ONION IS HOST FOR TWO PHYTOPLASMA LINEAGES, SUBGROUPS 16SrI-A AND 16SrI-(B/L)L, IN LITHUANIA: A *Hinf*I SITE REVEALED A SNP MARKING DIVERGENT BRANCHES OF EVOLUTION

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SUMMARY

Onions (*Allium cepa* L.) grown for seed production in the Kaunas region of Lithuania exhibited mild yellowing of leaves and stems, stunting, phyllody, and proliferation of flowers. RFLP and sequence analysis of PCR-amplified 16S rRNA, ribosomal protein (rp), and *secY* genes revealed the presence of phytoplasmas belonging to subgroups 16SrI-A (rpI-A) and 16SrI-L (rpI-B, secYI-B). The results indicated that phytoplasma strains in subgroup16SrI-A (rpI-A) have potential to damage onions in Europe, as well as in North America, and for the first time demonstrated onion as a host for subgroup 16SrI-L. Subgroup 16SrI-L was distinguished based on a composite *Hinf*I RFLP pattern of 16S rDNA that revealed the presence of two sequence heterogeneous rRNA operons in this subgroup, thus showing the significance of composite RFLP patterns for phytoplasma identification and classification. A single nucleotide polymorphism (SNP) in the first base of one *Hinf*I recognition site (5'-GANTC-3') marked the divergence of major phylogenetic branches, supporting the concept that SNPs provide powerful molecular markers of phytoplasma evolution.

INTRODUCTION

Phytoplasmas are unique prokaryotic microbes that lack cell walls and possess highly reduced AT-rich genomes, having discarded genes encoding diverse metabolic pathways in an ongoing process of evolutionary adaptation to intracellular parasitism (Davis *et al*., 2005; Oshima *et al.*, 2004). As transkingdom parasites and pathogens of insects and plants, phytoplasmas inhabit specialized cells in plant phloem tissue and are transmitted from plant to plant by insect vectors, mainly leafhoppers and psyllids (Bertaccini, 2007; Lee *et al*., 2000; Hogenhout *et al*., 2008; Weintraub and Beanland, 2006). Infected plants exhibit symptoms such as stunting, shoot proliferation, and phyllody that may be due in part to imbalance of plant growth regulators (Hoshi *et al*., 2009). Inability to isolate and cultivate phytoplasmas in cell-free media has hindered progress in ascertaining characters used in the identification and classification of cultivable bacteria. Thus, molecular analysis of ribosomal (r) RNA gene sequences forms the major basis for comprehensive phytoplasma classification (Lee *et al*., 1998; Wei *et al*., 2008).

Despite development of powerful molecular techniques for phytoplasma classification (Lee *et al*., 1998, 2006; Martini *et al*., 2007; Wei *et al*., 2008; Zhao *et al*., 2009), challenges still exist. One challenging area is related to the presence in a phytoplasma genome of two rRNA operons, sometimes sequence heterogeneous. Intragenomic rRNA heterogeneity observed in some bacteria including phytoplasmas can pose significant difficulties in using rRNA genes as tools for classification (Boucher *et al*., 2004; Davis *et al*., 2003). For example, analysis of a phytoplasma strain's "sequence heterogeneous rRNA operons in mutual isolation could result in erroneous assignment of the same phytoplasma to two different 16S rRNA subgroups, or putative taxa, in current classification schemes that are based on RFLP patterns" (Davis *et al*., 2003).

Studies on the diversity of phytoplasmas infecting plant species around the world are based principally upon 16S rRNA gene sequence analysis, but rRNA interoperon sequence heterogeneity has the potential to lead to overestimations of phytoplasma diversity, as noted for other bacteria (Acinas *et al*., 2004). Where phytoplasmal rRNA intragenomic sequence heterogeneity lies within recognition sites for restriction endonucleases, PCR products can yield obvious composite RFLP patterns that signal the existence of a unique phytoplasma subgroup and distinct lineage (Davis *et al*., 2003; Jomantiene *et al*., 2002; Lee *et al*., 1998; Marcone *et al*., 2000).

In summer 2008, we noticed symptoms characteristic of phytoplasmal disease in onions grown for seed production in the Kaunas region of Lithuania. Nucleotide sequence and RFLP analysis of 16S rDNA revealed that the onions were infected by two different types of group

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16SrI, '*Candidatus* Phytoplasma asteris'-related, strains. One yielded a type of *Hinf*I RFLP pattern that was previously interpreted as a composite of rDNAs from two sequence heterogeneous rRNA operons in a subgroup 16SrI-L strain (Marcone *et al*., 2000). The nucleotide sequence was previously determined for rDNA from just one such operon (GenBank No. AY180957) of subgroup 16SrI-L strain AV2192.

It was suggested that, based on similarities in *tuf* and rDNA sequences, subgroup 16SrI-L should not be distinguished from subgroup 16SrI-B (Marcone *et al*., 2000). In this communication we report subgroups 16SrI-A and 16SrI-L in diseased onion plants, analyze nucleotide sequences from both rRNA operons of subgroup 16SrI-L, unveil a 16S rDNA SNP that distinguishes major branches of phytoplasma evolution, analyze subgroup 16SrI-L ribosomal protein and *secY* gene sequences, and assert that phytoplasma strains classified in subgroup 16SrI-L represent a distinct evolutionary lineage.

MATERIALS AND METHODS

Plant samples, PCR, RFLP and sequence analysis, and phytoplasma classification. Samples of symptomatic onion tissues, including flowers and leaves**,** were collected in the Kaunas region. DNA was extracted from the collected tissues using a genomic DNA purification kit (Fermentas, Lithuania) and used as template in PCR assays for amplification of 16S ribosomal (r) RNA, ribosomal protein, and *secY* gene sequences. In nested PCRs for amplification of rRNA gene sequences, the first reaction was primed by primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995). Products obtained in the first PCR were diluted 1:50 with sterile water and used as template in the second (nested) PCR primed by primer pair R16F2n/R16R2 (F2n/R2) (Gundersen and Lee, 1996). Ribosomal protein (rp) gene sequences were amplified using primer pair rpF1/rpR1 (Lee *et al*., 1998). Amplification of *secY* gene sequences was primed by primer pair AYsecYF1/AYsecYR1 (Lee *et al*., 2006).

Amplifications were conducted under the same conditions (94°C for 1 min, 55°C for 2 min, 72°C for 3 min) for 35 cycles (first denaturation was at 94°C for 3 min, and extension in final cycle was at 72°C for 10 min) in Fermentas PCR buffer with $(NH_4)SO_4$, 0.25 mM dNTPs, 0.4 µM of each primer, and 1 unit of recombinant Taq polymerase per 50 µl of reaction mixture. Annealing temperature for amplification of rp and *secY* gene sequences was 50°C (Lee *et al*., 1998, 2006). Products of the nested PCR (1.2 kbp F2nR2 fragment) were subjected to enzymatic RFLP analysis using restriction endonucleases *Alu*I, *Bfa*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*II, *Kpn*I, *Mse*I, and *Rsa*I (Fermentas, Lithuania). Digested products were analyzed using electrophoresis through 5% acrylamide gel for rDNA and for ribosomal protein gene. DNA bands were stained with ethidium bromide and visualized using a UV transilluminator. Phytoplasmas were classified in groups and subgroups through comparisons of RFLP patterns with patterns previously published, in accordance with the classification schemes of Lee *et al.* (1998, 2004) and Wei *et al.* (2008).

Cloning of PCR products and analysis of cloned DNA sequences. PCR-amplified phytoplasmal rDNA and rp gene sequences were cloned in *Escherichia coli* using T/A Instal cloning kit (Fermentas, Lithuania) according to manufacturer's instructions. *SecY* gene sequences were cloned in *E. coli* using TOPO TA Cloning Kit (Invitrogen, USA). Recombinant plasmids were screened for the presence of cloned rRNA, rp, and *secY* gene sequences by amplification of DNA in PCRs primed by F2n/R2, rpF1/rpR1, and AYsecYF1/AYsecYR1, respectively, followed by RFLP analysis of the amplified DNA. The RFLP profiles were compared with those obtained from analysis of uncloned PCR products. The nucleotide sequences of cloned rRNA, rp, and *secY* genes were determined by automated sequencing of both strands to achieve a minimum of 3-fold coverage per base position, and the sequences were deposited in the GenBank database. Nucleotide sequence alignments were generated and sequence similarities evaluated using LaserGene software MegAlign program (DNASTAR, USA). The nucleotide sequences were subjected to virtual RFLP analysis, and phytoplasma classification was assisted using *i*PhyClassifier (Wei *et al*., 2008; Zhao *et al*., 2009). For phylogenetic analyses, nucleotide sequences were aligned using Clustal X 1.63 b (Thompson *et al*., 1997), and trees were viewed using TreeViewPPC (Page, 1996).

RESULTS AND DISCUSSION

Detection and identification of phytoplasmas in diseased onions. About 5% of onion plants in the surveyed fields exhibited mild yellowing of leaves and stems, stunting, proliferation and phyllody of flowers (Fig .1).

DNA was amplified from eight symptomatic plants, in PCR primed by primer pairs P1/P7 and F2n/R2, showing that the symptomatic onion plants were infected by phytoplasma(s). Preliminary comparison of the RFLP patterns of amplified 16S rDNA with the RFLP patterns in the phytoplasma classification scheme of Lee *et al.* (1998) revealed that the onions were infected by at least two phytoplasma strain types, designated OnP1 (onion proliferation 1) and OnP2 (onion proliferation 2).

16S rRNA group and subgroup classification of phytoplasma strain OnP1. Three plants infected by strain

type OnP1 yielded the same collective RFLP patterns. Since the strain OnP1 16S rDNA RFLP patterns were most similar to those of 16S rDNA from '*Candidatus* Phytoplasma asteris'-related strains classified in group 16SrI subgroup A (Fig. 2A), strain OnP1 was assigned to subgroup 16SrI-A. Onion damaging phytoplasmas classified in subgroup 16SrI-A have previously been reported from Canada and Texas (USA) (Khadhair *et al*., 2002; Lee *et al*., 2003). This is, therefore, the first record of subgroup 16SrI-A in onions in Europe.

16S rRNA group and subgroup classification of phytoplasma strain OnP2. Five plants infected by strain type OnP2 yielded the same collective RFLP patterns. The OnP2 16S rDNA collective RFLP patterns were most similar to those characteristic of phytoplasmas classified in group 16SrI, subgroup B (Fig. 2B and data not shown), but the *Hinf*I RFLP profile differed from nearly all known *Hinf*I profiles for group 16SrI strains. The only similar 16S rDNA *Hinf*I RFLP profile of a group 16SrI phytoplasma was that for subgroup 16SrI-L (Marcone *et al*., 2000), which appeared to be a composite containing subgroup 16SrI-L signature DNA bands that represented two sequence heterogeneous rRNA operons. On the basis of the similar RFLP profiles of 16S rDNA, we classified the OnP2 phytoplasma in group 16SrI subgroup L.

Fig. 1. Diseased onion plant exhibiting symptoms of mild yellowing of leaves, and proliferation and phyllody of flowers.

Some doubt had previously been cast on the validity of recognizing subgroup 16SrI-L because of its close relatedness to subgroup 16SrI-B (Marcone *et al*., 2000). However, the *Hinf*I constant composite (CC) 16S rDNA RFLP pattern characteristic of subgroup 16SrI-L has been observed from phytoplasmas in different individual plants in a field, in diverse plant species, and in plants from different geographical regions (Table 1),

Table 1. Phytoplasma strains classified in subgroup 16SrI-L on the basis of a unique, composite *Hinf*I RFLP pattern of 16S rDNA.*

Phytoplasma	Strain	Origin	Host	Reference
Onion proliferation 2	OnP2	Lithuania	*Allium cepa*	This study
Hyacinth virescence	HyacVir	Lithuania	*Hyacinthus orientalis*	Alminaite *et al.* (2001)
Aster yellows	AV2192	Germany	*Callistephus chinensis*	Marcone *et al.* (2000)
Aster yellows	AV2226	Germany	*Callistephus chinensis*	Marcone *et al.* (2000)
Aster yellows	AV976	Germany	*Callistephus chinensis*	Marcone *et al.* (2000)
Bermudagrass white leaf	AYBG (BGWL)	Thailand	*Cynodon dactylon*	Marcone *et al.* (2000)
Aster yellows	PRIVA	Germany	*Primula* sp.	Marcone *et al.* (2000)
Rape phyllody	RapPh	Lithuania	*Brassica napus*	Valiunas (2003)
Gladiolus yellows	GlY	Lithuania	*Gladiolus* sp.	Valiunas (2003)
Oat yellows	OatY	Lithuania	*Avena sativa*	Urbanaviciene *et al.* (2007)
Ryegrass yellows	RgY	Lithuania	*Lolium multiflorum*	Urbanaviciene *et al.* (2007)
Grosheimia phytoplasma	NA**	Lithuania	*Grosheimia macrocephala*	Samuitiene et al. (2007)
Lunaria phytoplasma	NA	Lithuania	*Lunaria annua*	Samuitiene *et al.* (2007)
Armeria phytoplasma	NA	Lithuania	*Armeria alliaceae*	Samuitiene *et al.* (2007)
Aquilegia phytoplasma	NA	Lithuania	*Aquilegia vulgaris*	Samuitiene *et al.* (2007)
Aconitum virescence	AconVir	Lithuania	*Aconitum napellus*	Samuitiene *et al.* (2007)
Thalictrum proliferation	ThPr	Lithuania	*Thalictrum speciosissimum*	Samuitiene *et al.* (2007)
Veronica phyllody	VerPh	Lithuania	*Veronica teucrium*	Samuitiene *et al.* (2007)

*, strain designation 16SrI-L was modified to 16SrI-(B/L)L in this study. **NA, not assigned.

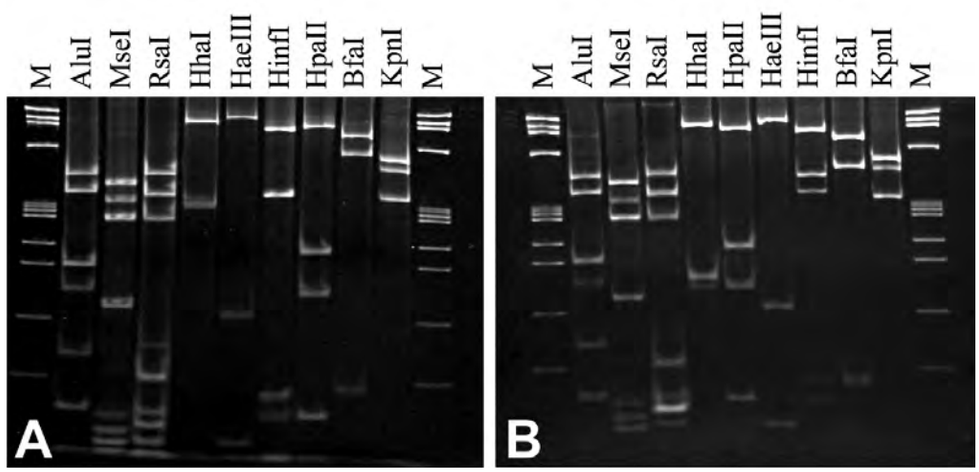

Fig. 2. RFLP analysis of 16S rDNA amplified from phytoplasma strain OnP1 (A) and OnP2 (B) in nested PCR primed by R16F2n/R16R2. M, size marker ΦX174 RFI DNA *Hae*III digest; fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

supporting the notion that subgroup 16SrI-L represents a distinct lineage. A possible biological distinction between subgroup 16SrI-L and 16SrI-B strains may be given by the list of host plants for 16SrI-L strains, on which there are no woody plants, whereas 16SrI-B strains infect both woody and herbaceous hosts. Furthermore, while subgroup I-B strains appear common in North America, subgroup I-L has not been found there (Table 1) (Jomantiene *et al*., unpublished information; Lee *et al*., 2003, 2004; Marcone *et al*., 2000; Valiunas *et al*., 2003).

Nucleotide sequence analysis of rDNA. Phytoplasmal rDNA amplified from strain OnP1 in P1/P7-primed PCR was cloned in *E. coli*, sequenced; and the sequence was deposited in GenBank under accession No. GU223210. Virtual RFLP analysis of the cloned rDNA confirmed that strain OnP1 was a member of subgroup 16SrI-A.

Previously, the nucleotide sequence of rDNA from one of the two sequence heterogeneous rRNA operons of subgroup 16SrI-L strain AV2192 had been determined (Lee *et al*., 2003). Our *i*PhyClassifier analysis of that sequence (GenBank accession No. AY180957) yielded collective RFLP patterns that did not match subgroup 16SrI-B (data not shown), although this sequence contained an undetermined base (base 1259 in AY180957) in the position corresponding to the first

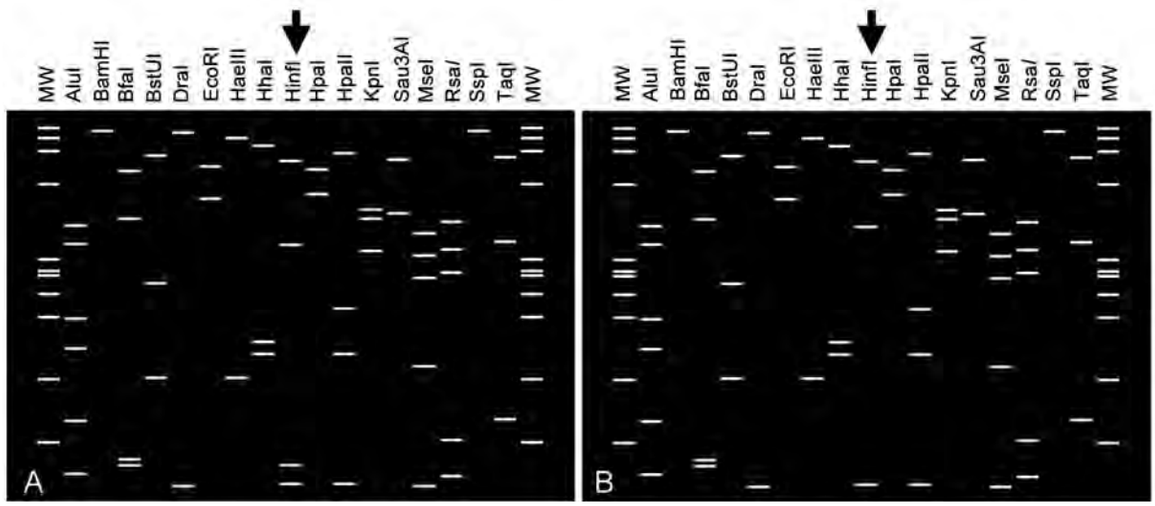

Fig. 3. Computer-simulated, virtual RFLP patterns derived from *in silico* digestions of 1.2 kb 16S rDNA based on recognition sites for 17 restriction endonucleases. Patterns from phytoplasma strain OnP2 operon rrnA (A) and operon rrnB (B). Operon rrnA exhibits a collective RFLP pattern (pattern type I-B) identical to those exhibited by the rDNAs of strains classified in subgroup 16SrI-B. Operon rrnB exhibits a unique collective RFLP pattern (pattern type I-L). Restriction fragments were resolved by *in silico* electrophoresis through 3% agarose gel. MW, ΦX174 DNA-*Hae*III digestion; fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

base of a *Hinf*I site (GANTC) that distinguished subgroup 16SrI-L. Classification based on this sequence alone could result in misidentification of subgroup 16SrI-L strain AV2192 as member of a previously undescribed subgroup.

In the present work, phytoplasmal rDNAs amplified in P1/P7-primed PCRs from strain OnP2 were cloned in *E. coli* and sequenced. Analysis of the cloned rDNAs revealed two *Hinf*I RFLP pattern types, consistent with their representing two different rRNA operons, rrnA

Table 2. 16S rRNA gene sequences from '*Candidatus* Phytoplasma'-related species used in this study.

'*Candidatus* Phytoplasma' species	16Sr group	GenBank accession No.
'*Ca*. Phytoplasma asteris'	I	M30790
'*Ca*. Phytoplasma aurantifolia'	II	U15442
'*Ca*. Phytoplasma australasia (australasiae)'	II	Y10097
'*Ca*. Phytoplasma pruni'†*	III	L04682
'*Ca*. Phytoplasma palmae'‡*	IV	U18747
'*Ca*. Phytoplasma cocostanzaniae'‡*	Unc.	X80117
'*Ca*. Phytoplasma ulmi'	V	AY197655
'*Ca*. Phytoplasma ziziphi'	V	AB052876
'*Ca*. Phytoplasma vitis'†*	V	AF176319
'*Ca*. Phytoplasma trifolii'	VI	AY390261
'*Ca*. Phytoplasma fraxini'	VII	AF092209
'*Ca*. Phytoplasma luffae'†*	VIII	AF086621
'*Ca*. Phytoplasma phoenicium'	IX	AF515636
'*Ca*. Phytoplasma mali'	X	AJ542541
'*Ca*. Phytoplasma pyri'	X	AJ542543
'*Ca*. Phytoplasma prunorum'	X	AJ542544
'*Ca*. Phytoplasma spartii'	X	X92869
'*Ca*. Phytoplasma oryzae'	XI	AB052873
'*Ca*. Phytoplasma australiense'	XII	L76865
'*Ca*. Phytoplasma japonicum'	XII	AB010425
'*Ca*. Phytoplasma fragariae'	XII	DQ086423
'*Ca*. Phytoplasma solani'†*	XII	AF248959
Mexican periwinkle virescence phytoplasma**	XIII	AF248960
Chinaberry yellows phytoplasma**	Unc.	AF495882
'*Ca*. Phytoplasma cynodontis'	XIV	AJ550984
'*Ca*. Phytoplasma brasiliense'	XV	AF147708
'*Ca*. Phytoplasma graminis'	XVI	AY725228
'*Ca*. Phytoplasma caricae'	XVII	AY725234
'*Ca*. Phytoplasma americanum'	XVIII	DQ174122
'*Ca*. Phytoplasma castaneae'	XIX	AB054986
'*Ca*. Phytoplasma rhamni'	XX	X76431
'*Ca*. Phytoplasma pini'	XXI	AJ632155
'*Ca*. Phytoplasma cocosnigeriae'‡*	XXII	Y14175
Buckland Valley grapevine yellows phytoplasma**	XXIII	AY083605
Sorghum bunchy shoot phytoplasma**	XXIV	AF509322
Weeping tea witches'-broom phytoplasma**	XXV	AF521672
Sugarcane phytoplasma D3T1**	XXVI	AJ539179
Sugarcane phytoplasma D3T2**	XXVII	AJ539180
Derbid phytoplasma**	XXVIII	AY744945
'*Ca*. Phytoplasma omanense'	XXIX	EF666051
'*Ca*. Phytoplasma tamaricis'	XXX	FJ432664
'*Ca*. Phytoplasma lycopersici'	Unc.	EF199549
'*Ca*. Phytoplasma allocasuarinae'	Unc.	AY135523

†Name proposed by the IRPCM Phytoplasma Working Team at the X International Congress of the International Organization of Mycoplasmology, Bordeaux, 1994.
‡ Name proposed by the IRPCM Phytoplasma/Spiroplasma Working Team at the XIV International Congress of the International Organization of Mycoplasmology, Vienna, 2002.
*According to Rule 28b of the Bacteriological Code, this is an incidental citation and does not constitute prior citation.
**No name suggested.
Unc., unclassified.

and rrnB. The 16S rDNA from rrnA (GU223208) had a *Hinf*I site that was missing in the 16S rDNA from rrnB, (GU223209) (Fig. 3). Conceptual superimposition of the two *Hinf*I RFLP pattern types resulted in a composite RFLP pattern identical to that seen in actual RFLP analysis of PCR products (Fig. 2 and 3). Thus, an observation of composite RFLP patterns from analysis of uncloned, PCR-amplified phytoplasmal rDNA should prompt work to separate sequence heterogeneous rDNAs by cloning and to sequence the separated DNAs.

The *Hinf*I RFLP pattern distinguished strain OnP2 operon rrnB from 16S rDNAs of phytoplasmas representing all group 16SrI subgroups in the *i*PhyClassifier database (data not shown). Not surprisingly, the OnP2 rrnA 16S rDNA sequence yielded a virtual *Hinf*I RFLP pattern that was characteristic of subgroup 16SrI-B (Fig. 3). In accordance with recent advances in phytoplasma classification, we denote the RFLP pattern types of the subgroup 16SrI-L operons rrnA and rrnB as 16SrI-(B)L (pattern type I-B) and 16SrI-(L)L (pattern type I-L), respectively. The capital letter in parentheses describes the RFLP pattern type of 16S rDNA; the last letter describes the subgroup lineage of the phytoplasma studied. In accordance with Zhao *et al.* (2009), we modify the subgroup 16SrI-L designation to subgroup 16SrI-(B/L)L.

Interestingly, the 16SrI-(L)L *Hinf*I RFLP pattern type was also observed in GenBank records of 16S rDNAs from representatives of groups 16SrII, III, V, VI, VII, VIII, X, XIII, XIV, XV, XX, XXIII, and XXIX, whereas, the 16SrI-(B)L pattern type was observed in 16S rDNAs from representatives of groups 16SrI, XII,

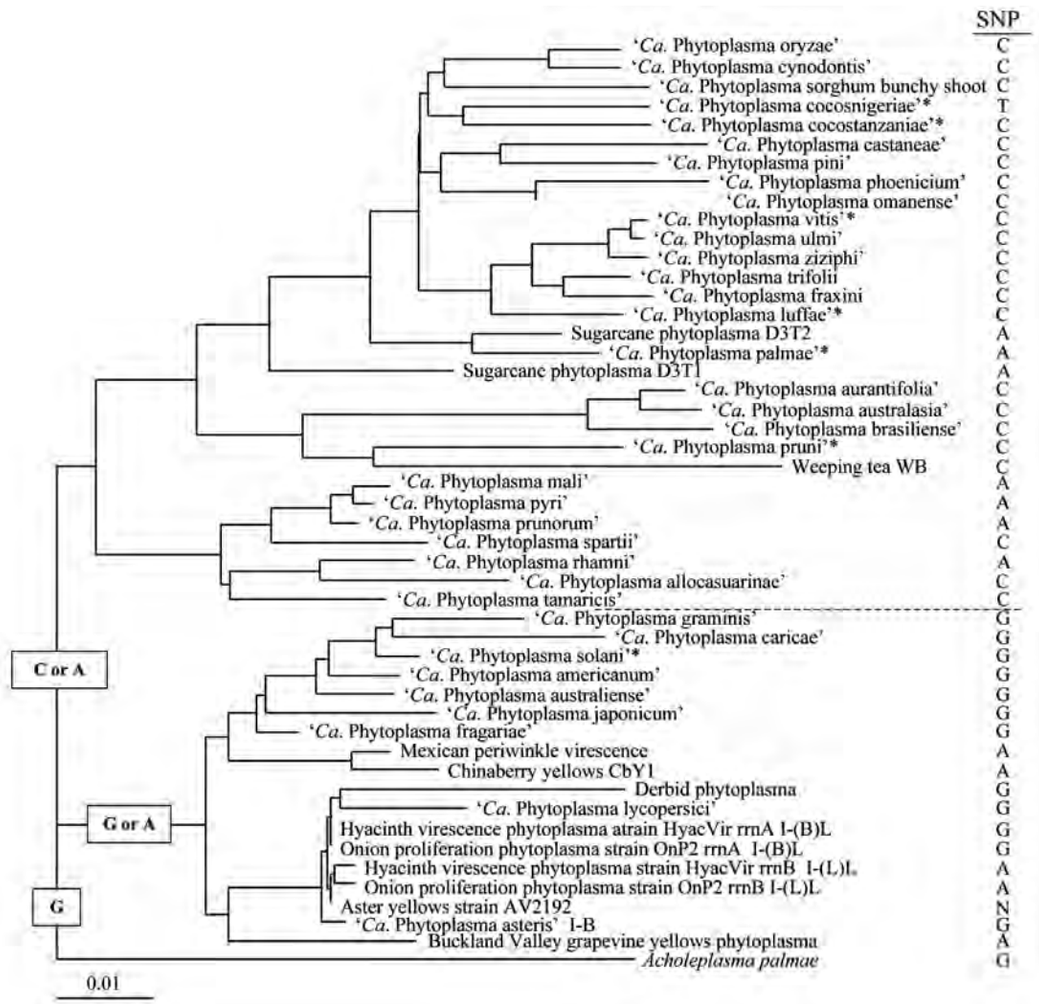

Fig. 4. Phylogenetic tree constructed by the Neighbor-Joining method of 16S rRNA gene sequences from 46 phytoplasmas, including named and putative '*Candidatus* Phytoplasma' species, and *Acholeplasma palmae*, employing *A. palmae* as the outgroup. Strains HyacVir, OnP2, and AV2192 are '*Ca*. Phytoplasma asteris'-related strains; 16Sr group and subgroup RFLP pattern types are given for each of these strains' respective 16S rDNA sequences. *This is an incidental citation and does not constitute prior citation, according to Rule 28b of the Bacteriological Code. SNP, single nucleotide polymorphism in the position corresponding to the first base of a *Hinf*I recognition site in 16S rDNA that distinguishes 16S rDNA RFLP pattern type I-L from pattern type I-B; the identity of the base in that position is given for each sequence. The SNP bases found in major phylogenetic branches are given in boxes.

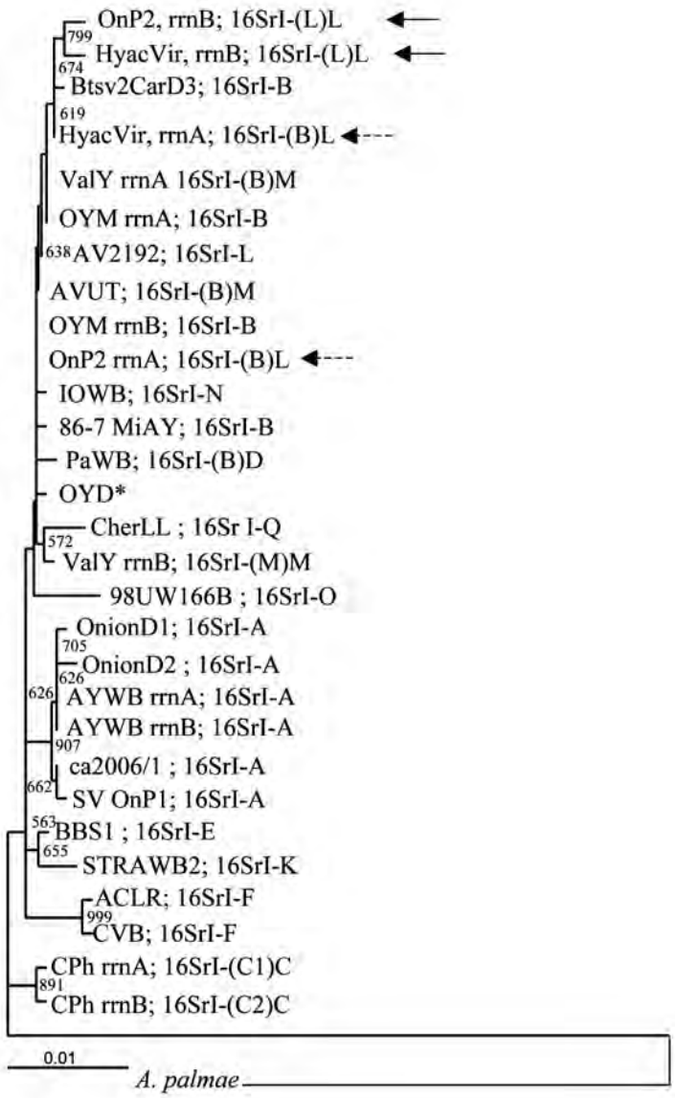

Fig. 5. Phylogenetic analysis of 16S rRNA gene sequences from 23 phytoplasma strains classified in diverse subgroups of group 16SrI ('*Candidatus* Phytoplasma asteris'-related strains), and *Acholeplasma palmae*, employing *A. palmae* as the outgroup. Phytoplasma classification is according to Lee *et al.* (1998), Marcone *et al.* (2000), and Samuitiene *et al.* (2007). Strains OnP1 and OnP2 were classified in the present study. Arrows indicate 16S rRNA gene sequences from strains, except AV2192, that are classified in subgroup 16SrI-L. Broken arrows indicate rRNA operon sequences of subgroup 16SrI-L strains that exhibited virtual RFLP patterns characteristic of subgroup 16SrI-B; rrnA and rrnB indicate the operons from which the analyzed 16S rDNA sequences were derived. For nucleotide sequences derived from intragenomic sequence-heterogeneous rRNA operons, the RFLP pattern type of the given nucleotide sequence is noted in parentheses. Subgroup classification of each strain is given as a letter not in parentheses. For strain AV2192 classified in subgroup 16SrI-L, nucleotide sequence is available for one of its two sequence-heterogeneous operons; classification is according to Marcone *et al.* (2000). Numbers at nodes are bootstrap (confidence) >500 values. *, unclassified. Phytoplasma strain names and GenBank accession numbers for rDNA sequences used in constructing the tree are given in Table 1.

XVI, XVII, XVIII, and XXVIII, according to analyses using *i*PhyClassifier. Phytoplasmas representing the two sets of 16Sr groups fall into two different phylogenetic subclades (Fig. 4 and Table 2), prompting the hypothesis that the base change accounting for the two pattern types occurred at or before divergence of these subclades.

Ribosomal protein (rp) and *secY* gene sequences. For further characterization of the phytoplasma strains from onion, we examined rp and *secY* gene sequences. Results from amplification, cloning, and virtual RFLP and sequence analysis of rp gene sequences (GU228515) showed that phytoplasma OnP1 belongs to subgroup rpI-A. The OnP1 *secY* gene was not sequenced.

Virtual RFLP analysis of rp gene sequences (GU228514) indicated that strain OnP2 belongs to subgroup rpI-B. The same RFLP pattern is exhibited by comparable rp gene sequences from strains classified in 16S rDNA subgroups 16SrI-B, 16SrI-L, and 16SrI-M (Martini *et al.*, 2007). The rp gene sequences from OnP2 were nearly identical to rp gene sequences from strain AV2192 (accession No. AY183708) and from subgroup 16SrI-B strains previously reported (Lee *et al.*, 2003; Martini *et al.*, 2007). We found that the *secY* gene sequence (GU228516) from phytoplasma strain OnP2 was identical to the *secY* sequences from phytoplasmas belonging to subgroup 16SrI-B, subgroup secY-IB (Lee *et al.*, 2006), and to that from the subgroup 16SrI-L strain AV2192 (AY803167). Thus, based on nucleotide sequence and RFLP similarities in 16S rDNA, rp, and *secY* gene sequences, strain OnP2 and other strains classified in subgroup 16SrI-(B/L)L are closely related to members of subgroup 16SrI-B, in agreement with previous work (Marcone *et al.*, 2000), but subgroup 16SrI-(B/L)L might not be a recently emerged lineage. Does the distribution of I-B and I-L rDNA RFLP pattern types among two phylogenetic subclades indicate the sequence heterogeneous rRNA operons of subgroup 16SrI-(B/L)L as preserved evidence of an early divergence event?

Phylogenetic analysis. On the basis of analysis of 16S rRNA gene sequences, a phylogenetic tree was constructed to show relationships among *Acholeplasma palmae* and 23 phytoplasmas, including strains OnP1 and OnP2 from onions (Fig. 5 and Table 3). The 16S rDNA from strain OnP1 clustered with that from other strains classified in subgroup 16SrI-A. The branching order distinguished 16S rDNAs that exhibited a *Hinf*I RFLP pattern characteristic of *rrn*B of subgroup I-(B/L)L strains from 16S rDNAs of other phytoplasmas belonging to group 16SrI. Moreover, 16S rDNA from operon *rrn*A of the subgroup 16SrI-(B/L)L strains clustered with 16S rDNA of subgroup B strains. These results are consistent with a relatively close relatedness between

Table 3. Phytoplasma strains belonging to group 16SrI and their rRNA gene sequences used in this study.

Phytoplasma	Strain	Origin	16S rDNA Group-subgroup	RFLP pattern of 16S rDNA*	GenBank No. of rRNA gene sequence
Onion proliferation 1	OnP1	Lithuania	16SrI-A	I-A	GU223210
Aster yellows witches' broom	AYWB	Ohio	16SrI-A	I-A (rrnA) I-A (rrnB)	CP000061 CP000061
Aster yellows	OnionD1	Texas	16SrI-A	I-A	AY180948
Aster yellows	OnionD2	Texas	16SrI-A	I-A	AY180931
Carrot phytoplasma	ca2006/1	Serbia	16SrI-A	I-A	EU215424
Blueberry stunt	BBS1	Michigan	16SrI-A	I-A	AY265220
Aster yellows	Btsv2CarD3	Texas	16SrI-B	I-B	AY180945
Onion yellows mild	OY-M	Japan	16SrI-B	I-B (rrnA) I-B (rrnB)	AP006628 AP006628
Oenothera AY phytoplasma	86-7	Michigan	16SrI-B	I-B	M30790
Clover phyllody	CPh	Canada	16SrI-C	I-C1 (rrnA) I-C2 (rrnB)	AF222065 AF222066
Paulownia witches' broom	PaWB	Taiwan	16SrI-D	I-B	AY265206
Aster yellows	ACLR-AY	Germany	16SrI-F	I-F	AY265211
Aster yellows	CVB	Germany	16SrI-F	I-F	AY265212
Strawberry phytoplasma	STRAWB2	Florida	16SrI-K	I-K	U96616
Onion proliferation 2	OnP2	Lithuania	16SrI-L†	I-B (rrnA) I-L (rrnB)	GU223208 GU228209
Hyacinth virescence	HyacVir	Lithuania	16SrI-L†	I-B (rrnA) I-L (rrnB)	AY744071 AY744072
Aster yellows	AV2192	Germany	16SrI-L†	I-B	AY180957
Aster yellows	AVUT	Germany	16SrI-M	I-B	AY265209
Valeriana yellows	ValY	Lithuania	16SrI-M	I-B (rrnA) I-M (rrnB)	AY102274 AY102273
Aster yellows	IOWB	Taiwan	16SrI-N	I-N	AY265205
Soybean purple stem	98UW166B	Wisconsin	16SrI-O	I-O	AF268405
Cherry little leaf	CherLL	Lithuania	16SrI-Q	I-Q	AY034089
Onion yellow dwarf	OYD	South Korea	16SrI-Unc.	Unc.‡	AB292849

†Subgroup 16SrI-L is redesignated as 16SrI-(B/L)L (this paper). ‡Unc., unclassified. *Ribosomal RNA operons rrnA and rrnB are indicated in parentheses.

subgroup 16SrI-(B/L)L and subgroup 16SrI-B, while the phylogenetic position of *rrn*B from two subgroup 16SrI-(B/L)L strains is consistent with the concept that subgroup 16SrI-(B/L)L represents a distinct lineage.

***Hinf*I RFLP patterns revealed a new SNP marker of phylogenetic divergence.** Unexpectedly, two major evolutionary branches were marked by a SNP that corresponded in position to the first base of the *Hinf*I site (5'-GANTC-3') at positions 1259 to 1263 in 16SrI-(B/L)L rrnA (GU223208) (Fig. 4 and Table 2). *Acholeplasma* species in the *A. palmae* and *A. laidlawii* clusters contain G in the corresponding position of 16S rDNA (data not shown), as do nearly all phytoplasma species that clustered on the major phylogenetic branch that also contained subgroup 16SrI-(B/L)L (Fig. 4 and Table 2). The interchange of predominantly C in place of G, marking the other major phytoplasma phylogenetic branch, represents a significant transversion, likely resulting from a rare event, since in other bacteria transversions are relatively infrequent (Lind and Andersson, 2008). Our current findings (this paper), recent study of SNPs in '*Ca.* Phytoplasma solani'-related strains (Quaglino *et al*., 2008), and the emerging use of SNPs in studies of walled bacteria (Watson and Lockwood, 2009; Pandya *et al*., 2009), suggest that SNPs will likely constitute a new generation of molecular markers of phytoplasma evolution.

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