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Bacteria and phytoplasmas/Bactéries et phytoplasmes

Identification and molecular characterization of the phytoplasma associated with peach rosette-like disease at the Canadian Clonal Genebank based on the 16S rRNA gene analysis

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Abstract: Peach trees exhibiting peach rosette-like disease symptoms and infected by a phytoplasma of group 16SrI '*Candidatus* Phytoplasma asteris' at the Canadian Clonal Genebank were further tested for the pathogen characterization based on the 16S rRNA gene. Nested PCR with phytoplasma universal primers R16mF2/R1 and R16F2n/R2 resulted in amplification of products of approximately 1.25 kb from all four symptomatic trees tested. Virtual RFLP of the R16F2n/R2 sequenced amplicons with selected restriction endonucleases showed unique RFLP patterns when compared to the described 16SrI phytoplasma subgroups; these data were confirmed by phylogenetic analyses. The phytoplasma was therefore assigned as a member of a new 16SrI subgroup (16SrI-W). Results represent the first report of a new phytoplasma 16SrI subgroup infecting peach in Canada, and provide a valuable tool for further epidemiological studies on this phytoplasma in peach.

Keywords: 16SrI-W phytoplasma, '*Candidatus* Phytoplasma asteris', *Prunus*, sequencing, RFLP

Résumé: Les pêchers, affichant les symptômes de la rosette du pêcher, infectés par un phytoplasme du groupe 16SrI 'Candidatus Phytoplasma asteris' à la Banque canadienne de clones, ont subi des tests plus poussés visant la caractérisation de l'agent pathogène à partir du gène 16S de l'ARNr. Un test, basé sur la PCR par amorces incluses utilisant les amorces universelles spécifiques des phytoplasmes R16mF2/R1 et R16F2n/R2, a permis l'amplification de fragments d'environ 1,25 kb à partir des quatre arbres symptomatiques ayant servi à l'analyse. Le RFLP virtuel des amplicons séquencés R16F2n/R2 avec endonucléases de restriction a affiché des profils uniques de RFLP lorsqu'ils ont été comparés aux sous-groupes de phytoplasmes 16SrI décrits. Ces données ont été confirmées par analyse phylogénétique. Le phytoplasme a par conséquent été désigné comme membre d'un nouveau sous-groupe 16SrI (16SrI-W). Les résultats constituent la première indication d'un nouveau sous-groupe de phytoplasme 16SrI infectant la pêche au Canada, et constituent un outil précieux quant à la poursuite d'études épidémiologiques sur ce phytoplasme chez la pêche.

Mots clés: '*Candidatus* Phytoplasma asteris', phytoplasme 16SrI-W, *Prunus*, RFLP, séquençage

Introduction

Peach, *Prunus persica* (L.) Batsch, is a member of the family Rosaceae, and is among the most important fruit crops within the genus *Prunus* in Canada. Approximately 3200 hectares (7900 acres), mainly located in Ontario, are devoted to peach cultivation in

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Canada. This contributes to 82% of the national total peach fruit yield (Statistics Canada, 2009). The Canadian Clonal Genebank (CCG) in Harrow, Ontario, maintains 83 accessions of peach for both research and propagation. Phytoplasmas are non-cultivable cell wall-less prokaryotes of the class *Mollicutes* (Lee *et al.*, 2000). They are naturally transmitted by phloem-feeding insects (Hemiptera: Auchenorryncha), and affect hundreds of plant species worldwide (Lee *et al.*, 2000; Bertaccini, 2007). Molecular-based analyses introduced during the last two decades have proven to be more accurate and reliable than biological criteria previously used for phytoplasma identification (Lee *et al.*, 2000; Bertaccini, 2007). Differentiation and classification of phytoplasmas rely on molecular analyses of conserved genes, in particular the 16S rRNA. Several hundred phytoplasma strains have been classified on the basis of distinct 16S rRNA gene RFLP patterns resolved on actual and/or virtual electrophoresis gel analysis (Lee *et al.*, 1998*b*; Marcone *et al.*, 2000; Davis & Dally, 2001; Jomantiene *et al.*, 2002; Lee *et al.*, 2007; Wei *et al.*, 2007; Cai *et al.*, 2008).

Recently, in Canada, eight different phytoplasma groups were identified associated with diseases in several crop and non-crop species (Olivier *et al.*, 2009). A phytoplasma of group 16SrI was identified in peach accessions showing rosette-like symptoms at the CCG (Zunnoon-Khan *et al.*, 2010), which represents a significant phytosanitary threat for other *Prunus* species considering that this is a phytoplasma group with a wide host range and a complex ecology (Lee *et al.*, 2004). A number of subgroups have been identified within group 16SrI. Therefore, the present study was undertaken to characterize this phytoplasma at the 16S rRNA gene subgroup level.

Materials and methods

Phytoplasma-infected peach material and controls

Two CCG peach accessions, PRU0382 (peach-almond cultivar 'Kando' from the Czech Republic), and PRU0445 (breeding line HW271 from Canada) exhibiting peach rosette-like symptoms at the CCG, and previously tested for phytoplasma presence during June to August 2009, were re-surveyed. Peach rosette-like symptoms observed included very short internodes, dehiscence of older shoot leaves, and flowers which rarely set fruit. Random leaves were collected from four symptomatic trees (two from each accession), as well as from two asymptomatic peach trees, accessions PRU0447 (breeding line HW273), and PRU0444 (breeding line HW270), and were subjected to molecular analyses.

Total DNA from phytoplasmas maintained in periwinkle (*Catharantus roseus L.*) and belonging to groups 16SrI (European aster yellows, ribosomal group 16SrI-B), 16SrII (peanut witches' broom, ribosomal subgroup 16SrII-A) (apple proliferation, ribosomal group 16SrX-A) and 16SrXII (stolbur from pepper from Serbia, ribosomal subgroup 16SrXII-A) was used as reference. Total DNA isolated from peach infected with the Canadian peach X-disease phytoplasma was used as a reference strain for group 16SrIII (X-disease) (Wang *et al.*, 2008).

Total DNA extraction and polymerase chain reaction

Total DNA was extracted from 100 mg of leaf midribs (DNeasy plant extraction kit, QIAGEN) and used as a template for a nested PCR with universal primers that target the phytoplasma 16S rRNA gene, R16mF2/R1 for the first PCR reaction and R16F2n/R2 for the nested reaction (Gundersen & Lee, 1996). For all PCR reactions, one μL of the DNA template (approximately 20 ng) was added to a 25 μL PCR reaction (illustra Pure Taq Ready-to-go-PCR-beads, GE Healthcare, UK). For the nested reaction, 1 μL of the first round PCR product was used. Thirty-five cycles were performed for all primer pairs in a DNA Engine Peltier thermal cycler Chromo 4 (Biorad). PCR cycling conditions for both primer pairs R16mF2/R1 and R16F2n/R2 were as follows: 1 min (2 min for the initial denaturation) at 94 °C, 2 min at 50 °C and 3 min (8 min for the final extension) at 72 °C. Five microlitres of the PCR products were separated in a 1.5% agarose gel, stained with GelRed Nucleic Acid Stain (Cat 41001, Biotium, Hayward, USA), and visualized with UV transilluminator in a gel documenter (red, Alpha Innotech, USA).

Cloning, sequencing and sequence analysis

One representative R16F2n/R2 PCR amplicon from each accession (PRU0382 and PRU0445) were purified on spin columns (Wizard PCR Clean-up, Promega, Madison, USA), cloned (pGEM-T Easy Vector, Promega, Madison, USA), and sequenced in both forward and reverse directions (Princess Margaret Hospital, Toronto, Canada). The 16S rDNA sequences were compared with reference sequences in GenBank, including representatives of each described subgroup within the group 16SrI, by BLAST (Altschul *et al.*, 1990). Sequences obtained were aligned using Clustal W (Thompson *et al.*, 1994) and phylogenetic trees were constructed using the neighbour-joining method (Saitou & Nei, 1987) with the program MEGA version 3.1 (Kumar *et al.*, 2004) with default values and 1000 replicates for bootstrap analysis.

In silico *restriction fragment length polymorphism (RFLP)*

The trimmed and aligned R16F2n/R2 sequence of the phytoplasma detected in peach rosette-like infected trees and those of representative phytoplasmas of each of the reported 15 16SrI subgroups, 16SrI-A, 16SrI-B, 16SrI-C, 16SrI-D, 16SrI-E, 16SrI-F, 16SrI-N, 16SrI-O, 16SrI-P, 16SrI-Q, 16SrI-R, 16SrI-S, 16SrI-T, 16SrI-U and 16SrI-V were exported to the *in silico* restriction analysis and virtual gel plotting program pDRAW32, developed by AcaClone software (http://www.acaclone.com). Each aligned DNA fragment was digested *in silico* with *Alu*I, *Bfa*I, *BstU*I (*Tha*I), *Hae*III, *Hha*I, *Hinf*I, *Hpa*II, *Mse*I and *Tsp509*I restriction endonucleases. After *in silico* restriction digestion, a virtual 3.0% agarose gel electrophoresis image with minimum 50 bp was plotted automatically to the computer screen. Restriction endonucleases yielding unique RFLP patterns for the peach rosette-like phytoplasma were selected for actual RFLP profile validation. The virtual RFLP patterns were compared and a similarity coefficient (F) was calculated for each pair of phytoplasma strains according to the formula: $F = 2Nxy/(Nx+Ny)$, in which Nx and Ny are the total number of bands resulting from digestions by the seven restriction enzymes in strains x and y, respectively, and Nxy is the number of bands shared by the two strains (Nei & Lee, 1979; Lee *et al.*, 1998*b*).

Results

Identification of phytoplasmas by PCR and 16S rDNA sequence analysis

Amplicons of approximately 1250 kb were visible after nested PCR from the DNA of all the symptomatic peach accessions PRU0382 and PRU0445 with the primer combination R16F2n/R2 (data not shown). No amplification products were obtained from the asymptomatic peach trees. The R16F2n/R2 sequences of the phytoplasmas detected in PRU0382 and PRU445 were 100% identical. The consensus sequence was submitted to GenBank under Acc. No. HQ450211, and the PRU0382 phytoplasma was used as the study reference strain in all further analyses. BLAST analysis R16F2n/R16R2 16S rDNA sequence of the PRU0382 phytoplasma yielded a 99% of identity with those of phytoplasma members of subgroup 16SrI-B.

In silico *restriction fragment length polymorphism (RFLP)*

After virtual digestion with *Alu*I, *Bfa*I, *Hae*III, *Hpa*II and *Tsp509*I, the PRU0382 phytoplasma exhibited RFLP profiles different from those of phytoplasmas of groups 16SrII (U15442), 16SrIII (AF533231), 16SrV (AY197655), 16SrVI (AY390261), 16SrVII (AF092209), 16SrX (AJ542541) and 16SrXII (L76865), and identical to those of the 16SrI phytoplasma (NC_005303) (Fig. 1).

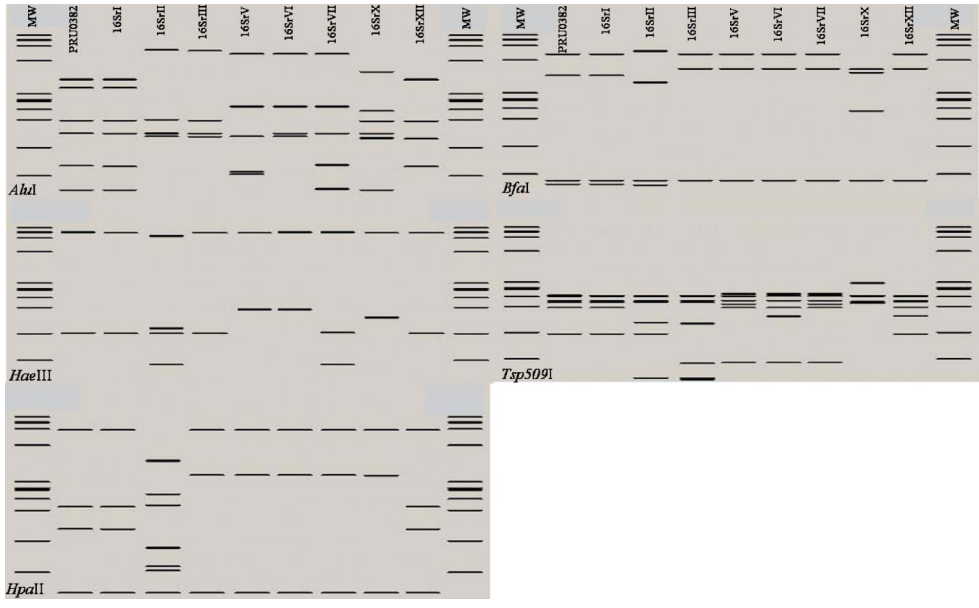

Fig. 1. Virtual RFLP generated with program pDRAW32 from *in silico* digestion of the 16S rDNA R16F2n/R2 fragments of the PRU0382 phytoplasma and 16Sr phytoplasma groups (16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, 16SrVII, 16SrX, 16SrXII) using *Alu*I, *Bfa*I, *Hae*III, *Hpa*II and *Tsp509*I restriction endonucleases. MW: ϕX174DNA-*Hae*III digest DNA molecular weight marker.

The PRU0382 phytoplasma showed unique RFLP patterns with the restriction endonucleases *Bst*UI (*Tha*I), *Hinf*I, *Hha*I and *Mse*I that clearly differentiated it from those of 16SrI phytoplasma subgroups, 16SrI-A (L33760), 16SrI-B (NC_005303); 16SrI-C (L33762); 16SrI-D (FJ263621); 16SrI-E (AY265220); 16SrI-F (AY265211); 16SrI-N (AY265205); 16SrI-O (AF268405); 16SrI-P (AF503568); 16SrI-Q (AY034089); 16SrI-R (AY102275); 16SrI-S (FJ914654); 16SrI-T (FJ914639); 16SrI-U (FJ914650); and 16SrI-V (FJ914642) (Fig. 2). The RFLP profile obtained on the PRU0382 phytoplasma amplicon is shown in Fig. 3. Similarity coefficients derived from virtual RFLP analysis of the R16F2n/R2 16S rDNA sequence of the PRU0382 phytoplasma were compared with those of 16S rDNA sequences of selected 16SrI phytoplasma subgroups (Table 1). The similarity coefficient values for the PRU0382 phytoplasma scored values less than 0.97, the threshold similarity coefficient for delineation of a new subgroup RFLP pattern type within a given group (Wei *et al.*, 2007), which supported the designation of the PRU0382 phytoplasma as a member of a new 16SrI subgroup, termed 16SrI-W.

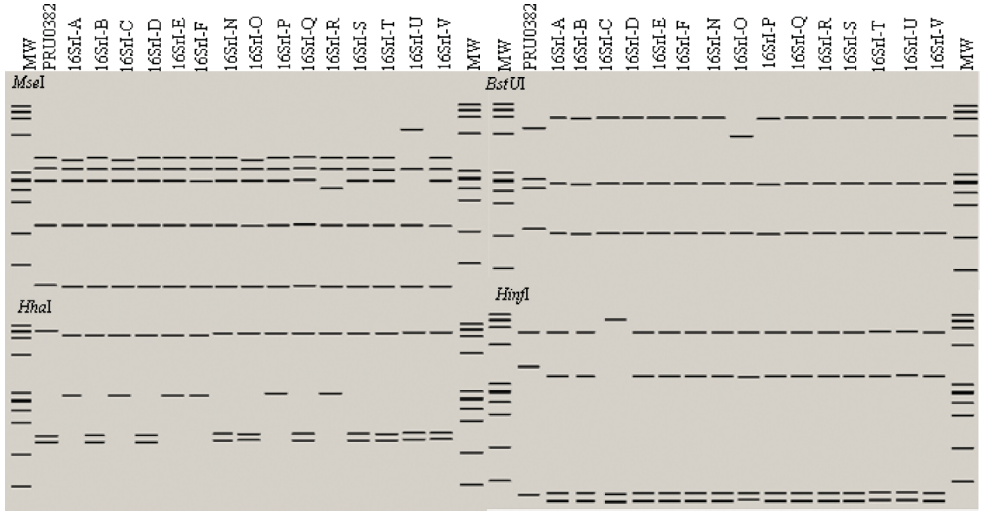

Fig. 2. Virtual RFLP generated with program pDRAW32 from *in silico* digestion of the 16S rDNA R16F2n/R2 fragments of the PRU0382 phytoplasma and other 16SrI subgroups using *Bst*UI (*Tha*I), *Hha*I, *Hinf*I and *Mse*I restriction endonucleases. MW: ϕX174DNA-*Hae*III digest DNA molecular weight marker.

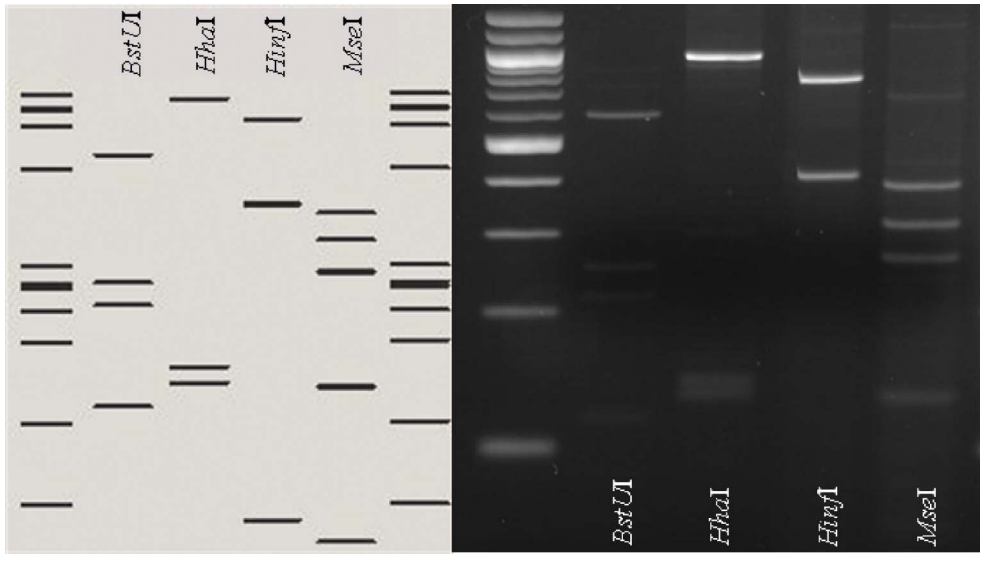

Fig. 3. Virtual and actual RFLP profiles of the PRU0382 phytoplasma R16F2n/R2 16S rDNA fragment with *Bst*UI (*Tha*I), *Hha*I, *Hinf*I and *Mse*I restriction endonucleases. Lane 1: MW 100 bp (BioLabs, USA).

Table 1. Similarity coefficients derived from RFLPs based on putative restriction-site analysis of nucleotide 16S rRNA gene sequences of PRU0382 phytoplasma (16SrI-W) and phytoplasmas representative of 16SrI subgroups.

Phytoplasma	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. PRU0382(I-W)	1.0	0.95	0.96	0.93	0.95	0.93	0.95	0.95	0.93	0.92	0.93	0.93	0.93	0.95	0.94	0.94
2. L33760(I-A)		1.0	0.95	0.99	0.95	0.98	0.98	0.95	0.95	0.98	0.98	1.0	0.95	0.95	0.98	0.98
3. NC005303(I-B)			1.0	0.94	1.0	0.98	0.98	1.0	0.95	0.98	1.0	0.98	1.0	1.0	0.98	0.98
4. L33762(I-C)				1.0	0.94	0.95	0.95	0.94	0.98	0.95	0.95	1.0	0.94	0.94	0.93	0.95
5. FJ263621(I-D)					1.0	0.98	0.98	0.95	1.0	0.98	1.0	0.98	1.0	1.0	0.98	0.98
6. AY265220(I-E)						1.0	1.0	0.98	0.94	1.0	1.0	1.0	0.98	0.98	0.95	0.95
7. AY265211(I-F)							1.0	0.98	0.94	1.0	1.0	1.0	0.98	0.98	0.95	0.95
8. AY265205(I-N)								1.0	0.95	0.98	1.0	0.98	1.0	1.0	0.98	0.98
9. AF268405(I-O)									1.0	0.93	1.0	0.98	0.95	0.95	0.93	0.93
10. AF503568(I-P)										1.0	1.0	1.0	1.0	0.98	0.95	0.95
11. AY034089(I-Q)											1.0	1.0	1.0	1.0	1.0	1.0
12. AY102275(I-R)												1.0	0.98	0.98	0.95	1.0
13. FJ914654(I-S)													1.0	0.95	0.98	0.93
14. FJ914639(I-T)														1.0	0.98	0.98
15. FJ914650(I-U)															1.0	0.95
16. FJ914642(I-V)																1.0

Phylogenetic analysis

comparison of the R16F2n/R2 16S rDNA sequences of the PRU0382 phytoplasma with phytoplasma reference sequences yielded the phylogenetic tree shown in Fig. 4. Phylogenetic analysis supported the *in silico* RFLP results. The phytoplasma identified in the peach accession PRU0382 grouped in the phylogenetic branch that encloses the phytoplasma strains from group 16SrI, and it is most closely related to the phytoplasma reference of subgroup 16SrI-B. Different 16SrI subgroups separate in distinct single branches like those containing phytoplasmas in subgroups 16SrI-O/16SrI-Q, 16SrI-E/16SrI-P, 16SrI-S/16SrI-U, 16SrI-T/16SrI-V and 16SrI-C/16SrI-R, further supporting PRU0382 phytoplasma as belonging to a distinct subgroup 16SrI-W.

Discussion

A number of peach diseases have been associated with phytoplasmas belonging to different ribosomal groups, including peach-X-disease and peach yellow leaf roll in USA and Canada (16SrIII group); peach decline in China (16SrV group); peach chlorotic leafroll (16SrX group), and peach rosette in USA and in Europe (16SrI group) (Marcone *et al.*, 1995; Lee *et al.*, 1998*a*), and a peach yellow-like disease in Jordan (16SrI group) (Anfoka & Fattesh, 2004), which indicates that different phytoplasmas are able to infect peach trees worldwide.

Aster yellows (AY, 16SrI group, '*Candidatus* Phytoplasma asteris') is the most widespread plant disease among those known to be associated with phytoplasma (Lee *et al.*, 2003). It was described in Canada as early as 1915 in carrot and from the 1930s in lettuce and celery (Olivier *et al.*, 2009), and in potato affected by potato purple top disease (MacLeod, 1939). This phytoplasma group affects a wide diversity of crops and ornamentals in Canada (Olivier *et al.*, 2009). A peach rosette-like disease was previously associated with a 16SrI phytoplasma in peach in Canada and tentatively placed as a member of the phytoplasma subgroup 16SrI-B (Zunnoon-Khan *et al.*, 2010). The current classification of phytoplasmas, however, is based on the analysis of a single, unique R16F2n/R2 16S rRNA gene sequence (> 1200 bp) (Lee *et al.*, 1993, 1998*b*, 2007; IRPCM, 2004), which has been also adopted for their virtual RFLP classification (Wei *et al.*, 2007, 2008).

Based on the R16F2n/R2 sequence and virtual RFLP patterns obtained after *in silico* enzymatic digestion of the R16F2n/R2 16S rDNA fragment, the PRU0382 phytoplasma detected in the peach rosette-like affected peaches was clearly confirmed as a member of the 16SrI group and differentiated from phytoplasmas previously reported in Canada (groups 16SrII, 16SrIII, 16SrV, 16SrVI, 16SrVII, 16SrX and 16SrXII) (Olivier *et al.*, 2009). It was designated as a member of a new RFLP subgroup within the group 16SrI, termed 16SrI-W. The sequence and virtual RFLP analysis of the R16F2n/R2 sequence proved a suitable approach for the identification and characterization of the PRU0382 phytoplasma as for many other phytoplasmas worldwide (IRPCM, 2004; Wei *et al.*, 2007), and confirmed the effectiveness of the R16F2n/R2 sequence as the phytoplasma phylogenetic marker (Lee *et al.*, 2007;

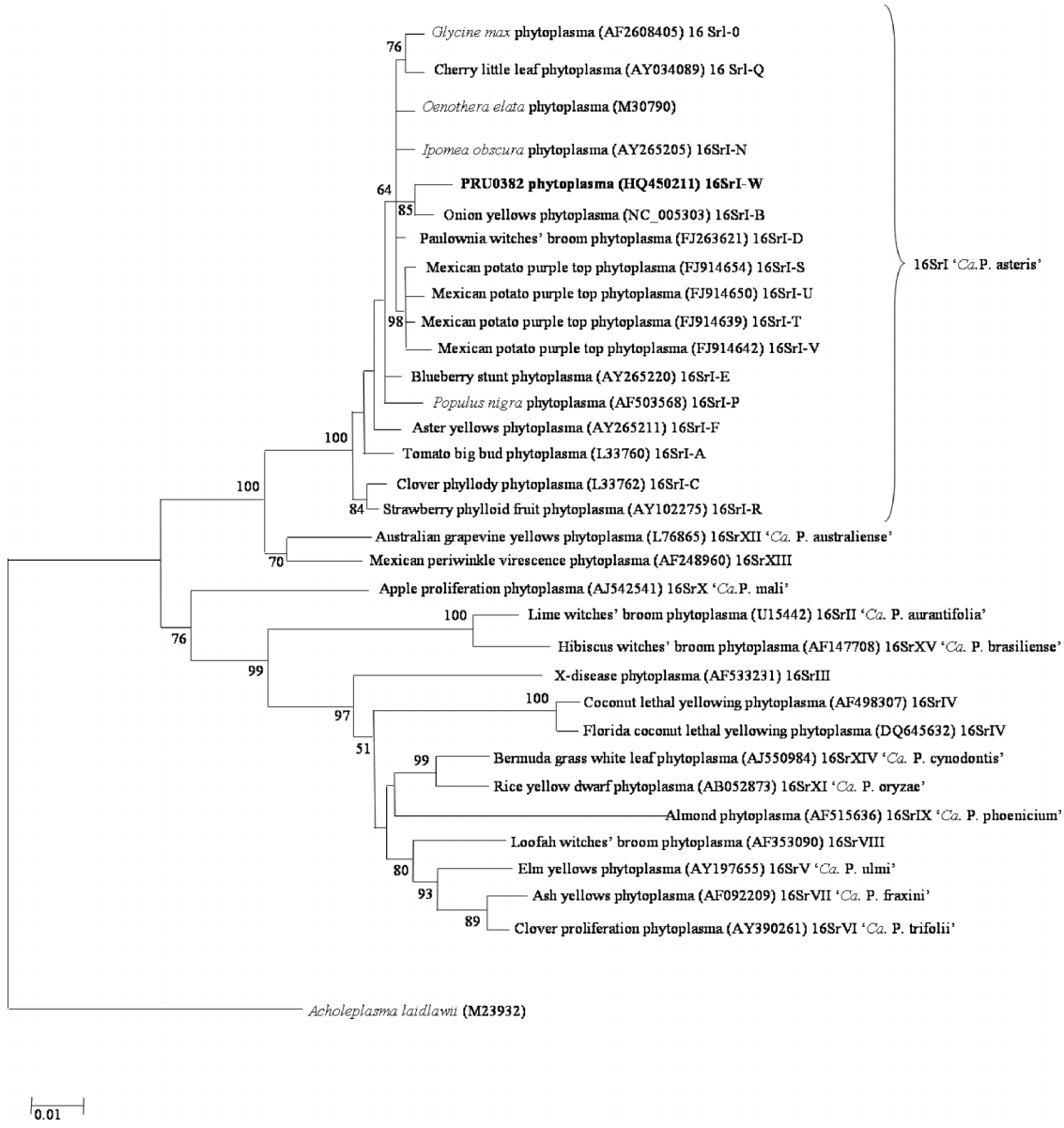

Fig. 4. Phylogenetic tree constructed using the neighbour-joining algorithm based on the R16F2n/R2 16S rDNA sequences of the PRU0382 phytoplasma and selected reference phytoplasma groups. *Acholeplasma laidlawii* is the outgroup to root the tree. '*Ca.* P': '*Candidatus* Phytoplasma sp.'.

Wei *et al.*, 2007, 2008). A number of new 16SrI subgroups have been identified and classified using the combination of R16F2n/R2 virtual RFLP and sequence analyses including subgroups 16SrI-Q, associated with a proliferation disease in cherry in Lithuania (Valiunas *et al.*, 2009), as well as subgroups 16SrI-S, 16SrI-T, 16SrI-U and 16SrI-V associated with potato purple top disease in Mexico (Santos-Cervantes *et al.*, 2008).

In North America, AY diseases are attributed primarily to phytoplasma strains belonging to subgroups 16SrI-A (termed Eastern AY) and 16SrI-B (termed California AY or Western AY) within the 16SrI group (Lee *et al.*, 2003). In Canada, 16SrI-A, 16SrI-B and 16SrI-C are the most common subgroups identified, associated with clover phyllody, strawberry green petal, grapevine yellows and diseases of cereals, forage grasses, herbs, spices and *Brassica* spp. (Olivier *et al.*, 2009). The results from this study indicate peach as a new host for a new phytoplasma subgroup of group 16SrI (16SrI-W) in Ontario, Canada. The emergence of this new subgroup in the peach host suggests ongoing evolution in adaptation of the AY phytoplasma to a new ecological niche. Moreover, a possible

genetic mutation for the peach rosette-like phytoplasma could result in the appearance of a new strain with a different epidemic capacity. The new 16SrI-W subgroup could represent a serious threat not only for the peach industry, but also for other *Prunus* species in Canada. The extent of infection of peach trees in the main production areas in Ontario by this new 16SrI-W subgroup should be assessed. The fact that only a few peach trees were infected by this 16SrI-W phytoplasma may contribute to identifying potential sources of phytoplasma resistance in peach, and provide new tools for CCG to prevent disease spread.

The molecular identification and characterization of a possible agent of peach rosette-like disease should contribute to expanding the knowledge of the genetic diversity and epidemiology of 16SrI phytoplasmas in Canada and worldwide, and to developing new approaches to better control 16SrI phytoplasma-associated diseases in peach and other *Prunus* species. Moreover, the molecular methodology used for the identification and characterization of the 16SrI-W phytoplasma in peach is a suitable tool to evaluate samples from other *Prunus*-cultivated areas of Ontario, and to identify potential vectors, which could assist in developing effective disease management strategies.

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