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Identification of new 16SrIX subgroups, -F and -G, among ‘*Candidatus Phytoplasma phoenicium*’ strains infecting almond, peach and nectarine in Lebanon

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Summary. Almond witches’-broom is a lethal almond disease associated with ‘*Candidatus Phytoplasma phoenicium*’, subgroups 16SrIX-B and -D. In Lebanon, where the disease was first reported, analogous diseases affecting peach and nectarine were recently associated with presence of ‘*Ca. P. phoenicium*’. In the present study, genetic diversity among 24 ‘*Ca. P. phoenicium*’ strains infecting almond, peach and nectarine plants in diverse geographic regions of Lebanon was investigated by virtual restriction fragment length polymorphism (RFLP) analysis of 16S rDNA nucleotide sequences. Calculation of virtual restriction similarity coefficients revealed the presence of two new subgroups, -F and -G, in group 16SrIX that were confirmed by real RFLP analyses. Obtained results open new opportunities for in-depth studies of the distribution of ‘*Ca. P. phoenicium*’ strains in plant hosts and insect vector populations from different geographic areas of Lebanon.

Key words: Single Nucleotide Polymorphisms (SNPs), genetic diversity, phytoplasma classification, restriction fragment length polymorphism (RFLP).

Introduction

Phytoplasmas of ribosomal group 16SrIX (IX) (pigeon pea witches’-broom group) are associated with diseases affecting crop and wild plants in different geographic areas worldwide (Kenyon *et al.*, 1998; Verdin *et al.*, 2003; Khan *et al.*, 2007; Davis *et al.*, 2010). ‘*Candidatus Phytoplasma phoenicium*’ (‘*Ca. P. phoenicium*’) strains, ribosomal subgroups IX-B and -D, are the agents of a severe disease of almond trees (almond witches’-broom, AlmWB) in Lebanon and in Iran (Choueiri *et al.*, 2001; Abou-Jawdah *et al.*, 2002; Verdin *et al.*, 2003; Salehi *et al.*, 2006). A similar disease, induc-

ing almond axillary proliferation, was reported in Iran (Verdin *et al.*, 2003) in association with phytoplasmas closely related to those responsible for *Knautia arvensis* phyllody (KAP), subgroup IX-C (Khan *et al.*, 2007). Furthermore, other phytoplasmas inducing different symptoms, such as small yellow leaves, on almond trees were detected in Iran (Zirak *et al.*, 2009).

By 2002, more than 100,000 almond trees had died from AlmWB in Lebanon. In 2009, ‘*Ca. P. phoenicium*’ was identified also in association with a severe disease of peach and nectarine in southern Lebanon (Abou-Jawdah *et al.*, 2009) and more than 40,000 newly diseased trees were observed in 2010 throughout the country. Grafting experiments and molecular analyses have revealed that ‘*Ca. P. phoenicium*’ does not affect plum, apricot and cherry trees (Abou-Jawdah *et al.*, 2003). Nevertheless, the rapid spread of the

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pathogen in peach and nectarine orchards confirmed the alarm about the risk it poses for stone fruit production in Lebanon and in all neighboring countries.

The most characteristic symptoms of AlmWB on almond trees are the shoot proliferation at several points on the main trunks of affected trees, with an appearance of witches'-broom, the perpendicular development of many auxiliary buds on the branches, with small and yellowing leaves, the general decline of affected trees, and severe die-back. A total loss of production happens 1–2 years after the initial appearance of the symptoms. In the case of peach and nectarine trees infected by '*Ca. P. phoenicium*', the first symptom observed is the early flowering (15 to 20 days earlier than normal), followed by the early development of all the buds of the infected branches. In addition, some months after the normal flowering period, phyllody and serrate, slim, light green leaves develop on the plant branches and witches'-brooms develop from the trunks and crowns of affected trees.

Phytoplasmas live exclusively in the phloem tissue of plants and they are normally transmitted by vegetative propagation or grafting (seedlings, scions, rootstocks) and by insect vectors (Lee *et al.*, 2000). The rapid spread of '*Ca. P. phoenicium*' over large geographical areas in North Lebanon suggested the presence of an efficient vector (Abou-Jawdah *et al.*, 2009). However, this vector has not been identified. In order to better understand the disease epidemiology and achieve effective disease management, more research on '*Ca. P. phoenicium*' genetic diversity, host range and vectors is required. For this reason, a development project financed by Italian Cooperation focusing on investigating epidemics and biology of '*Ca. P. phoenicium*' is being implemented by the Association of Volunteers in International Service (AVSI) Foundation in Lebanon (Molino Lova *et al.*, 2010). In the present study, data on genetic diversity among '*Ca. P. phoenicium*' strains infecting almond, nectarine and peach plants from diverse Lebanese regions are reported.

Materials and methods

Sample collection

Preliminary field surveys on the incidence of

'*Ca. P. phoenicium*'-associated disease were carried out from 2008 to 2009 in orchards located in Sarada, Feghal, and Marjayoun regions of Lebanon. Leaf, and in some cases flower, samples were collected in 15 orchards from 24 plants (nine almonds, four peaches, and 11 nectarines) showing symptoms such as witches'-broom, phyllody, virescence and chromatic alterations on the leaves.

Phytoplasma identification and characterization

Total DNA was extracted from 100 mg of leaf veins using a modified protocol from Doyle and Doyle (1990). Phytoplasma detection was carried out by means of amplification of 16S rDNA, in nested polymerase chain reactions (PCRs) primed by phytoplasma-universal primer pairs P1/P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996) and R16F2n/R16R2 (Gundersen and Lee, 1996). PCRs were performed by using *Taq* polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA) in an automated thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). Presence of PCR amplicons was verified by electrophoresis through 1% agarose gel. DNAs extracted from phytoplasma strains EY1 ('*Candidatus* Phytoplasma ulmi', subgroup 16SrV-A), STOL (stolbur group, subgroup 16SrXII-A), and AY1 ('*Ca. P. asteris*', subgroup 16SrI-B) served as reference controls; the phytoplasmas were maintained in periwinkle [*Catharanthus roseus* (L.) G. Don.]. DNA from healthy periwinkle plants and reaction mixture without DNA template were used as negative controls. Amplicons from nested PCRs were sequenced to achieve at least 4× coverage per base position. DNA sequencing was performed in an ABI PRISM 377 automated DNA sequencer (Applied Biosystems). The nucleotide sequence data were assembled by employing the Contig Assembling program of the sequence analysis software BIOEDIT, version 7.0.0 (<http://www.mbio.ncsu.edu/Bioedit/bioedit.html>). Sequences were compared with the GenBank database using the software BlastN (<http://www.ncbi.nlm.nih.gov/BLAST/>) with the aim of searching possible identity. Nucleotide sequences of '*Ca. P. phoenicium*' strains identified in the present study were deposited in the National Center of Biotechnology Information (NCBI) GenBank database at accession numbers HQ407512 to HQ407535.

Virtual RFLP analysis and calculation of similarity coefficients

A total of 37 16S rRNA gene sequences of phytoplasma group IX (13 from GenBank and 24 obtained in the present study), plus sequences from phytoplasma strains representative of known 16Sr subgroups, were each trimmed to an approximately 1.25-Kb fragment (delimited by R16F2n and R16R2 primer annealing positions), as previously described (Wei *et al.*, 2007), and exported to the program pDRAW32 (AcaClone Software, <http://www.acaclone.com>). Each DNA sequence was analyzed through an automated *in silico* restriction assay, and digestion results were plotted on virtual gels as described by Wei *et al.* (2007). In detail, each DNA fragment was digested *in silico* with 17 restriction enzymes used previously in actual enzymatic digestions by Lee *et al.* (1998): *AluI*, *BamHI*, *BfaI*, *BstUI* (*ThaI*), *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI* (*MboI*), *MseI*, *RsaI*, *SspI*, and *TaqI*. After *in silico* restriction digestion, a virtual 3.0% agarose gel electrophoresis image was plotted and captured as a device-independent PDF file. The virtual RFLP patterns were compared and a similarity coefficient (F) was calculated for each pair of phytoplasma strains according to the formula described previously (Nei and Li, 1979; Lee *et al.*, 1998), $F = 2N_{xy} / (N_x + N_y)$, in which x and y are two given strains under study; N_x and N_y are the total number of bands resulting from digestions by 17 enzymes in strains x and y , respectively; and N_{xy} is the number of bands shared by the two strains.

Phylogenetic analysis

Phytoplasma 16S rDNA gene sequences from this study and from GenBank were used to construct phylogenetic trees. Minimum evolution analysis was carried out using the Neighbor-Joining method and bootstrap replicated 1000 times with the software MEGA4 (<http://www.megasoftware.net/index.html>) (Tamura *et al.*, 2007).

Results and discussion

Identification of '*Ca. P. phoenicium*'

Primer pair R16F2n/R16R2, which is known to prime amplification of 16S rDNA from all described '*Candidatus* Phytoplasmas', primed am-

plification of DNA from templates derived from all samples studied (data not shown). Control PCRs containing template DNA from healthy periwinkle, or water in place of DNA, yielded no visible DNA amplification. Comparison of 16S rDNA sequences to the GenBank accessions revealed that phytoplasma strains here identified shared 99-100% of sequence identity with '*Ca. P. phoenicium*' (accession number AF515636), ribosomal group IX (data not shown).

New subgroups in group 16SrIX in Lebanon

Computer-simulated restriction analyses were carried out on R16F2n/R16R2 sequences from 24 '*Ca. P. phoenicium*' strains in Lebanon. Visualization and comparison of virtual gel plotted images revealed three different RFLP patterns, indicating genetic diversity among '*Ca. P. phoenicium*' strains in Lebanon (Table 1). One pattern was exhibited by DNAs from 15 '*Ca. P. phoenicium*' strains; this pattern was indistinguishable from that characteristic of strains classified in the '*Ca. P. phoenicium*', subgroup IX-D (Figure 1).

The remaining two virtual RFLP patterns differed from the pattern of the previously described subgroup IX-D and shared similarity coefficients of 93 to 97%, confirming their affiliation with group IX; according to Wei *et al.* (2007) and Lee *et al.* (2007), each of the two new RFLP patterns possibly identifies a new subgroup in group IX.

The 16S rDNAs from '*Ca. P. phoenicium*' strains SarN5 and MarN27-2 exhibited identical virtual RFLP patterns from use of 17 restriction enzymes. Since the *BstUI* RFLP pattern distinguished (similarity coefficient $\leq 97\%$) strains SarN5 and MarN27-2 from strains in all previously described subgroups in group IX, these two strains are classified in new subgroup IX-F (Figure 1 and 2; Table 2). The 16S rDNAs from '*Ca. P. phoenicium*' strains SarN1-2, FegA1-1, FegA13-1, FegA18-1, FegP3-1, FegA3, and FegA4 exhibited identical virtual RFLP patterns, which distinguished (similarity coefficient $\leq 97\%$) these strains from strains in all previously described subgroups, including new subgroup IX-F, on the basis of digestion with *TaqI* (Figure 1 and 2; Table 2). Hence, '*Ca. P. phoenicium*' strains SarN1-2, FegA1-1, FegA13-1, FegA18-1, FegP3-1, FegA3, and FegA4 are placed in a new subgroup, IX-G. Actual gel electrophoresis-RFLP analyses, car-

Table 1. Occurrence of '*Ca. P. phoenicium*' strains, belonging to distinct 16SrIX subgroups, in orchards of Lebanon regions. New subgroups confirmed by actual RFLP analyses are indicated in bold.

Strain	Origin	Orchard No.	Host	Subgroup IX
SarN1-2	Sarada	01	Nectarine	-G
SarN5	Sarada	01	Nectarine	-F
SarN8-1	Sarada	02	Nectarine	-D
SarN9-7	Sarada	01	Nectarine	-D
SarN10-8	Sarada	03	Nectarine	-D
SarP10(297)	Sarada	04	Peach	-D
MarN13-1	Marjayoun	05	Nectarine	-D
MarN14-1	Marjayoun	06	Nectarine	-D
MarN27-2	Marjayoun	07	Nectarine	-F
MarN28-1	Marjayoun	07	Nectarine	-D
FegA1-1	Feghal	08	Almond	-G
FegA11-4	Feghal	09	Almond	-D
FegA13-1	Feghal	09	Almond	-G
FegA16-4	Feghal	08	Almond	-D
FegA18-1	Feghal	10	Almond	-G
FegP1-2	Feghal	11	Peach	-D
FegP2-6	Feghal	11	Peach	-D
FegP3-1	Feghal	11	Peach	-G
FegPL3-1	Feghal	11	Almond	-D
FegA3	Feghal	12	Almond	-G
FegA4	Feghal	13	Almond	-G
KKN18-1	Kerbet Kanafar	14	Nectarine	-D
KKN19-1	Kerbet Kanafar	14	Nectarine	-D
KKN29-1	Kerbet Kanafar	15	Nectarine	-D

ried out using the distinguishing enzymes *Bst*UI and *Taq*I on R16F2n/R16R2 PCR products from strains MarN27-2, SarN5, SarN1-2, FegA1-1, FegA13-1, FegA18-1, FegP3-1, FegA3, and FegA4 confirmed the virtual RFLP patterns (Figure 3).

AlmWB-associated Lebanese '*Ca. P. phoenicium*' strains, whose sequences were retrieved from the GenBank, shared a virtual RFLP similarity coefficient > 98% with '*Ca. P. phoenicium*' strains of subgroup IX-D, while the Iranian phytoplasma strains associated with AlmWB and almond broomings shared a similarity coefficient > 99% with phytoplasmas of subgroup IX-C (Table 2).

Prior to the present study, five subgroups had been described in the group IX: Pigeon Pea Witches-Broom (PPWB) subgroup -A (Wei *et al.*, 2007), *Pichris echioides* yellows (PEY) subgroup -C (Khan *et al.*, 2007), '*Ca. P. phoenicium*' subgroups -B and -D (Verdin *et al.*, 2003), and Ju-

niper witches-broom subgroup -E (Davis *et al.*, 2010). The results of the present study add two new, confirmed subgroups -F and -G from almond, nectarine and peach plant hosts.

Phylogenetic relationships

In BLAST searches all of the 24 phytoplasma 16S rDNA sequences from the present study yielded best hits with '*Ca. P. phoenicium*', subgroup IX-D (data not shown). A minimum-evolution (ME) phylogenetic analysis of 16S rRNA gene sequences showed that phytoplasma strains of all 16SrIX subgroups cluster together on a separate tree branch within the same group (Figure 4). Inside the group IX branch, the 24 '*Ca. P. phoenicium*' strains identified in this study, clustered along with previously characterized Lebanese '*Ca. P. phoenicium*' strains, associated with AlmWB, in a phylogenetic subclade with the representative '*Ca. P. phoenicium*' strain

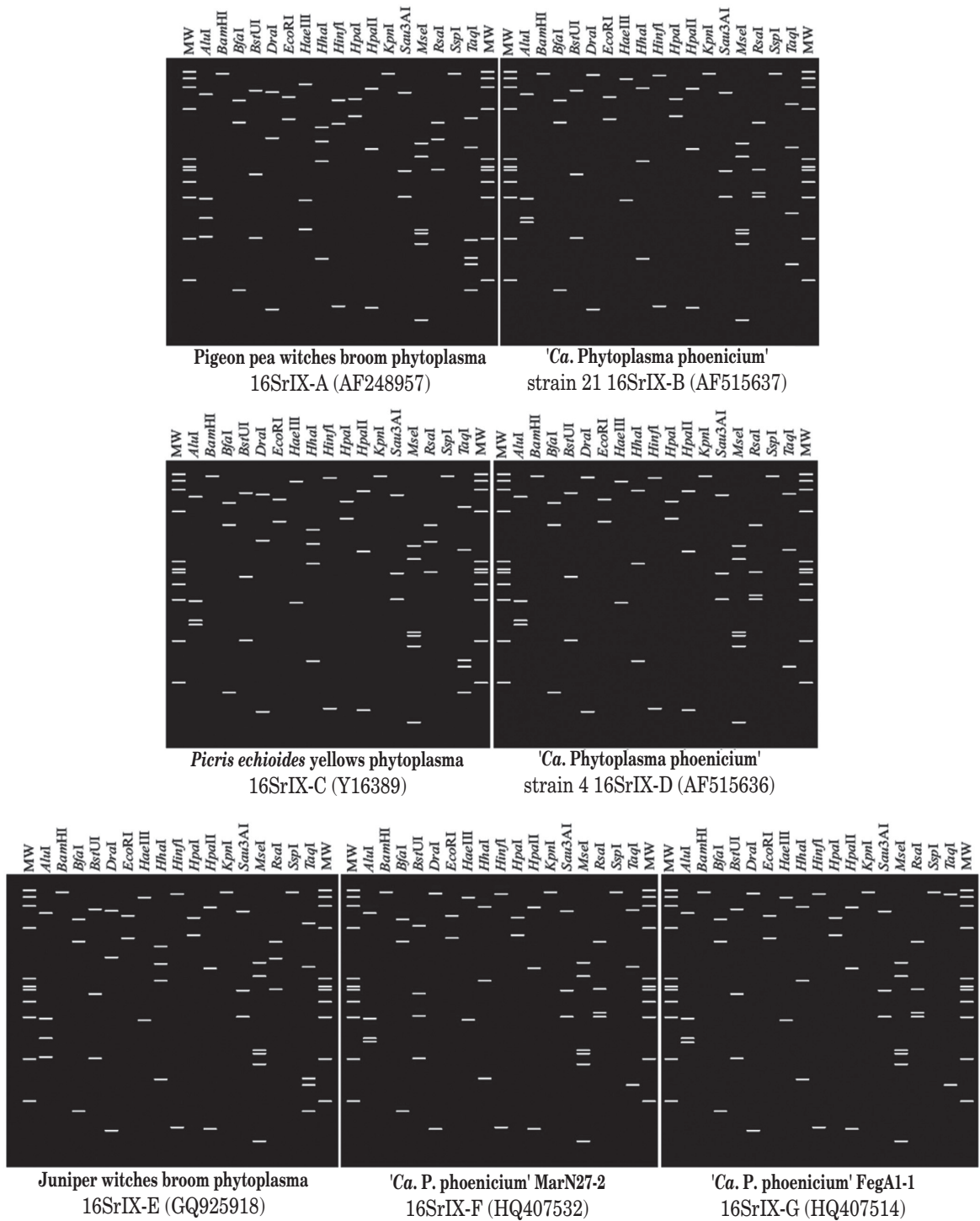


Figure 1. Virtual R16F2nR2 RFLP patterns of representative strains of 16SrIX subgroups. Recognition sites for the following 17 restriction enzymes were used in the simulated digestions: *AluI*, *BamHI*, *BfaI*, *ThaI* (*BstUI*), *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI* (*MboI*), *MseI*, *RsaI*, *SspI*, and *TaqI*. MW, FX174DNA digested by *HaeIII*, fragment size (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

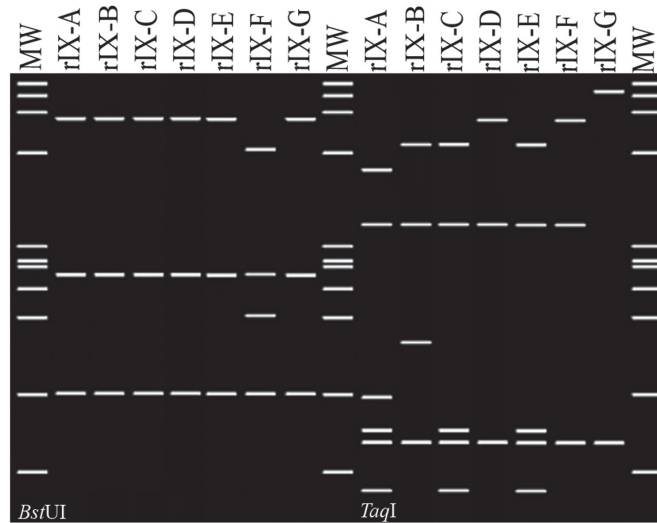


Figure 2. Virtual R16F2nR2 RFLP patterns by key enzymes *Bst*UI and *Taq*I for distinguishing among 16SrIX subgroups. MW, FX174DNA digested by *Hae*III, fragment size (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

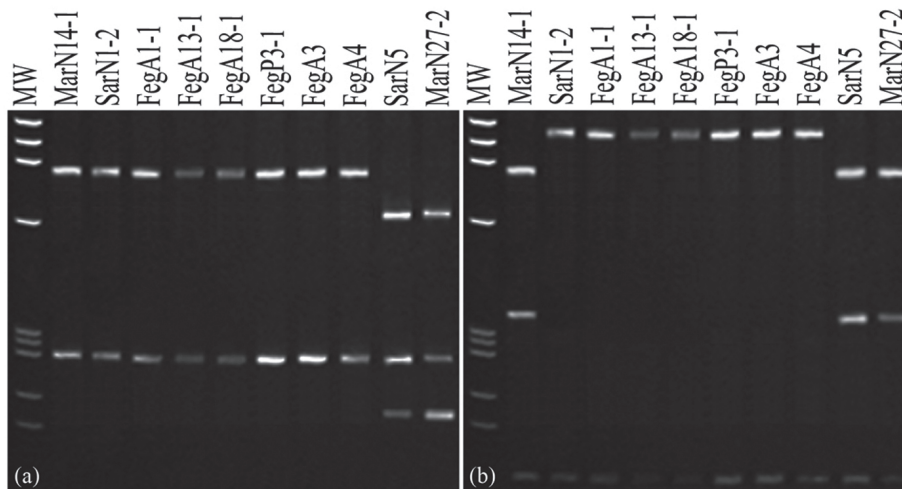


Figure 3. Actual gel-based *Bst*UI- and *Taq*I-RFLP patterns of R16F2nR2 amplicons from ‘*Ca. P. phoenicium*’ strains. Acronyms are described in Table 1; MW, FX174DNA digested by *Hae*III, fragment size (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. Restriction fragments were visualized by electrophoresis through 3% agarose gel.

A4, subgroup IX-D. ‘*Candidatus P. phoenicium*’ strains of new confirmed subgroup IX-G clustered together in a separate subclade within that of ‘*Ca. P. phoenicium*’ (subgroup IX-D). On the other hand, Iranian phytoplasma strains associated with AlmWB clustered in a separate

subclade with the representative strain of the subgroup IX-C.

Genetic diversity and ecology of ‘*Ca. P. phoenicium*’ strains

Identification of two new IX subgroups opens

Table 2. Similarity coefficients obtained through virtual RFLP analysis of 16S rDNA sequences from 24 'Ca. P. phoenicium' strains and from representative strains of group 16SrIX. New subgroups are indicated in bold.

Strain	Accession No.	Subgr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29									
01 PPWB	AF248957	-A	100																																					
02 CPP ^a strain 21	AF515637	-B	76	100																																				
03 PEY	Y16889	-C ^b	89	87	100																																			
04 CPP ^a strain A4	AF515636	-D ^b	77	97	86	100																																		
05 JunWB	GQ925918	-E	89	85	96	84	100																																	
06 SarN1-2	HQ407512	-G	75	95	84	97	82	100																																
07 MarN14-1	HQ407513	-D	75	93	84	97	82	93	100																															
08 FegA1-1	HQ407514	-G	75	95	84	97	82	100	93	100																														
09 FegA13-1	HQ407515	-G	75	95	84	97	82	100	93	100	100																													
10 FegA18-1	HQ407516	-G	75	95	84	97	82	100	93	100	100	100																												
11 FegP2-6	HQ407517	-D	77	97	86	100	84	97	97	97	97	97	97	100																										
12 FegP3-1	HQ407518	-G	75	95	84	97	82	100	93	100	100	100	97	100	97	100																								
13 FegA3	HQ407519	-G	75	95	84	97	82	100	93	100	100	100	97	100	100	100																								
14 KKN19-1	HQ407520	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100																								
15 FegA4	HQ407521	-G	75	95	84	97	82	100	93	100	100	100	97	100	97	100	97	100																						
16 SarN5	HQ407522	-F	74	93	83	97	80	93	93	93	93	93	97	93	93	97	93	93	97	93	100																			
17 FegA11-4	HQ407523	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100																				
18 SarN10-8	HQ407524	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100																			
19 SarN8-1	HQ407525	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100																			
20 MarN28-1	HQ407526	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100																			
21 FegP1-2	HQ407527	-D	73	90	82	93	80	90	90	90	90	90	90	93	90	93	90	93	90	93	93	100																		
22 SarN9-7	HQ407528	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100	93	100																	
23 KKN29-1	HQ407529	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100	93	100	100																
24 KKN18-1	HQ407530	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100	93	100	100	100															
25 FegA16-4	HQ407531	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100	93	100	100	100	100														
26 MarN27-2	HQ407532	-F	74	93	83	97	80	93	93	93	93	93	97	93	93	97	93	93	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	
27 FegPL3-1	HQ407533	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100	93	100	100	100	100	100													
28 SarP10(297)	HQ407534	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100	93	100	100	100	100	100													
29 MarN13-1	HQ407535	-D	77	97	86	100	84	97	97	97	97	97	97	100	97	100	97	100	97	100	100	93	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

^aCPP means 'Ca. P. phoenicium'.

^bLebanese 'Ca. P. phoenicium' strains AlmWB1, AlmWB2, AlmWB3, AlmWB4, AlmWB-P1, and AlmWB-N1, whose 16S rDNA nucleotide sequences were retrieved from GenBank, were grouped in subgroup IX-D; Iranian AlmWB and Khafr AlmWB strains, whose 16S rDNA nucleotide sequences were retrieved from GenBank, were grouped in subgroup IX-C.

new opportunities for in-depth studies of the distribution of ‘*Ca. P. phoenicium*’ strains in weeds, insect vector populations, and plant hosts from orchards located in different geographic areas. Application of automated virtual restriction analysis should facilitate such studies. For example, genetic diversity among ‘bois noir’ (BN) phytoplasma strains infecting *Vitis vinifera* L. in Italy was de-

scribed, on the basis of automated virtual RFLP analysis of 16S rDNA (Quaglino *et al.*, 2009).

Thirty-seven percent (9/24) of ‘*Ca. P. phoenicium*’ strains exhibited virtual and actual RFLP patterns distinct from those of IX known subgroups. In detail, 33% (2/6) of the strains from Sarada region belongs to subgroups IX-F and -G; 25% (1/4) of the strains from Marjayoun region belongs to

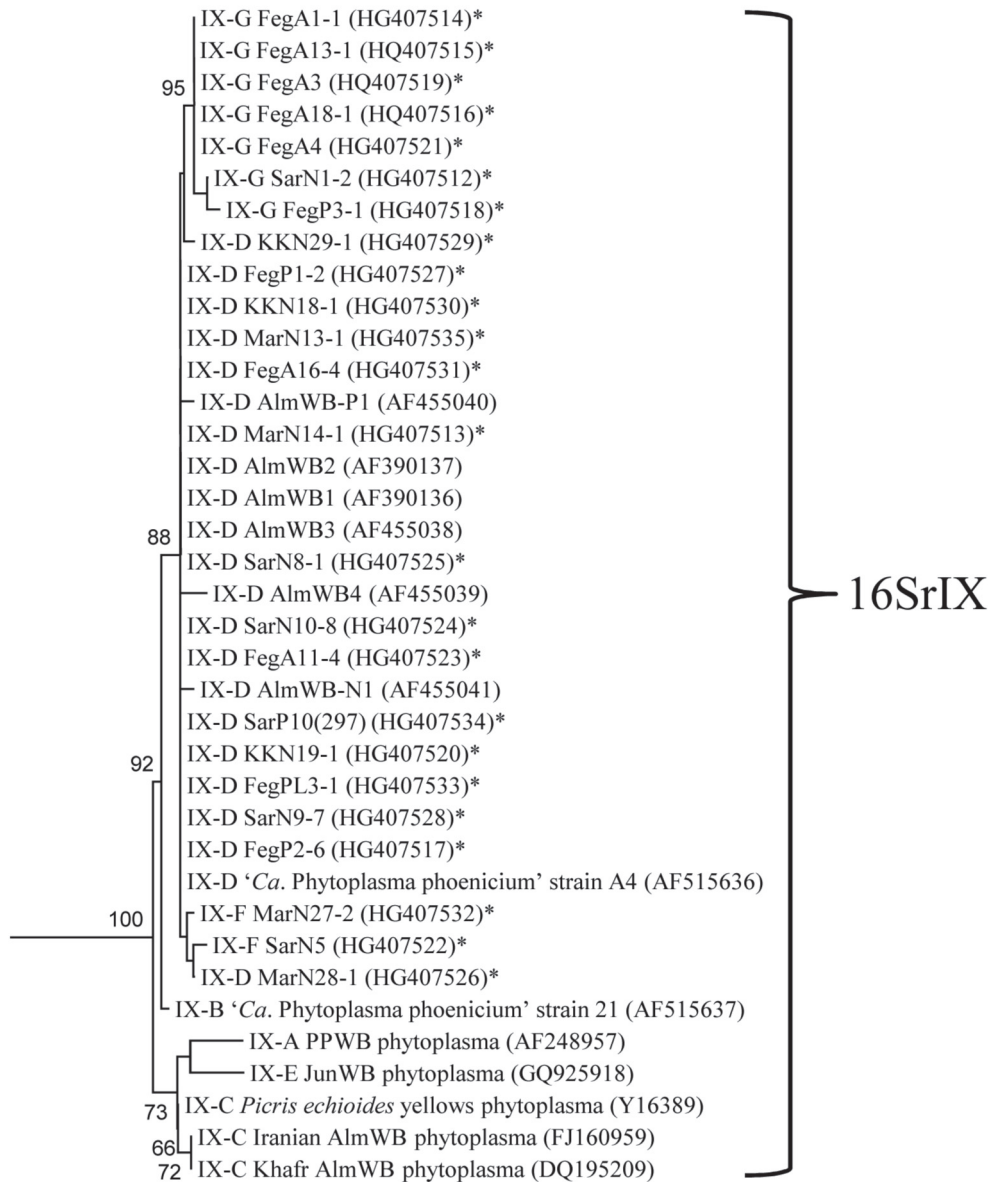


Figure 4. Group 16SrIX branch in phylogenetic tree inferred from phytoplasma 16S rDNA R16F2nR2 sequences. Bootstrap values are displayed at tree nodes. GenBank accession numbers of nucleotide sequences are shown along with the name of phytoplasma strains. Nucleotide sequences determined in this study are indicated by asterisks.

subgroup IX-F, and 54% (6/11) of the strains from Feghal belongs to the subgroup IX-G, whereas all the strains from Kerbet Kanafar (3/3) belong to the already described subgroup IX-D. '*Candidatus P. phoenicium*' strains of subgroup IX-F were found in nectarine plants, while the strains of subgroup IX-G were identified in almond, nectarine, and peach plants (Table 1). In addition, co-presence of '*Ca. P. phoenicium*' strains of diverse IX subgroups was found in individual orchards (i.e., orchards No. 1, 7, 8, 9, and 11) (Table 1). These data are evidence for diversity of '*Ca. P. phoenicium*' in Lebanon, particularly in Sarada regions, where three IX subgroups (-D, -F, and -G) co-exist and infect nectarine plants. Furthermore, phylogenetic analyses, performed in this study demonstrated divergence between Lebanese and Iranian '*Ca. P. phoenicium*' strains, both associated with AlmWB. This genetic diversity among '*Ca. P. phoenicium*' strains suggests possible influence of different ecological and/or climatic niches on phytoplasma population composition. This hypothesis is reminiscent of work by Cai *et al.* (2008), who found that genetic heterogeneity among cactus witches'-broom (CaWB) phytoplasma strains in China was correlated to environmental conditions. For example, temperature as well as other climatic factors could strongly influence the life cycle and behaviour of the insect vectors (Johannesen *et al.* 2008), conceivably altering their host plant feeding preferences and selection of '*Ca. P. phoenicium*' strains. These factors could also influence the variety and number of weeds hosting the phytoplasmas in and around orchards. Investigation of whether particular '*Ca. P. phoenicium*' subgroup(s) are correlated with symptom severity, are associated with specific plant hosts, efficiency and specificity of vector transmission of strains, or frequency in Lebanese regions and in neighboring countries, are all areas likely to provide valuable information on epidemiology of AlmWB.

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