Phytopathol. Mediterr. (2011) 50, 273–282

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

Marina MOLINO LOVA[1], Fabio QUAGLINO[1], Yusuf ABOU-JAWDAH[2], Elia CHOUEIRI[3], Hana SOBH[2], Paola CASATI[1], Rosemarie TEDESCHI[4], Alberto ALMA[4] and Piero Attilio BIANCO[1]

[1]Di.Pro.Ve.-Sezione Patologia Vegetale, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy
[2]Faculty of Agricultural and Food Sciences (FAFS), American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon
[3]Department of Plant Protection, Lebanese Agricultural Research Institute, Tal Amara, PO Box 287, Zahlé, Lebanon
[4]DIVAPRA, Entomologia e Zoologia applicate all'Ambiente, Università di Torino, Via Leonardo Da Vinci 44, 10095 Grugliasco (Torino), Italy

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, inducing 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

Corresponding authors: P.A. Bianco
Fax: +39 02 50316781
E-mail: piero.bianco@unimi.it

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 dieback. 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.nim.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): *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. 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), $F = 2Nxy/(Nx+Ny)$, in which *x* and *y* are two given strains under study; *Nx* and *Ny* are the total number of bands resulting from digestions by 17 enzymes in strains *x* and *y*, respectively; and *Nxy* 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 amplification 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 *Bst*UI RFLP pattern distinguished (similarity coefficient ≤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 ≤97%) these strains from strains in all previously described subgroups, including new subgroup IX-F, on the basis of digestion with *Taq*I (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 Juniper 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

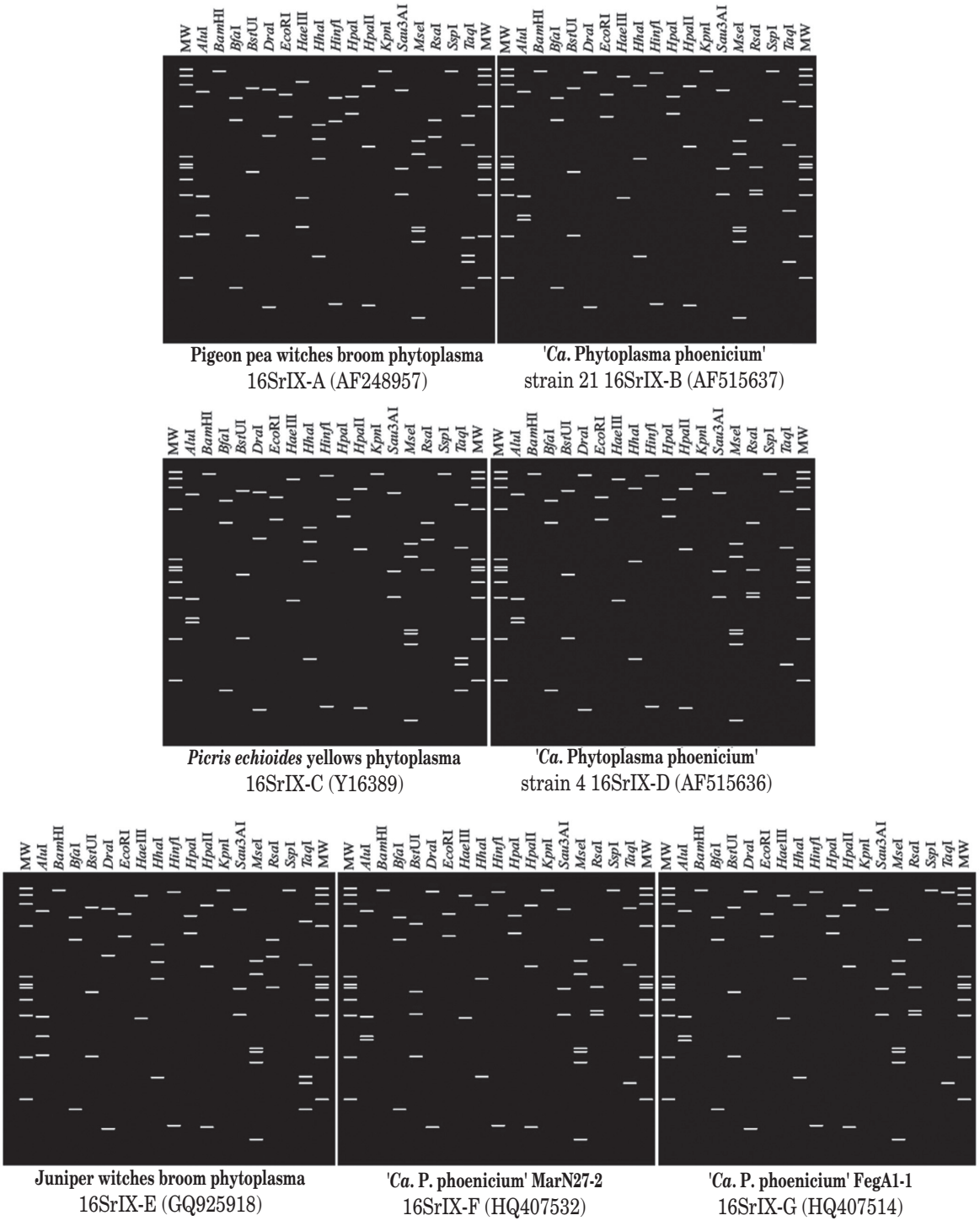

Figure 1. Virtual R16F2nR2 RFLP patterns of representative strains of 16SrIX subgroups. Recognition sites for the following 17 restriction enzymes were used in the simulated digestions: *Alu*I, *Bam*HI, *Bfa*I, *Tha*I (*Bst*UI), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. MW, FX174DNA digested by *Hae*III, fragment size (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

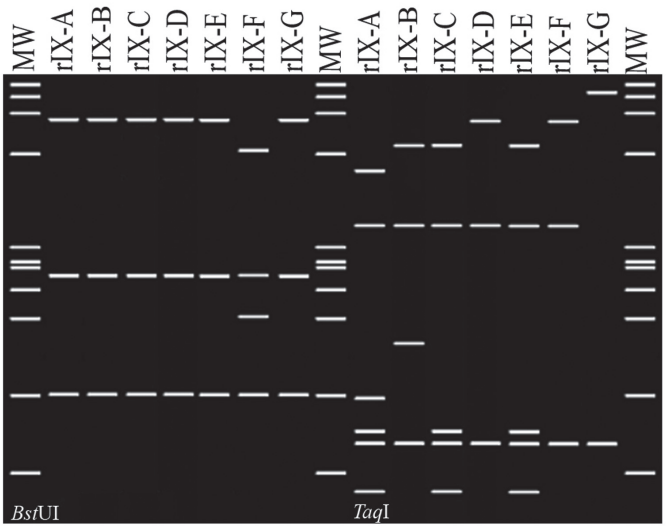

Figure 2. Virtual R16F2nR2 RFLP patterns by key enzymes *Bst*UI and *Taq*I for distinguishing among 16SrIX subgroups. MW, FX174DNA digested by *Hae*III, fragment size (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

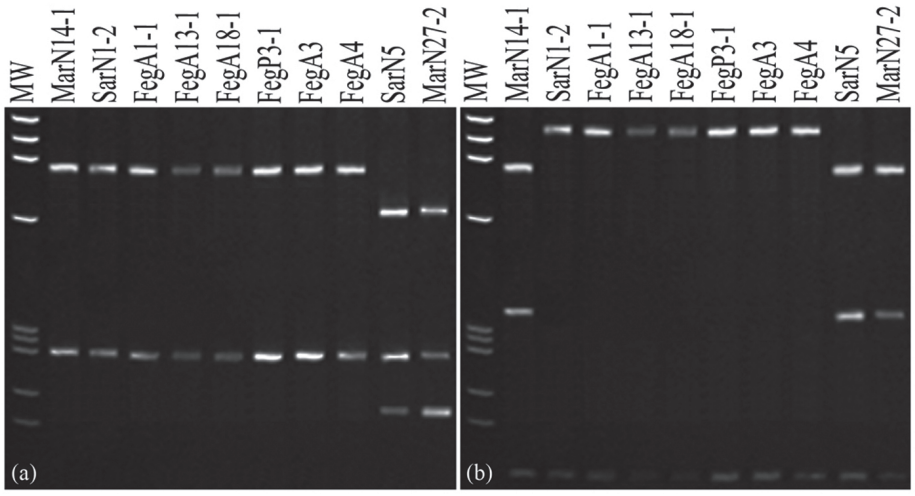

Figure 3. Actual gel-based *Bst*UI- and *Taq*I-RFLP patterns of R16F2nR2 amplicons from '*Ca.* P. phoenicium' strains. Acronyms are described in Table 1; MW, FX174DNA digested by *Hae*III, fragment size (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. Restriction fragments 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	Y16389	-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	100																		
12	**FegP3-1**	HQ407518	**-G**	75	95	84	97	82	100	93	100	100	100	97	100																	
13	**FegA3**	HQ407519	**-G**	75	95	84	97	82	100	93	100	100	100	97	100	100																
14	KKN19-1	HQ407520	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100															
15	**FegA4**	HQ407521	**-G**	75	95	84	97	82	100	93	100	100	100	97	100	100	97	100														
16	**SarN5**	HQ407522	**-F**	74	93	83	97	80	93	93	93	93	93	97	93	93	97	93	100													
17	FegA11-4	HQ407523	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100												
18	SarN10-8	HQ407524	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100											
19	SarN8-1	HQ407525	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100										
20	MarN28-1	HQ407526	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100									
21	FegP1-2	HQ407527	-D	73	90	82	93	80	90	90	90	90	90	93	90	90	93	90	90	93	93	93	93	100								
22	SarN9-7	HQ407528	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100							
23	KKN29-1	HQ407529	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100						
24	KKN18-1	HQ407530	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100	100					
25	FegA16-4	HQ407531	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	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	100	97	97	97	97	90	97	97	97	97	100			
27	FegPL3-1	HQ407533	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100	100	100	97	100		
28	SarP10(297)	HQ407534	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100	100	100	97	100	100	
29	MarN13-1	HQ407535	-D	77	97	86	100	84	97	97	97	97	97	100	97	97	100	97	97	100	100	100	100	93	100	100	100	100	97	100	100	100

[a]CPP means '*Ca.* P. phoenicium'.

[b]Lebanese '*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 described, 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

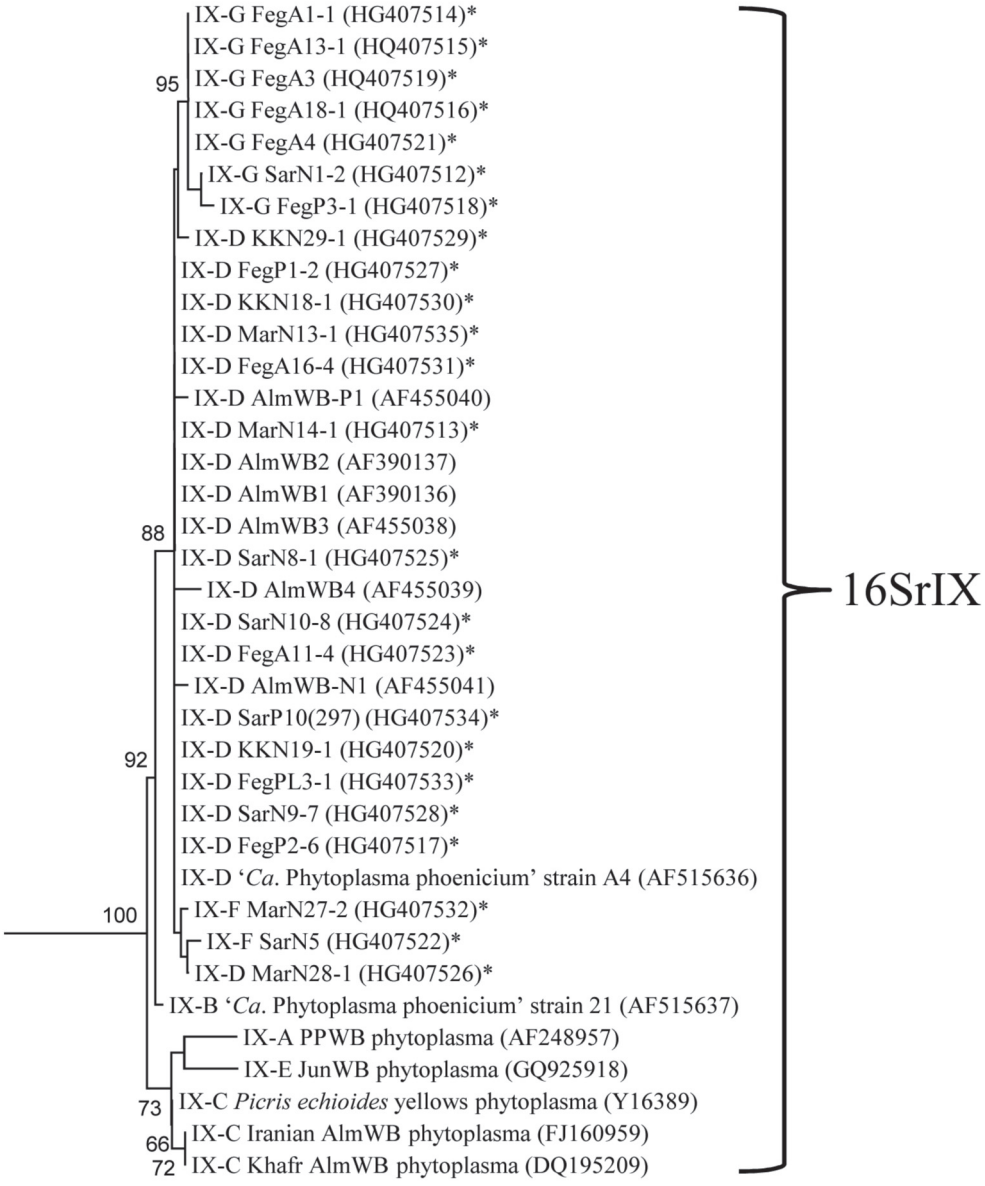

Figure 4. Group 16SrIX branch in phylogenetic tree inferred from phytoplasma 16S rDNA R16F2nR2 sequences. Bootstrap values are displayed at tree nodes. GenBank accession numbers of nucleotide sequences are shown along with the name of phytoplasma strains. Nucleotide sequences determined in this study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

Acknowledgments

We gratefully thank Dr Yan Zhao (Agricultural Research Service, United States Department of Agriculture, Beltsville, MD, USA) for his helpful technical assistance on virtual RFLP analyses, and Prof Assunta Bertaccini (DiSTA-Patologia Vegetale, Università Alma Mater Studiorum, Bologna, Italy) and Prof Carmine Marcone (University of Salerno, Italy) for providing respectively the phytoplasma reference strains NAXOS and PEY (subgroup 16SrIX-C). This study has been supported by the Italian NGO "Fondazione AVSI", in the project "Integrated management of the stone fruit Phytoplasma in Lebanon. L09A0500", financed by the Italian Cooperation.

Literature cited

Abou-Jawdah Y., A. Karakashian, H. Sobh, M. Martini and I-M. Lee, 2002. An epidemic of almond witches'-broom in Lebanon: classification and phylogenetic relationship of the associated phytoplasma. *Plant Disease* 86, 477–484.

Abou-Jawdah Y., H. Dakhil, S. El-Mehtar and I-M. Lee, 2003. Almond witches'-broom phytoplasma, a potential threat to almond, peach and nectarine. *Canadian Journal of Plant Pathology* 25, 28–32.

Abou-Jawdah Y., H. Sobh and M. Akkary, 2009. First report of Almond witches'-broom phytoplasma ('*Candidatus* Phytoplasma phoenicium') causing a severe disease on nectarine and peach trees in Lebanon. *Bulletin OEPP/EPPO* 39, 94–98.

Cai H., W. Wei, R.E. Davis, H. Chen and Y. Zhao, 2008. Genetic diversity among phytoplasmas infecting *Opuntia*: virtual RFLP analysis identifies new subgroups in the peanut witches'-broom phytoplasma group. *International Journal of Systematic and Evolutionary Microbiology* 58, 1448–1457.

Choueiri E., F. Jreijiri, S. Issa, E. Verdin, J. Bové and M. Garnier, 2001. First report of a phytoplasma disease of almond (*Prunus amygdalus*) in Lebanon. *Plant Disease* 85, 802.

Davis R.E., E.L. Dally, Y. Zhao, I-M. Lee, R. Jomantiene, A.J. Detweiler and M.L. Putnam, 2010. First report of a new subgroup 16SrIX-E ('*Candidatus* Phytoplasma phoenicium'-related) phytoplasma associated with juniper witches' broom disease in Oregon, USA. *New Disease Reports* 20, 35.

Deng S. and C. Hiruki, 1991. Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridyzation and polymerase chain reaction. *Phytopathology* 81, 1475–1479.

Doyle J.J. and J.L. Doyle, 1990. Isolation of plant DNA from fresh tissue. *Focus* 12, 13–15.

Gundersen D.E. and I-M. Lee, 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia mediterranea* 35, 144–151.

Kenyon L., N.A. Harrison, G.R. Ashburner, E.R. Boa and P.A. Richardson, 1998. Detection of a pigeon pea witches'-broom-related phytoplasma in trees of *Gliricidia sepium* affected by little-leaf disease in Central America. *Plant Pathology* 47, 671–680.

Khan A.J., A.M. Al-Subhi, A. Calari, N.A. Al-Saady and A. Bertaccini, 2007. A new phytoplasma associated with witches' broom of *Cassia italica* in Oman. *Bulletin of Insectology* 60, 269–270.

Johannesen J., B. Lux, K. Michel, A. Seitz and M. Maixner, 2008. Invasion biology and host specificity of the grapevine yellows disease vector *Hyalesthes obsoletus* in Europe. *Entomologia Experimentalis et Applicata* 126, 217–227.

Lee I-M., D.E. Gundersen-Rindal, R.E. Davis and I.M. Bartoszyk, 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology* 48, 1153–1169.

Lee I-M., R.E. Davis and D.E. Gundersen-Rindal, 2000. Phytoplasma: phytopathogenic mollicutes. *Annual Review of Microbiology* 54, 221–255.

Lee I-M., Y. Zhao, R.E. Davis, W. Wei and M. Martini, 2007. Prospects of DNA-based systems for differentiation and classification of phytoplasmas. *Bulletin of Insectology* 60, 239–240.

Molino Lova M., Y. Abou-Jawdah, E. Choueiri, L. Geagea, H. Abdul-Nour, N. Geze, H. Sobh, S. El Zammar, R. El Khoury, M. Nehme, R. El Amil, R. Fakhr, A. Haidar, C. Mortada, A. Alma, L. Picciau, R. Tedeschi, A. Vercesi, P. Casati, M. Perini, R. Maacaroun, C. Mahfoud, E. Mouannes, W. Sakr and P.A. Bianco, 2010. '*Candidatus* Phytoplasma phoenicium' in Lebanon. *Petria* 20, 694–696.

Nei M. and W.-H. Li, 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences of the United States of America* 76, 5269–5273.

Quaglino F., Y. Zhao, P.A. Bianco, W. Wei, P. Casati, G. Durante and R.E. Davis, 2009. New 16Sr subgroups and distinct SNP lineages among grapevine Bois noir phytoplasma populations. *Annals of Applied Biology* 154, 279–289.

Salehi M., K. Izadpanah and J. Heydarnejad, 2006. Characterization of a new almond witches'-broom phytoplasma in Iran. *Journal of Phytopathology* 154, 386–391.

Smart C.D., B. Schneider, C.L. Blomquist, L.J. Guerra, N.A. Harrison, U. Ahrens, K.H. Lorenz, E. Seemüller and B.C. Kirkpatrick, 1996. Phytoplasma-specific PCR primers based on sequences of the 16-23S rRNA spacer region. *Applied and Environmental Microbiology* 62, 2988–2993.

Tamura K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596-1599.

Verdin E., P. Salar, J. Danet, E. Choueiri, F. Jreijiri, S. El Zammar, B. Gélie, J.M. Bové, and M. Garnier, 2003. '*Candidatus* phytoplasma phoenicium' sp. nov., a novel phytoplasma associated with an emerging lethal disease of almond trees in Lebanon and Iran. *International Journal of Systematic and Evolutionary Microbiology* 53, 833–838.

Wei W., R.E. Davis, I-M. Lee and Y. Zhao, 2007. Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *International Journal of Systematic and Evolutionary Microbiology* 57, 1855–1867.

Zirak L., M. Bahar and A. Ahoonmanesh, 2009. Characterization of phytoplasmas associated with almond diseases in Iran. *Journal of Phytopathology* 157, 736–741.

Accepted for publication: May 19, 2011