European Journal of Plant Pathology **108:** 507–517, 2002.

New group 16SrIII phytoplasma lineages in Lithuania exhibit rRNA interoperon sequence heterogeneity

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Accepted 3 April 2002

Key words: 16S rDNA, rRNA operon, phytopathogenic mollicutes, PCR

Abstract

Previously undescribed phytoplasmas were detected in diseased plants of dandelion (*Taraxacum officinale*) exhibiting virescence of flowers, thistle (*Cirsium arvense*) exhibiting symptoms of white leaf, and a *Gaillardia* sp. exhibiting symptoms of stunting and phyllody in Lithuania. On the basis of restriction fragment length polymorphism (RFLP) analysis of 16S rDNA amplified in PCR, the dandelion virescence (DanVir), cirsium whiteleaf (CirWL), and gaillardia phyllody (GaiPh) phytoplasmas were classified in phylogenetic group 16SrIII (X-disease phytoplasma group), new subgroups III-P and III-R and subgroup III-B, respectively. RFLP and nucleotide sequence analyses revealed 16S rRNA interoperon sequence heterogeneity in the two rRNA operons, rrnA and rrnB, of both DanVir and CirWL. Results from phylogenetic analysis based on nucleotide sequences of 16S rDNA were consistent with recognition of the two new subgroups as representatives of distinct new lineages within the group 16SrIII phytoplasma subclade. The branching order of rrnA and rrnB sequences in the phylogenetic tree supported this interpretation and indicated recent common ancestry of the two rRNA operons in each of the phytoplasmas exhibiting interoperon heterogeneity.

Abbreviations: PCR – polymerase chain reaction; rDNA – gene for 16S rRNA; RFLP – restriction fragment length polymorphism.

Introduction

Phytoplasmas are unique cell wall-less prokaryotic pathogens that cause numerous diseases of plants worldwide (McCoy et al., 1989). Since it is not possible to isolate and study phytoplasmas in pure culture, molecular methods have been applied to detect them in plants and insect vectors and to construct a system for phytoplasma identification and classification. This approach has led to new understanding of phytoplasma diversity and phylogenetic relationships, and to a provisional taxonomy involving descriptions of '*Candidatus* Phytoplasma species'. Phylogenetic analyses have revealed that phytoplasmas are descended from Gram-positive bacterial ancestors and form a monophyletic clade in the class Mollicutes (Lim and Sears, 1989; Namba et al., 1993; Seemüller et al., 1994; Schneider et al., 1995; Gundersen et al., 1994). On the basis of analyses of 16S rDNA, phytoplasmas have been classified into at least 15 groups and over 38 subgroups (Lee et al., 1998; Montano et al., 2001).

Phytoplasmas belonging to six major 16S rRNA gene groups (groups 16SrI, 16SrIII, 16SrV, 16SrX, 16SrXI, and 16SrXII, respectively), representing at least six putative species, have been reported in Europe (Bertaccini et al., 1993; Bianco et al., 1993; Davis et al., 1993; Daire et al., 1993; Seemüller et al., 1994; Lee et al., 1995,1998). However, in some parts of Europe, including former Soviet Union countries, little is known of the geographical and ecosystem occurrence of phytoplasmas and about the host species that they inhabit.

Molecular investigation of phytoplasmas in the region of the Baltic states began recently, and knowledge concerning the genetic and biological diversity of phytoplasmas in this region is emerging (Jomantiene et al., 2000a–c; Staniulis et al., 2000; Valiunas et al., 2000, 2001a,b). The results of the present study provide new knowledge about group 16SrIII phytoplasmas in the Baltic region, the ecological niches that they occupy, and their phylogenetic relationships with phytoplasmas reported in other geographical regions.

Materials and methods

Plant samples, PCR, RFLP analysis, and phytoplasma classification

Samples of leaf tissue were collected in uncultivated fields of the Kaunas region, Lithuania, from plants of dandelion (*Taraxacum offinale*) exhibiting symptoms of virescence of flowers and thistle (*Cirsium arvense*) exhibiting symptoms of white leaf. Samples of leaf and flower tissue were collected in a private garden in Kursiu Neringa region from plants of a *Gaillardia* sp. exhibiting symptoms of phyllody and stunting. Template DNA was extracted from the tissues using a Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and used in a nested polymerase chain reaction (PCR) for amplification of sequences from phytoplasmal ribosomal (r) RNA operons. In nested PCR, the first reaction was primed by phytoplasma-universal 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). Both amplifications were conducted under the same conditions (94° for 1 min, 55° for 2 min, 72° for 3 min) for 35 cycles (first denaturation was at 94° for 3 min, and extension in final cycle was at 72° for 10 min), in Perkin Elmer PCR buffer, 0.25 mM dNTPs, 0.4 μM of each primer, and 1 unit of recombinant *Taq* polymerase per 50 μl of reaction mixture.

Products (1.2 kbp) of the nested PCR, primed by primer pair F2n/R2, were subjected to enzymatic restriction fragment length polymorphism (RFLP) analysis using restriction endonucleases *Alu*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Mse*I, *Rsa*I, *Sau*3AI, and *Taq*I (MBI Fermentas, Vilnius, Lithuania) and electrophoresis through 5% acrylamide gel. 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 scheme of Lee et al. (1998).

Cloning of PCR products and analysis of cloned rRNA operon sequences

We cloned phytoplasmal rDNA products, that had been amplified in direct (non-nested) PCRs primed by P1/P7 or in nested PCR primed by F2n/R2, in *Escherichia coli* using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Recombinant plasmids were screened for the presence of cloned rRNA operon fragments by amplification of DNA from recombinant plasmids in PCRs primed by F2n/R2 followed by RFLP analysis of the amplified DNA. The RFLP profiles were compared with those obtained from analysis of uncloned PCR products.

Nucleotide sequencing and putative restriction site analysis

Both strands of cloned DNA sequences (1.2 kbp or 1.8 kbp) from phytoplasmal rRNA operons were sequenced using standard dideoxy termination methods and automated DNA sequencing. The nucleotide sequences determined in this study were deposited in the GenBank database. Gene sequences were analyzed to estimate sequence similarities and to identify recognition sequences for selected restriction enzymes. Putative restriction site maps were generated by using the DNASTAR program MapDraw option. Alignments of pairs of sequences were generated and sequence similarities evaluated by using the DNASTAR program Align option.

Phylogenetic analysis

16S rRNA gene sequences (1.2 kb in size, representing the sequence between annealing sites of primer pair F2n/R2) from six phytoplasma strains from Lithuania and 23 other phytoplasma strains representing fifteen 16S rRNA phytoplasma groups (*sensu* Lee et al., 1998), and *Acholeplasma laidlawii* and *A. palmae* were aligned using Clustal X version 1.63b (Thompson et al., 1997) and DNASTAR. Analyses

were performed with the computer program Clustal X. A phylogenetic tree was constructed by the Neighbor-Joining method and the tree was viewed by using TreeViewPPC (Page, 1996). *Acholeplasma laidlawii* was selected as the outgroup to root the tree. GenBank accession numbers of the phytoplasma and *Acholeplasma* nucleotide sequences are given in Table 1.

Results

Identification of phytoplasma in diseased Gaillardia

The RFLP patterns of phytoplasmal 16S rDNA amplified from a phyllody-diseased *Gaillardia* sp. indicated that *Gaillardia* was infected by a phytoplasma

Table 1. GenBank accession numbers of phytoplasmal 16S rRNA gene sequences used in this study

Phytoplasma	rRNA operon[1]	Group-subgroup[2]	Accession no.	Reference[3]
Aster yellows (AY1)		I-B	AF322644	Unpubl.
Maize bushy stunt (MBS)		I-B	AF487779	Unpubl.
Alfalfa stunt (AlfS)		I-B	AF177384	Jomantiene et al., 2000
Michigan aster yellows (MIAY)		I-B	M30970	Lim and Sears, 1989
Clover phyllody (CPh)	rrnA	I-C	AF222065	Unpubl.
	rrnB		AF222066	Unpubl.
'*Candidatus* Phytoplasma aurantifolia'		II	U15442	Zreik et al., 1995
'*Ca*. P. japonicum'		Unc.[4]	ABO10425	Sawayanagi et al., 1999
Canada X-disease (CX)		III-A	L33733	Gundersen et al., 1994
Clover yellow edge (CYE)		III-B	AF175304	Davis and Dally, 2001
Clover yellow edge (CYE-L)		III-B	AF173558	Staniulis et al., 2000
Soybean veinal necrosis (SVN)		III-B	AF177383	Jomantiene et al., 2000
Gaillardia phyllody (GaiPh)		III-B	AY049029	This paper
Dandelion virescence (DanVir)	rrnA	III-P	AF370119	This paper
	rrnB		AF370120	This paper
Cirsium white leaf (CirWL)	rrnA	III-Q	AF373105	This paper
	rrnB		AF373106	This paper
Italian clover phyllody (ICPh)		III	X77482	Firrao et al., 1996
Coconut lethal yellows (LY)		IV-B	U18747	Tymon et al., 1998
Coconut lethal yellows (LDT)		IV-C	X80117	Harrison and Richardson, 1994
Elm yellows (EY1)		V-A	AF189214	Davis and Dally, 2001
Clover proliferation (CP)		VI-A	L33761	Gundersen et al., 1994; Davis and Dally, 2001
'*Ca*. P. fraxini'		VII-A	AF189215	Davis and Dally, 2001
Loofah WB (LfWB)		VIII-A	AF248956	Davis and Dally, 2001
Pigeon pea WB (PPWB)		IX-A	AF248957	Davis and Dally, 2001
Apple proliferation (APU)		X-A	AF248958	Davis and Dally, 2001
Rice yellow dwarf (RYD)		XI-A	D12581	Namba et al., 1993
Leafhopper-borne BVK		XI-C	X76429	Seemüller et al., 1994
Stolbur (STOL)		XII-A	AF248959	Davis and Dally, 2001
'*Ca*. P. australiense		XII-B	L76865	Davis et al., 1997
Mexican periwinkle virescence (MPV)		XIII-A	AF248960	Davis and Dally, 2001
Bermudagrass whiteleaf (BGWL)		XIV	AF248961	Davis and Dally, 2001
'*Ca*. P. brasiliense'		XV	AF147708	Montano et al., 2001
Acholeplasma palmae		N.A.[5]	L33734	Weisburg et al., 1989
A. laidlawii		N.A.	M23932	Weisburg et al., 1989

[1]The two sequence heterogeneous rRNA operons in a phytoplasma are indicated as rrnA and rrnB, respectively.
[2]16S rRNA RFLPgroup and subgroup affiliation of indicated phytoplasma. Group and subgroup assignments are based on RFLP analysis of DNA sequences corresponding to fragments amplified in PCRs primed by oligonucleotide pair R16F2n/R16R2, following the classification system of Lee et al. (1998).
[3]Reference to sequence data.
[4]Unc., unclassified.
[5]N.A., not applicable.

(GaiPh phytoplasma) belonging to group 16SrIII (group III, X-disease phytoplasma group), subgroup B (III-B, clover yellow edge phytoplasma subgroup). This phytoplasma subgroup has been reported in diseased clover (*Trifolium pratense*) in Lithuania (Staniulis et al., 2000). The other phytoplasmas discovered in this study represented two new subgroups in group 16SrIII.

New subgroup of group 16SrIII phytoplasmas in dandelion, T. officinale

A previously unknown phytoplasma was discovered in dandelion (*T. officinale*) exhibiting symptoms of virescence. This phytoplasma was designated dandelion virescence phytoplasma strain DanVir. Collective RFLP patterns of phytoplasmal 16S rDNA, amplified in nested PCR from DanVir-infected dandelion, indicated that this phytoplasma was a member of group 16SrIII (group III, X-disease phytoplasma group). It clearly differed from group 16SrI phytoplasma strains previously reported in dandelion in Canada (Wang and Hiruki, 2001) and Italy (Firrao et al., 1996), and it represented a new subgroup in group 16SrIII (Figure 1).

The unique *Hha*I pattern of its 16S rDNA was similar to, but not identical to, those of rDNA from previously described phytoplasma strains GR1 from goldenrod (*Solidago rugosa*), SP1 from spiraea (*Spiraea tomentosa*), or MW1 from milkweed (*Asclepias syriaca*) in New York State. GR1, SP1, and MW1 are representative of group III, subgroups III-D, III-E, and III-F, respectively (Griffiths et al., 1994). The *Hinf*I RFLP pattern differed from *Hinf*I patterns previously reported for 16S rDNA from any previously known group III phytoplasma (Figure 1). The sizes of DNA fragments in the *Hinf*I pattern were approximately 1.2, 0.8, and 0.4 kbp, respectively, which totaled a size greater than the 1.2 kbp size expected in PCR primed by F2n/R2. These results are interpreted as a case of ribosomal interoperon sequence heterogeneity in this new phytoplasma, DanVir. Based on the unique RFLP patterns of 16S rDNA, the DanVir phytoplasma is classified in group 16SrIII, new subgroup P (III-P).

We cloned the 1.8 kbp rDNA sequence that was amplified in direct (non-nested) PCR primed by P1/P7 from DanVir phytoplasma. This DNA sequence from the DanVir rRNA operons represented most of the 16S rRNA gene, the 16S-23S rRNA intergenic spacer region, and the 5′-end of the 23S rRNA gene. We analyzed the cloned insert rDNA through RFLP analysis of 16S rDNA that was amplified from the clones in

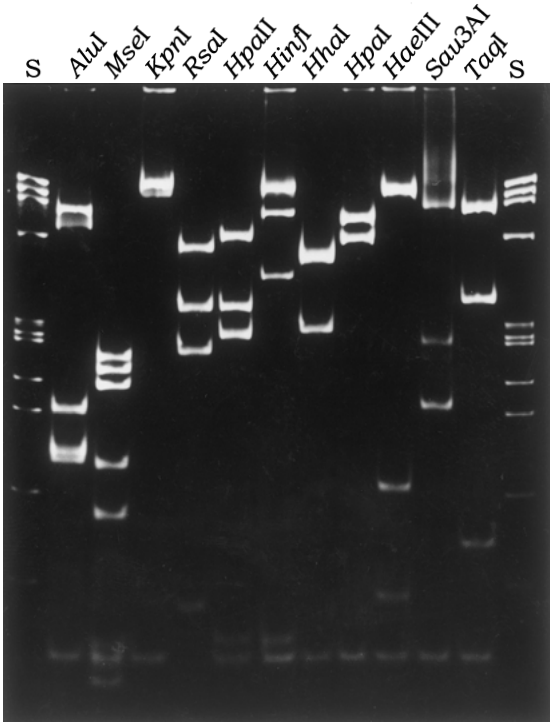

Figure 1. RFLP analysis of 16S rDNA amplified in nested PCR primed by oligonucleotide pair R16F2n/R2 from dandelion virescence phytoplasma strain DanVir infecting a naturally diseased dandelion plant. First round of PCR was primed by P1/P7 followed by reamplification of target DNA in nested PCR primed by R16F2n/R2. DNA products from the second, nested PCR were digested with restriction endonucleases *Alu*I, *Mse*I, *Kpn*I, *Rsa*I, *Hpa*II, *Hin*fI, *Hha*I, *Hpa*I, *Hae*III, *Sau*3AI, and *Taq*I. S, Phi X174 *Hae*III digest size standard.

PCRs primed by F2n/R2. Two classes, classes I and II, of collective RFLP patterns of the cloned rRNA operon sequences were observed following the use of *Hinf*I and *Hha*I (data not shown). Superimposition of these patterns yielded the same RFLP pattern as that observed from analysis of uncloned rDNA. The results were interpreted to indicate that the cloned sequences represented two sequence heterogeneous rRNA operons, designated rrnA [represented by clone DanVir204] and rrnB (represented by clone DanVir203).

The nucleotide sequences of clones DanVir203 and DanVir204 were determined and deposited in the GenBank database (Table 1). Results from putative restriction site analysis are shown in (Figure 2). Sizes of DNA fragments expected on the basis of putative restriction site maps within the F2n-R2 fragment were in agreement with results from enzymatic digestion of rDNA shown in Figure 1.

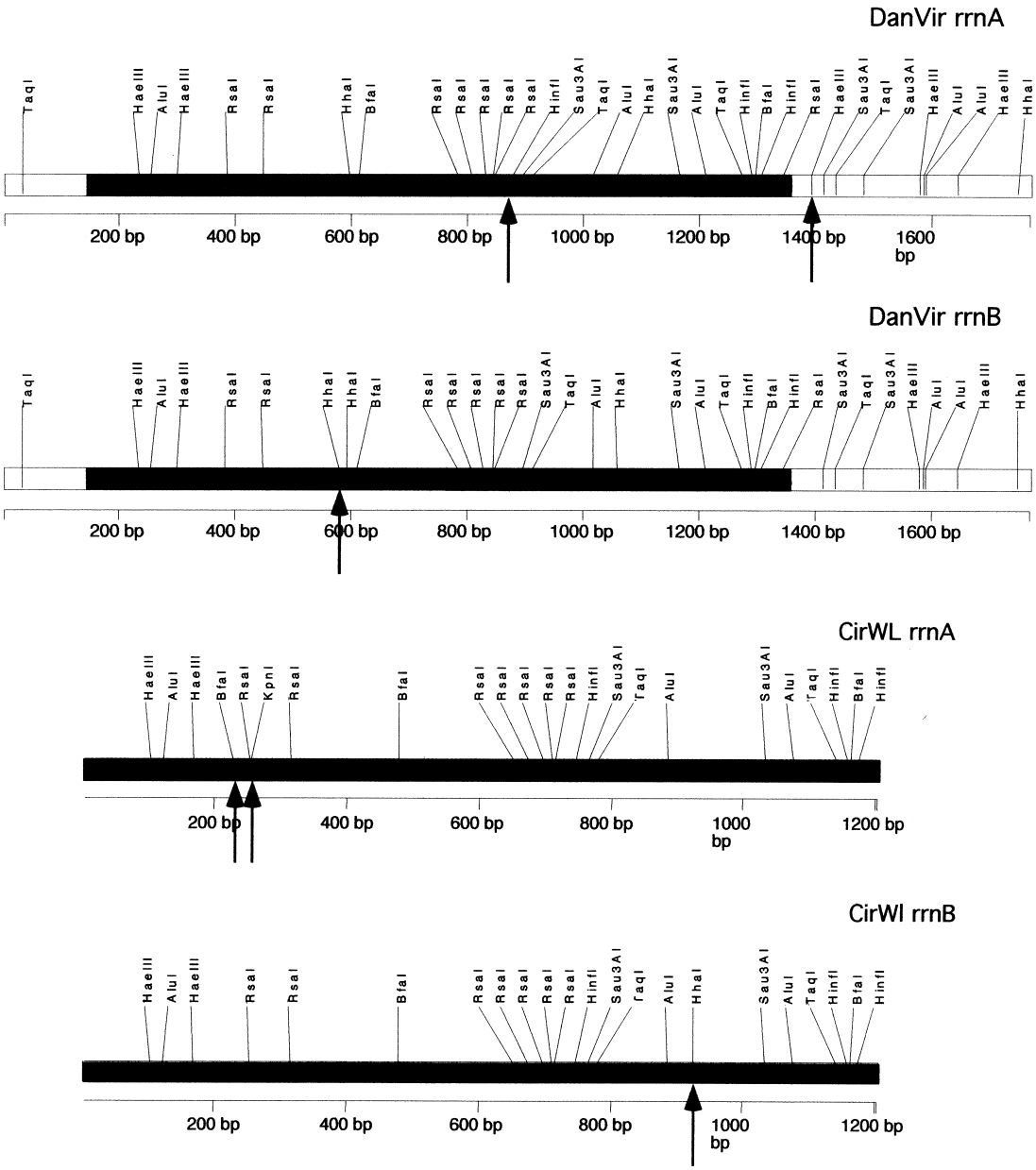

Figure 2. Analysis of putative restriction sites in cloned DNA sequences from ribosomal RNA operons rrnA and rrnB of dandelion virescence phytoplasma DanVir and of cirsium whiteleaf phytoplasma CirWL. The sequences from DanVir phytoplasma are comprised of most of the 16S rRNA gene, the 16S-23S rRNA spacer region, and the 5′-end of the 23S rRNA gene. The sequences from CirWL phytoplasma are 16S rRNA gene sequences. Arrows indicate restriction sites by which operons rrnA and rrnB of each phytoplasma may be distinguished. The solid bar represents the sequence amplified in PCR primed by oligonucleotide pair F2n/R2; phytoplasma classification into 16S rDNA groups and subgroups is based upon restriction sites in this sequence.

A group III phytoplasma has been reported in diseased dandelion in Italy, but the nucleotide sequence of its 16S rDNA was not reported. Firrao et al. (1996) implied that this phytoplasma (here termed Italian dandelion yellows, IDanY, phytoplasma) may be closely related to Italian clover phyllody (ICPh) phytoplasma. Since the 16S rDNA sequence from ICPh is available, we compared ICPh with DanVir.

Nucleotide sequence comparisons revealed at least 15 base differences in 16S rDNA between DanVir and ICPh (data not shown), indicating that these phytoplasmas, and by implication possibly DanVir and IDanY, are distinct.

New subgroup of group 16SrIII phytoplasmas in thistle (Cirsium arvense)

In diseased thistle exhibiting white leaf disease symptoms, we detected a previously undescribed phytoplasma, designated cirsium white leaf (CirWL) phytoplasma. Sufficient rDNA for RFLP analysis was obtained only through use of nested PCR primed by F2n/R2. Thus, 16S rDNA amplified in the nested PCR was analyzed. RFLP patterns of 16S rDNA amplified from CirWL were most similar to those of 16S rDNA from phytoplasmas classified in group 16SrIII (Figure 3A). On this basis, phytoplasma CirWL was classified in group 16SrIII. RFLP analysis also revealed that 16S rDNA of CirWL phytoplasma possessed a recognition site for *Kpn*I (Figure 3A), an unusual characteristic for members of group 16SrIII. Based on the unique RFLP patterns of 16S rDNA, we classify CirWL phytoplasma in group 16SrIII, new subgroup R (III-R).

The *Kpn*I RFLP pattern was composed of DNA fragments of 1.2, 0.9 and 0.3 kbp (Figure 3A), which totaled a size larger than that (1.2 kbp) expected from PCR primed by primer pair F2n/R2. Similarly, digestion of 16S rDNA with *Hha*I resulted in DNA fragments totaling greater than 1.2 kbp in size (Figure 3A). The results appear to be due to sequence heterogeneity of rRNA operons, as noted above in the case of the group 16SrIII phytoplasma strain DanVir, and found in other phytoplasmas (Davis et al., 1998; Lee et al., 1998; Liefting et al., 1996). Analysis of cloned CirWL rDNA confirmed this interpretation.

The 16S rDNA sequences from the two rRNA operons in CirWL phytoplasma were separated by cloning. The RFLP profiles obtained by digestion of cloned CirWL DNA showed that rrnA contained one site for *Kpn*I and that rrnB contained no site for *Kpn*I (Figure 3B). In addition, the operons were distinguished by digestion of cloned 16S rDNA with *Hha*I (Figure 3B). Thus, RFLP analysis using both restriction enzymes indicated sequence heterogeneity between the two rRNA operons in the genome of CirWL phytoplasma.

The nucleotide sequences of the clones CirWL#3 (representative of rrnA) and CirWL#1 (representative of rrnB) were determined and deposited in the GenBank database (Table 1). Putative restriction site analysis revealed the unique *Kpn*I site in rrnA and the *Hha*I site distinguishing rrnA and rrnB, in agreement with enzymatic RFLP analysis of the cloned DNAs, as well as a *Bfa*I site distinguishing the two operons (Figure 2).

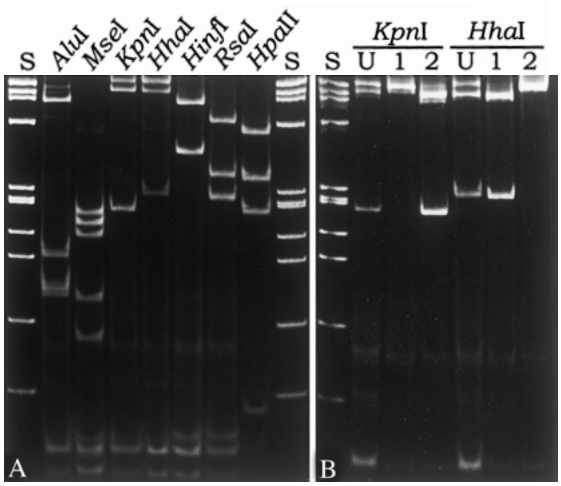

Figure 3. RFLP analysis of cloned and uncloned 16S rDNA from cirsium white leaf phytoplasma strain CirWL. A, 16S rDNA amplified in PCR primed by primer pair F2n/R2. B, *Kpn*I and *Hha*I RFLP analysis of 16S rDNA cloned from the two rRNA operons in the phytoplasma. Cloned DNA sequences were comprised of two RFLP classes. Class I RFLP patterns representing rrnB are seen in lanes *Kpn*I-1 and *Hha*I-1. Class II RFLP patterns representing rrnA are seen in lanes *Kpn*I-2 and *Hha*I-2. U, uncloned rDNA from CirWL phytoplasma. S, Phi X174 *Hae*III digest size, standard.

Sequence similarity of 16S rDNAs among group 16SrIII phytoplasmas

CirWL phytoplasma operons rrnA and rrnB share 99.3% nucleotide sequence similarity of 16S rDNA. Operons rrnA and rrnB of DanVir share 99.6% nucleotide sequence similarity of 16S rDNA. Both operons of CirWL phytoplasma share 98.8% nucleotide sequence similarity with 16S rDNA from the group 16SrIII phytoplasma strain CYE-L (this study), which Staniulis et al. (2000) have shown is widespread in Lithuania. CirWL operon rrnA shares 98.8% and 98.9% sequence similarity with 16S rDNA from operons rrnA and rrnB, respectively, of DanVir phytoplasma. CirWL operon rrnB shares 98.9% and 99.0% sequence similarity with 16S rDNA

from operons rrnA and rrnB, respectively, of DanVir phytoplasma.

Phylogenetic analysis

On the basis of analysis of 16S rRNA gene sequences, a phylogenetic tree was constructed to show relationships among previously known phytoplasmas and the new group III phytoplasmas, DanVir and CirWL (Figure 4). The branching order of the tree indicates that the sequences of rrnA and rrnB in DanVir phytoplasma evolved from a common ancestral sequence. Similarly, the branching order of the tree indicates that rrnA and rrnB of CirWL phytoplasma evolved from a recent common ancestral sequence that was distinct from that of the rRNA operons in DanVir

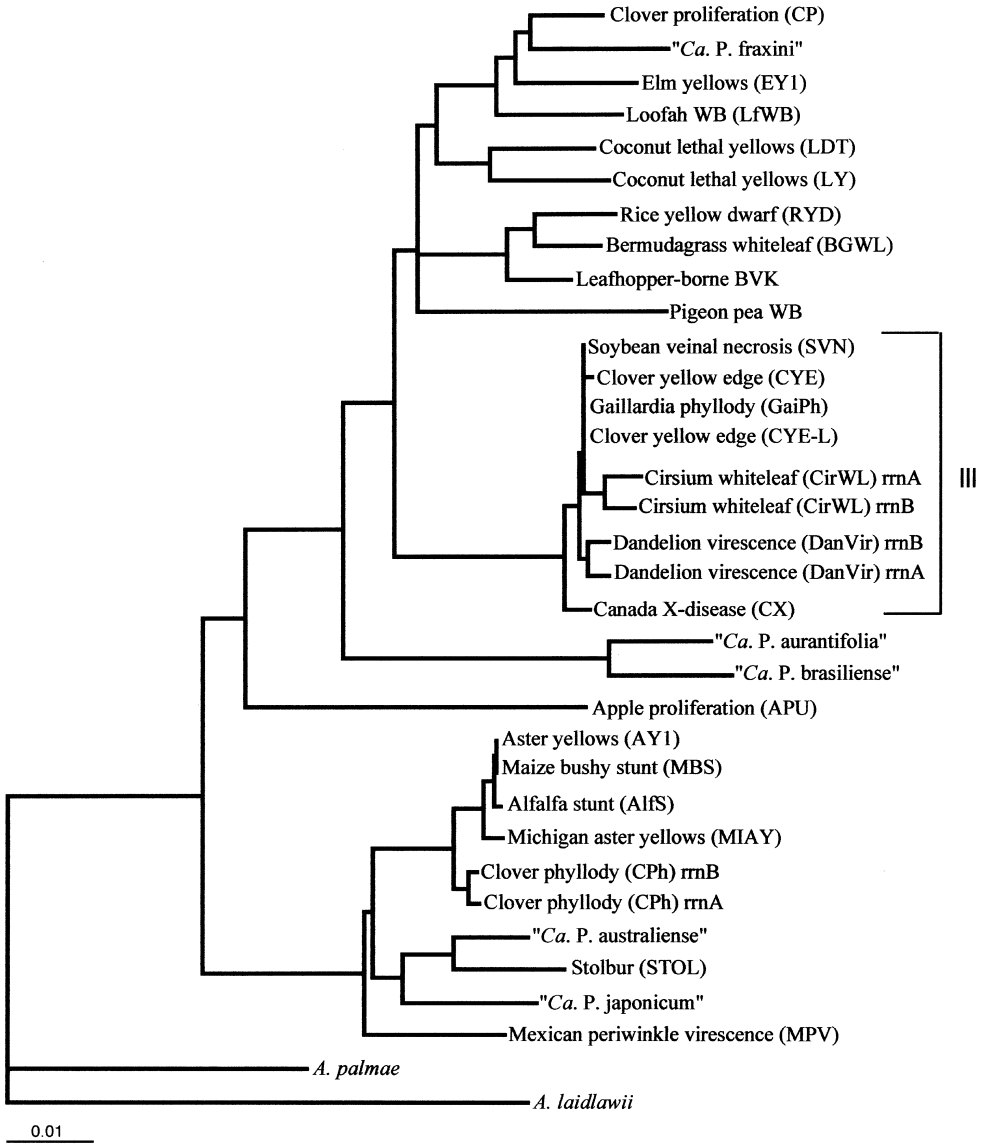

Figure 4. Phylogenetic tree constructed by the Neighbor-Joining method of 16S rRNA gene sequences from 29 phytoplasmas and *A. laidlawii* and *A. modicum*, employing *A. laidlawii* as the outgroup. Roman numeral III indicates Group III (16SrIII, X-disease phytoplasma group).

phytoplasma. The phylogenetic positions of DanVir and CirWL are consistent with the recognition of these two phytoplasmas as representatives of distinct new lineages within the group III phytoplasma subclade.

Discussion

In this communication, *Gaillardia* sp. is shown to be a natural plant host for a subgroup III-B phytoplasma, and two previously unknown phytoplasmas are classified into two new subgroups (III-P and III-R, respectively) within phylogenetic group 16SrIII (group III, X-disease phytoplasma group). This classification was based, according to the scheme of Lee et al. (1998), upon collective RFLP patterns of a 1.2 kbp segment of 16S rDNA that was amplified in PCR primed by F2n/R2. Phylogenetic analysis based on nucleotide sequences of the 1.2 kbp segments of 16S rDNA supported the distinction of DanVir and CirWL phytoplasmas from each other, as well as from other phytoplasmas belonging to group III and phytoplasmas belonging to other groups.

Interestingly, both dandelion and thistle may be naturally infected by several distinct lineages of phytoplasmas. For example, two different phytoplasmas were reported in diseased dandelion in Canada (Wang and Hiruki, 2001). Whereas, DanVir phytoplasma is a member of group III, both phytoplasmas associated with dandelion yellows in Canada are members of group I. Thus, DanVir phytoplasma clearly represents a taxon that is phylogenetically distinct from the taxon or taxa represented by the phytoplasma strains associated with dandelion yellows (DanY) in Canada. A different phytoplasma was reported in diseased dandelion in Italy (Firrao et al., 1996). This phytoplasma (here termed Italian dandelion yellows, IDanY, phytoplasma) was reported as a member of group III, but it was not classified according to subgroup, and the nucleotide sequence of its 16S rDNA was not reported (Firrao et al., 1996). Therefore, we could not directly compare DanVir with IDanY. However, if DanY phytoplasma is closely related to ICPh, as implied by Firrao et al. (1996), the results from 16S rDNA sequence comparisons between DanVir and ICPh in the present study suggest that IDanY and DanVir are mutually distinct.

CirWL represents a phytoplasma lineage, and taxon, that is distinct from others that are known to infect Canada thistle. For example, cirsium yellows (CirY) phytoplasma, previously reported in diseased Canada thistle in Lithuania, is a member of group I (Jomantiene et al., 2000). A different phytoplasma, cirsium phyllody (CIRP) phytoplasma, was reported in diseased Canada thistle in Germany (Marcone et al., 1997b; Schneider et al., 1997). Phylogenetic analysis based on 16S rDNA sequences indicated that CIRP phytoplasma was most closely related to group 16SrXI (group XI, rice yellow dwarf phytoplasma group, *sensu* Lee et al., 1998), although CIRP phytoplasma was distinct from this and other groups and probably represents a new phytoplasma lineage (Schneider et al., 1997). Thus in Europe, Canada thistle is a natural host for distinct phytoplasma lineages represented by CirWL (group III) and CirY (group I) phytoplasmas in Lithuania and by CIRP, a new lineage closely related to group XI, in Germany.

Previous work has shown that the phytoplasma genome contains two sets of rRNA genes (Schneider and Seemüller, 1994). This study revealed that the two new phytoplasmas discovered in Lithuania exhibit interoperon sequence heterogeneity in their respective 16S rRNA genes. The phylogenetic positions of the 16S rRNA gene sequences of DanVir phytoplasma probably indicate recent common ancestry of the two rRNA operons in this phytoplasma. A similar observation is made concerning the two rRNA operons of CirWL phytoplasma.

Several other studies have also shown that RFLP analysis of rDNA, as in this study, can provide the first indication of sequence heterogeneous rRNA operons in a single phytoplasma genome (Lee et al., 1993; Liefting et al., 1996; Davis et al., 1998 and unpublished; Davis and Dally, 2001a; Ho et al., 2001; Schneider et al., 1997). Support for the conclusion that certain RFLP data reflect interoperon heterogeneity, and not the presence of two different phytoplasma genomes in the same plant, comes from several lines of evidence. For example, the RFLP patterns of rDNA from clover phyllody (CPh) phytoplasma were interpreted as resulting from interoperon heterogeneity because the same RFLP patterns were observed in studies of rDNA from related phytoplasma strains found in various individual plants in a field, in different plant species, and in plants from different geographical regions (Lee et al., 1993; Gundersen and Lee, 1996; Staniulis et al., 2000; Harrison et al., 1997; Schneider et al., 1997). If the RFLP patterns were derived from two distinct phytoplasmas, one would expect to observe either

operon, but not both, in some plants. Moreover, the 16S rDNA sequences from heterogeneous operons in a phytoplasma genome differ from one another by no more than a few base positions and are closely related phylogenetically (Liefting et al., 1996; Davis and Dally, 2001; Davis et al., 1998). In contrast, mixed phytoplasma infections of individual plants have been reflected by RFLP patterns of rDNA clearly derived from well-established, phylogenetically distinct phytoplasma groups (Bianco et al., 1993; Lee et al., 1995; Staniulis et al., 2000). In addition, extraction of DNA from different plant parts consistently yielded the same two amplification products representing two sequence heterogeneous rRNA operons in the case of DanVir and CirWL phytoplasmas (R. Jomantiene, unpublished). In contrast, the well-known, uneven distribution of phytoplasmas in plants would be expected to yield detection of one, but not both operons, in some part(s) of an infected plant if the operons were derived from two distinct phytoplasmas in mixed infection.

The discovery of DanVir and CirWL phytoplasmas adds to the growing number of group III strains known in Lithuania. Together with phytoplasmas previously described in clover, soybean, and lupine in Lithuania, DanVir and CirWL increase knowledge about the diversity of group III phytoplasmas reported around the world. It is reasonable to expect that additional subgroups in this group will be discovered as research on the geographical and ecological distribution of phytoplasmas continues. Undoubtedly, several group III subgroups will eventually be recognized as distinct species level lineages, and it seems likely that subgroups III-P (DanVir) and III-R (CirWL) will be among those so recognized. The apparently recent common ancestry of the two rRNA operons in DanVir phytoplasma, as indicated by the branching order of its two 16S rRNA gene sequences in the phylogenetic tree, underscores the distinctness of this phytoplasma from others in group III. The distinctness of CirWL phytoplasma is similarly underscored. We postulate that such phytoplasmas represent divergent lineages that may be more clearly resolved through study of genes less highly conserved than the 16S rRNA gene. Future studies of less highly conserved genes and of the biology of varied group III phytoplasmas should aid in the recognition and description of multiple distinct species within the group, including DanVir and CirWL phytoplasmas. Development of genetic markers of biological properties will be valuable for understanding the role of these phytoplasmas in agricultural and natural ecosystems.

Acknowledgements

We gratefully acknowledge Juozas B. Staniulis for support and encouragement of this work and Ellen L. Dally for helpful suggestions in preparation of the manuscript.

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