYIII α strains is ATATTGAAGGTGGCATAAGATGAAAA TTGTAAT. Based on these observations, we annotate the *secY* gene of strain MD6 and other sequevar NAGYIII β strains with GTG as the translational start codon. Thus, the *secY* genes of the NAGYIII phytoplasma strains contained either ATG or GTG as the translational start codon; the start codon of *secY* genes in the sequevar NAGYIII α lineage was ATG, whereas the start codon of *secY* genes in the

sequevar NAGYIII β lineage was GTG. All sequevar NAGYIII α *secY* genes contained an ATG start codon and an in-frame ATT triplet at bases 7 to 9, whereas, all sequevar NAGYIII β *secY* genes contained GTG as the start codon and an ATG triplet at bases 7 to 9. By contrast, the *secY* genes of all studied strains of '*Ca. Phytoplasma pruni*' had ATG as the translational start codon and contained an in-frame ATG triplet at bases 7 to 9.

Deduced amino acid sequences and structures of SecY proteins.

The SecY-deduced amino acid sequences encoded by sequevars NAGYIII α and NAGYIII β differed from that of '*Ca. Phytoplasma pruni*' SecY (GenBank number AEY80160) by seven residues (at positions 3, 90, 167, 193, 197, 264, and 339) and six residues (at positions 4, 90, 167, 197, 264, and 339), respectively, and differed from each other by three amino acid residues (positions 3, I [isoleucine]→M [methionine]; 4, V [valine]→A [alanine]; and 193, I→V). Because differences in mature SecY amino acid sequences may have functional implications for phytoplasmas (Wei et al. 2011), we compared the predicted 3D SecY structures of sequevars NAGYIII α and NAGYIII β SecY with that of '*Ca. Phytoplasma pruni*' (strain PX11CT1^R). The predicted 3D structures (Fig. 4) indicated that most of the altered amino acid residues in the NAGYIII α and NAGYIII β SecY proteins were located at the surface of the folded protein. The surface-exposed regions of the NAGYIII α and NAGYIII β SecY proteins differed in 3D structure from the SecY of '*Ca. Phytoplasma pruni*'.

SNPs in the *rp* gene locus of NAGYIII phytoplasmas strains.

Sequevars NAGYIII α and NAGYIII β were distinguished from '*Ca. Phytoplasma pruni*' by SNPs in the *rp* gene locus (Fig. 3; data not shown). Some base changes resulted in altered amino acid

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composition of encoded S3 proteins. Amino acid substitutions distinguishing the two sequevars from '*Ca. Phytoplasma pruni*' included the residues T (threonine) and Y (tyrosine) in '*Ca. Phytoplasma pruni*' versus K (lysine) and S (serine) in all NAGYIII α and NAGYIII β strains examined, at positions 96 and 247, respectively in the S3 protein. An amino acid substitution (W [tryptophan] versus L [leucine] at position 205 in the S3 rp protein) distinguished between the sequevars, consistent with delineation of the two sequevar lineages (this study) on the basis of SNPs in the 16S rRNA and *secY* gene loci.

Phylogenetic clustering of NAGYIII phytoplasma strains. Relationships inferred from phylogenetic analyses were consistent with the concept that the NAGYIII phytoplasma strains are distinct from strains recognized (Davis et al. 2013) within '*Ca. Phytoplasma pruni*' (Fig. 5). Phylogenetic analysis of 16S rRNA genes placed the NAGYIII α and NAGYIII β sequevars in close relationship with '*Ca. Phytoplasma pruni*', consistent with high gene sequence similarity noted above, and indicated that the NAGYIII strains and '*Ca. Phytoplasma pruni*' shared a common ancestor (Fig. 5A). In the expanded view of the region containing the two NAGYIII sequevars and '*Ca. Phytoplasma pruni*', none of the NAGYIII strains clustered with '*Ca. Phytoplasma pruni*' (Fig. 5B). Phylogenetic analysis based on amino acid sequence of SecY proteins also placed the NAGYIII α and NAGYIII β sequevars in close relationship with '*Ca. Phytoplasma pruni*' (Fig. 5C), and the two NAGYIII sequevars each formed a branch distinct from that of '*Ca. Phytoplasma pruni*' (Fig. 5D).

Discussion

In this communication, we present evidence of phytoplasma infection in diverse wine grape cultivars exhibiting symptoms of NAGY across six states. The occurrence of NAGY in diverse red and white wine cultivars points to broad susceptibility of cultivated *V. vinifera* to NAGY phytoplasma infection, and the distribution of the disease from New York south to Pennsylvania, Maryland, and Virginia and west to Ohio and Missouri suggests that it may occur over a much wider area than presently known.

Because symptoms cannot distinguish NAGY from GY diseases that occur in other regions of the world, correct diagnosis required

genotyping of the associated phytoplasma strains. The findings delineated multilocus molecular markers of NAGY group 16SrIII strains in Virginia; provided the first definitive identification of NAGY phytoplasma strains in New York State; and provided the first documented evidence of NAGY in cultivated grapevines in Pennsylvania, Missouri, Ohio, and Maryland. Because NAGY phytoplasma strains studied here have been classified in group 16SrIII (peach X-disease phytoplasma group), the implication might be drawn that NAGY is caused by X-disease phytoplasma; however, data herein indicate otherwise. Our study provides evidence that the NAGY group 16SrIII phytoplasma strains differ from strains of '*Ca. Phytoplasma pruni*' *sensu* Davis et al. (2013). First, the NAGYIII phytoplasma strains belong to variant subgroup 16SrIII-A*, whereas '*Ca. Phytoplasma pruni*' is classified in subgroup 16SrIII-A. Furthermore, the NAGYIII phytoplasma strains represent two novel sequevars, both of which are distinct from '*Ca. Phytoplasma pruni*'. 16S rDNA base differences distinguishing the two sequevars (NAGYIII α and NAGYIII β) from '*Ca. Phytoplasma pruni*' include one that accounts for the presence of a recognition site for *Mse*I that is not present in the 16S rDNA of '*Ca. Phytoplasma pruni*'. Significantly, three 16S rDNA base differences occurred in regions corresponding to '*Ca. Phytoplasma pruni*'-specific sequences designated in the original description of this species (Davis et al. 2013). In accordance with current guidelines for recognizing and naming '*Ca. Phytoplasma*' spp., which state that, "Strains in which even minimal differences in the 16S rRNA gene sequences from the reference strain are detected do not 'belong' to the *Candidatus* species, but are 'related' to it" (IRPCM 2004), the NAGYIII strains should not be termed strains of '*Ca. Phytoplasma pruni*'. Instead, the novel NAGYIII sequevars are appropriately termed '*Ca. Phytoplasma pruni*'-related strains. This consequence implies that NAGYIII phytoplasma strains may differ from '*Ca. Phytoplasma pruni*' in important yet undescribed biological properties that could illuminate the taxonomic level appropriate for the NAGYIII strains.

As considered previously (Davis and Sinclair 1998), a small difference in 16S rRNA genes can possibly signal differences in other genomic regions and point to lineage divergence that yields distinct gene pools. Here, a single 16S rRNA gene SNP (SNP790T/C) was

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predictive of correlative SNPs in *secY* and *rp* genes of the NAGYIII phytoplasma strains. For example, sequevar NAGYIII α strains utilize the common ATG start codon for the *secY* gene, whereas sequevar NAGYIII β strains utilize the alternative start codon GTG. Further, 3D modeling of the encoded SecY proteins indicated that the two NAGYIII sequevars also differed from one another and from 'Ca. *Phytoplasma pruni*' in the identities of SecY amino acids near or exposed at the surface of the phytoplasma membrane in the assembled Sec secretion system. Wei et al. (2011) suggested that differences in amino acid sequence and deduced structure of SecY proteins between phytoplasma strains may have functional implications that are correlated with differences in biology. Thus, differences among NAGYIII sequevars and 'Ca. *Phytoplasma pruni*' observed in putatively surface-exposed regions of the SecY subunit may differentially impact phytoplasma–host interactions.

Although our observations indicate that different phytoplasma genotypes are associated with NAGYIII and *Prunus* X-disease, it is feasible that some insect vectors of X-disease phytoplasmas may transmit NAGYIII phytoplasma strains. Beanland et al. (2006) reported spatial and temporal distribution of possible leafhopper vectors of NAGY phytoplasmas in Virginia. Some belonged to species previously reported as X-disease vectors and exhibited a seasonal woodland-to-vineyard trend in migration throughout the growing season, consistent with the pattern of NAGY disease development within vineyards. The molecular markers documented herein should aid work aimed at elucidating the identities of the NAGY insect vectors. It will be interesting to learn whether the two NAGYIII sequevars differ from one another in specific insect vectorship or plant host range.

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