WILD PLANTS COULD PLAY A ROLE IN THE SPREAD OF DISEASES ASSOCIATED WITH PHYTOPLASMAS OF PIGEON PEA WITCHES'-BROOM GROUP (16SrIX)

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SUMMARY

Phytoplasmas are cell wall-less prokaryotic parasites associated with diseases affecting hundreds of plant species. In the Middle East, phytoplasmas of taxonomic subgroups 16SrIX-B and -C are the etiological agents of important diseases of almond and sesame, and are transmitted from plant-to-plant by leafhoppers and planthoppers. In the present study, surveys on wild plants as reservoirs of 16SrIX phytoplasmas have been carried out in North and South Lebanon. During field surveys, leaf samples were collected from 261 wild plants belonging to 41 species within 25 families. PCR-based analyses allowed the detection of 16SrIX phytoplasmas in the leaf samples of 24 plants belonging to 12 species. Eight of such species have been reported as phytoplasma hosts for the first time. Molecular characterization by nucleotide sequence analysis of *16S rRNA* and *rplV-rpsC* genes revealed that the infected wild plant species harbored 16SrIX-C phytoplasma strains genetically undistinguishable. *In silico* enzymatic digestion of 16S rDNA sequences of phytoplasma strains, described in previous works, allowed the recognition of two new tentative subgroups (16SrIX-G and -H) within group 16SrIX. Results of the present study highlighted the wide host range of 16SrIX-C phytoplasma, associated with almond broomings, an almond witches'-broom-like disease in Iran, and with sesame phyllody in Iran and Turkey. The potential adaptation of 16SrIX-C phytoplasma to numerous wild plants highlights the elevated risk of its spread throughout the Middle East and neighbouring geographic regions.

Key words: '*Candidatus* Phytoplasma phoenicium', almond broomings, sesame phyllody, 16S rDNA, epidemiology

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INTRODUCTION

Phytoplasmas are phloem-restricted, cell wall-less prokaryotic parasites belonging to the class *Mollicutes* (Lee *et al*., 2000). They are associated with diseases affecting hundreds of plant species and are transmitted by phloem-sucking insects (Weintraub and Beanland, 2006). Their identification and differentiation is achieved mainly by DNA-based techniques. The analysis of *16S rRNA* gene nucleotide sequences allowed the delineation of two distinct classification schemes of phytoplasmas: (i) based on 16S rDNA sequence identity level, phytoplasmas were attributed to distinct species within the genus '*Candidatus* Phytoplasma' (IRPCM, 2004); (ii) based on actual and virtual restriction fragment length polymorphism (RFLP) analyses of 16S rDNA sequence, phytoplasmas were attributed to diverse 16Sr groups/subgroups (Lee *et al*., 1998; Wei *et al*., 2007; Zhao *et al*., 2009).

Phytoplasmas of taxonomic group 16SrIX (pigeon pea witches'-broom group) are associated with diseases affecting crops and wild plants in different geographic areas worldwide (Kenyon *et al*., 1998; Verdin *et al*., 2003; Khan *et al*., 2007; Davis *et al*., 2010). In the Middle East, '*Candidatus* Phytoplasma phoenicium', taxonomic subgroup 16SrIX-B (Abou-Jawdah *et al*., 2002; Lee *et al*., 2012), and its genetic variants (Molino Lova *et al*., 2011) are the etiological agents of a lethal devastating disease of almond, peach and nectarine trees (almond witches'-broom, AlmWB) in Lebanon and Iran (Choueiri *et al*., 2001; Abou-Jawdah *et al*., 2002, 2009; Verdin *et al*., 2003; Salehi *et al*., 2006). Moreover, phytoplasmas of subgroup 16SrIX-C have been associated with sesame phyllody in Turkey (Ikten *et al*., 2014) and almond broomings in Iran (Zirak *et al*., 2009), and phytoplasmas of subgroup 16SrIX-D have been reported as etiological agents of *Echinops* witches'-broom (EWB) (Al-Subhi *et al*., 2007) in Oman and *Lactuca sativa* phyllody in Iran (Salehi *et al*., 2007). In particular, phytoplasmas of subgroup 16SrIX-C have been reported

also in other continents in a large range of cultivated and wild plant hosts (Bertaccini and Duduk, 2009; Azadvar and Baranwal, 2010; Martini *et al*., 2012; Ferretti *et al*., 2014), showing a great ability of adaptation to different environments and consequently representing a candidate high-impact pathogen for agriculture worldwide. Recent studies demonstrated that phytoplasmas of subgroups 16SrIX-B and -C, associated respectively with AlmWB and sesame phyllody, are transmitted mainly by planthoppers (Hemiptera, Auchenorrhyncha, Cixiidae) and leafhoppers (Hemiptera, Auchenorrhyncha, Cicadellidae) (Abou-Jawdah *et al*., 2014; Ikten *et al*., 2014; Tedeschi *et al*., 2015). Crucially, the polyphagous feeding activity of these insects, particularly planthoppers (Weintraub and Beanland, 2006), suggested the possible role of wild plants as phytoplasma reservoirs involved in the pathogen diffusion, as reported for other phytoplasmas (Langer and Maixner, 2004; Filippin *et al*., 2009; Tedeschi *et al*., 2009; Mori *et al*., 2015). For example, concerning 16SrIX phytoplasmas, Tedeschi and colleagues (2015) showed that *Smilax aspera* L. and *Anthemis* sp. were infected by the same phytoplasma (16SrIX-B) associated with AlmWB and identified also in the insect vectors.

In the present study, surveys on wild plants as reservoirs of 16SrIX phytoplasmas have been carried out in Lebanon. Obtained data contributed to improve the knowledge of the epidemiology of diseases associated with phytoplasmas of taxonomic group 16SrIX.

MATERIALS AND METHODS

Wild plant recognition and collection. During Autumn 2011 and Spring 2012, in-depth investigation on wild plants was performed in northern and southern Lebanese areas. In the North, field surveys were carried out in Feghal, in the Caza (District) of Jbeil (Byblos), at about 165 m a.s.l., in almond orchards not irrigated and untreated with pesticides. In the South, field surveys were carried out in Sarada and Kfarkela, Caza of Marjayoun, at about 500 m a.s.l., in nectarine orchards drip irrigated and managed according to the integrated pest management principles. Since no symptoms typically associated with phytoplasma infection (i.e. yellowing, leaf malformation and discoloration, dwarfism, witches'-broom, hyper-proliferation, virescence, phyllody) were observed, in order to identify candidate plant hosts of 16SrIX phytoplasmas leaf samples were randomly collected from major asymptomatic wild plant species recognized within or at the periphery of orchards. At least one leaf sample of each species recognized was collected. Fresh leaf samples were stored at 4°C for not more than four days before total nucleic acids extraction.

Total nucleic acids extraction and 16SrIX phytoplasma detection by real-time PCR. Leaf samples collected from wild plants were prepared for total nucleic acids extraction by cutting the veins from the leaf lamina with a sterile scalpel. About 100 mg of material per sample were prepared and stored at –20°C until the extraction. Total nucleic acids were extracted using a protocol by Angelini *et al*. (2001) with some modifications. The nucleic acid pellet was air-dried, suspended in 50 µl of deionised sterile water and kept at –20°C until use.

One µl of undiluted or 1:10 diluted DNAs extracted from wild plants were used as templates in SYBR® Green real-time PCR assays, coupled with melting curve analysis, carried out to detect 16SrIX phytoplasmas. One µl of undiluted or 1:10 diluted DNAs extracted from plants infected by phytoplasma strains FegA11-4 ('*Ca*. P. phoenicium', subgroup 16SrIX-B) and PEY (*Picris echioides* yellows phytoplasma, subgroup 16SrIX-C), representatives of group 16SrIX, and phytoplasma strains EY1 ('*Ca*. P. ulmi', subgroup 16SrV-A), STOL ('*Ca.* P. solani', subgroup 16SrXII-A), and AY1 ('*Ca*. P. asteris', subgroup 16SrI-B), representatives of other 16Sr groups, served as reference controls; the phytoplasma strains PEY, EY1, STOL, and AY1 were maintained in periwinkle [*Catharanthus roseus* (L.) G. Don.], while the strain FegA11-4 was identified in AlmWB-diseased almond tree in a previous study (Molino Lova *et al*., 2011). DNA from healthy periwinkle plants and reaction mixtures without DNA template were used as negative controls. Each DNA was analyzed in triplicate. Amplification was carried out by using the 16SrIX phytoplasma group-specific primers ALW-F2 (5′-AGAGTAGCTACAACGTGAGTT-3′) and ALW-R2 (5′-GAGCTATAGGCCCAGGAT-3′) (Abou-Jawdah *et al*., 2003), amplifying a 390 bp-long fragment within the operon *rrnA*. Reaction mixture, performed in a volume of 20 µl, includes 1× Master Mix (Kapa SYBR® Fast qPCR, Kapa Biosystems, USA) and 0.4 µM of each primer. Reactions, carried out in 48-well plates in a StepOne™ System (Applied Biosystems, Italy), consisted of: one cycle at 95°C for 20 s, 40 cycles at 95°C for 3 s and 60°C for 1 min. Melting curves were analysed at the end of the real-time amplification. The PCR products were heated to 95°C for 10 s, cooled at 60°C for 1 min and then slowly heated back to 95°C at a rate of 0.3°C per second. Melting temperature (Tm) of each PCR product was calculated by StepOne™ Software v2.2.2 (Applied Biosystems, Italy). Tm of each sample was compared with those produced by amplification of the positive controls. Real-time PCR amplification was also verified by electrophoretic analysis on 1% agarose gel and visualization of DNA bands through a UV transilluminator after staining with ethidium bromide.

Moreover, in order to confirm the specificity of the amplification reaction, real-time PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit (Qiagen, Italy), cloned in plasmid vector pGEM®-T Easy (Promega, Italy) and propagated in *Escherichia coli* JM109 High Efficiency cells, following the manufacturer's instructions. DNA sequencing was performed in an ABI PRISM 377 automated DNA sequencer (Applied

Biosystems, Italy) by a commercial service (Primm, Italy). Both strands of cloned inserts were sequenced to achieve at least 4× coverage per base position. The nucleotide sequence data were assembled by employing the Contig Assembling program of the software BioEdit version 7.0.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) (Hall, 1999) and searched for sequence identity by BlastN (http://www.ncbi.nim.nih.gov/BLAST/) analysis.

16SrIX phytoplasma characterization by sequence analysis of the genes *16S rRNA* and *rplV-rpsC*. In order to determine the 16SrIX subgroup affiliation of phytoplasmas detected by real-time PCR, DNAs extracted from infected plants were used as templates in nested PCRs amplifying a partial region of 16S rDNA. In direct PCR, universal primer pair P1/P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996) was used. An aliquot of 2 µl of the diluted (1:40) P1/P7 PCR products was used as a template for the nested PCR performed with primer pair R16F2n/R16R2 (Gundersen and Lee, 1996), which amplifies a 1250 bp fragment of the gene *16S rRNA*.

A nested PCR using primer pair rpF1/rpR1 (Lim and Sears, 1992) followed by rpF1/rp(I)R1A (Martini *et al.*, 2007) was used to amplify a phytoplasma DNA segment (about 1.2 kb) of the ribosomal protein (rp) operon that encompassed genes *rplV* and *rpsC*.

All amplifications were performed with a thermocycler, Icycler (Bio-Rad, USA) in 20 µl reactions containing 200 mM each of the four dNTPs, 0.5 µM of each primer, 2 mM $MgCl_2$, 1× polymerase buffer, 1 unit of ABgene *Taq* DNA polymerase (Thermo Fisher Scientific, Germany) and 1 or 2 µl sample DNA for dPCR or nPCR, respectively. PCR reactions consisted of one cycle at 95°C for 5 min, 35 cycles at 95°C for 1 min, 50°C (dPCR) or 55°C (nPCR) for 2 min and 72°C for 3 min, and a final extension step at 72°C for 10 min. Amplified products (5 µl) were analyzed by electrophoresis in 1% agarose gel, followed by staining with ethidium bromide and visualization on UV transilluminator.

Amplicons obtained from F2n/R2 and rpF1/rp(I)R1A nested PCRs were sequenced by a commercial service (Primm, Italy) to achieve at least 4× coverage per base position. DNA sequencing was performed in an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Italy). The nucleotide sequence data were assembled by employing the Contig Assembling program of the sequence analysis software BioEdit.

Sequences were compared with those from the GenBank database by using the software BlastN (http://www.ncbi.nim.nih.gov/BLAST/). Nucleotide sequences of phytoplasmas identified in the present study (one sequence representative of each plant) were deposited in the National Center of Biotechnology Information (NCBI) GenBank database at accession numbers KP851762 to KP851774 for 16S rDNA, and from KP851775 to KP851787 for *rplV-rplC* genes.

16SrIX subgroup attribution of identified phytoplasmas by virtual RFLP analysis. Affiliation of identified phytoplasmas to taxonomic 16Sr group/subgroup was determined by *in silico* RFLP analyses of F2n/R2 amplicon nucleotide sequences carried out using the software iPhyClassifier (http://plantpathology.ba.ars.usda.gov/cgibin/resource/iphyclassifier.cgi, Zhao *et al*., 2009). The analysis was carried out also for nucleotide sequences of 16SrIX phytoplasmas recently deposited in GenBank. Briefly, 16S rDNA-amplified gene sequences, trimmed at F2n/R2 primer annealing sites, were 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, *Hinf*I, *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 (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 analyses. Nucleotide sequences of *16S rRNA* and *rplV-rpsC* genes of representative strains of group 16SrIX, obtained in the present study, and of previously described 16SrIX strains (reference strains), retrieved from GenBank, were employed for phylogenetic analyses. GenBank accession numbers are shown on the phylogenetic trees. *16S rRNA* and *rplV-rpsC* gene nucleotide sequences were compiled in FASTA format, trimmed to fragments delineated by primer annealing sites (F2n/R2 for 16S rDNA; rpF1/rp(I)R1A for *rplV-rpsC*), and aligned using the "ClustalW Multiple Alignment" application of the software BioEdit version 7.0.5. Minimum evolution analysis was carried out using the Neighbor-Joining method and bootstrap replicated 1000 times with the software MEGA6 (Tamura *et al*., 2013). Clover phyllody phytoplasma was used as out-group for rooting the trees.

RESULTS

Wild plant diversity recognized in Lebanese orchards. During the field surveys carried out in orchards of North and South Lebanon, 41 wild plant species (31 perennial and 10 annual), belonging to 25 families, were recognized. Among them, seven species were present both in northern and southern Lebanon, while 17 species were recognized only in northern Lebanon, and other 17 species only in southern Lebanon. Wild plants recognized in the examined orchards and leaf samples collected during the surveys are shown in Table 1.

Table 1. Wild plants recognized and sampled within and around orchards in Lebanon.

Species	Family	A / P	No. of samples			
			Feghal		Sarada / Kfarkela	
			Aut 2011	Spr 2012	Aut 2011	Spr 2012
Amaranthus gracilis Desf.	Amaranthaceae	A	0	0	2	0
Amaranthus graecizans L.	Amaranthaceae	A	0	0	6	0
Anagallis arvensis L.	Primulaceae	A	0	0	0	5
Apium nodiflorum (L.) Lag.	Apiaceae	P	0	0	0	1
Bryonia multiflora Boiss. & Heldr.	Cucurbitaceae	P	0	0	0	3
Capparis spinosa L.	Capparaceae	P	8	0	5	4
Cephalaria joppensis (Rchb.) Coult.	Caprofoliaceae	A	0	2	0	0
Eryngium creticum Lam.	Apiaceae	P	0	2	0	0
Erysimum bonannianum C. Presl	Brassicaceae	P	0	0	4	0
Ficus carica L.	Moraceae	P	5	0	0	0
Geranium purpureum Vill.	Geraniaceae	A	0	5	0	0
Hypericum triquetrifolium Turra	Clusiaceae	P	6	0	0	2
Inula viscosa (L.) Aiton	Asteraceae	P	5	0	6	14
Lactuca serriola L.	Asteraceae	P	0	0	6	0
Laurus nobilis L.	Lauraceae	P	6	0	0	0
Malus domestica Borkh.	Rosaceae	P	0	0	0	1
Malva sylvestris L.	Malvaceae	P	0	7	7	5
Neslia paniculata (L.) Desv.	Brassicaceae	A	0	0	1	0
Olea cuspidata Wall. & G.Don	Oleaceae	P	0	0	2	0
Olea europaea L.	Oleaceae	P	7	0	3	0
Ononis spinosa L.	Fabaceae	P	0	2	5	0
Origanum syriacum L.	Lamiaceae	P	9	0	0	0
Osyris alba L.	Santalaceae	P	6	0	0	0
Phillyrea latifolia L.	Oleaceae	P	0	2	0	0
Pistacia palaestina Boiss.	Anacardiaceae	P	7	4	0	0
Prunus domestica L.	Rosaceae	P	0	0	0	2
Rhamnus alaternus L.	Rhamnaceae	P	6	0	0	0
Rhamnus punctata Boiss.	Rhamnaceae	P	6	0	0	2
Rhus coriaria L.	Anacardiaceae	P	0	0	5	3
Rumex acetosella Koch.	Polygonaceae	P	0	0	2	0
Salvia hierosolymitana Boiss.	Lamiaceae	P	0	4	0	0
Scolymus maculatus L.	Asteraceae	A	0	0	5	0
Sinapis arvensis L.	Brassicaceae	A	0	0	0	4
Solanum nigrum L.	Solanaceae	A	9	5	6	5
Spartium junceum L.	Fabaceae	P	8	0	0	0
Tamus communis L.	Dioscoreaceae	P	0	0	0	5
Teucrium stachyophyllum P. H. Davis	Lamiaceae	P	5	0	0	0
Tribulus terrestris L.	Zygophyllaceae	A	0	0	0	4
Trifolium clypeatum L.	Fabaceae	P	0	4	0	0
Vitis vinifera L.	Vitaceae	P	1	0	0	0
Ziziphus jujuba Mill.	Rhamnaceae	P	0	5	0	0
Total		**41**	**94**	**42**	**65**	**60**

A, annual; P, perennial

Total nucleic acids were extracted from leaf samples for PCR-based molecular analyses carried out to detect the possible presence of infection by phytoplasmas of the group 16SrIX.

Detection of 16SrIX group phytoplasma by real-time PCR. SYBR Green real-time PCR assay performed using 16SrIX group-specific primer pair ALW-F2/ALW-R2 amplified DNA extracted from plants infected by phytoplasma strains FegA11-4 (16SrIX-B) and PEY (16SrIX-C) showing a Tm of 83.05°C for both the strains, and a Cq (quantification cycle) of 16 and 18 for the strains FegA11-4 and PEY, respectively. No amplification was observed for periwinkle plants infected by phytoplasma strains EY1, STOL, and AY1 and reaction mixture devoid of DNA. Sequence analysis of cloned ALW-F2/ALW-R2 fragment and comparison with GenBank database confirmed the 16SrIX group-specificity of the reaction but the impossibility to distinguish among 16SrIX subgroup strains by Tm analysis (data not shown). Thus, only PCR products, amplified from wild plants, showing a Tm of 83.05 ± 0.2°C and a Ct < 37 were associated with the presence of 16SrIX phytoplasmas in analyzed plants. Real-time amplification reactions showed the presence of 16SrIX phytoplasmas in 24 out of 261 analyzed samples belonging to 12 wild plant species (Table 2). In detail, six species of northern

Table 2. Wild plants infected by phytoplasmas of group 16SrIX in Lebanon.

Species	A / P[a]	Feghal		Sarada/Kfarkela		Phytoplasma subgroup		Representative strain[d]		
		Realtime PCR positive / total		Realtime PCR positive / total		16S rDNA[b]	*rplV-rpsC*[c]	Name	Accession Number	
		Autumn 2011	Spring 2012	Autumn 2011	Spring 2012				16S rDNA	*rplV-rpsC*
Bryonia multiflora Boiss. & Heldr.	P	-	-	-	1 / 3	16SrIX-C (1)	rpIX-C (1)	Brmul	KP851762	KP851775
Geranium purpureum Vill.	A	-	5 / 5	-	-	16SrIX-C (5)	rpIX-C (5)	Gepur	KP851763	KP851776
Inula viscosa L.	P	0 / 5	-	1 / 6	0 / 14	16SrIX-C (1)	rpIX-C (1)	Invis	KP851764	KP851777
Lactuca serriola L.	P	-	-	4 / 6	-	16SrIX-C (4)	rpIX-C (4)	Laser	KP851765	KP851778
Malus domestica Borkh.	P	-	-	-	1 / 1	16SrIX-C (1)	rpIX-C (1)	Madom	KP851766	KP851779
Malva sylvestris L.	P	-	1 / 5	0 / 7	0 / 5	16SrIX-C (1)	rpIX-C (1)	Masyl	KP851767	KP851780
Osyris alba L.	P	1 / 6	-	-	-	16SrIX-C (1)	rpIX-C (1)	Osalb	KP851768	KP851781
Pistacia palaestina Boiss.	P	-	1 / 11	-	-	16SrIX-C (1)	rpIX-C (1)	Pipal	KP851769	KP851782
Rhamnus punctata Boiss.	P	0 / 6	-	-	1 / 2	16SrIX-C (1)	rpIX-C (1)	Rhpun	KP851770	KP851783
Scolymus maculatus L.	A	2 / 5	-	-	-	16SrIX-C (2)	rpIX-C (2)	Scmac	KP851771	KP851784
Sinapis arvensis L.	A	-	-	-	4 / 4	16SrIX-C (4)	rpIX-C (4)	Siarv	KP851772	KP851785
Solanum nigrum L.	P	-	2 / 5	-	-	16SrIX-C (2)	rpIX-C (2)	Sonig	KP851774	KP851786

[a] A, annual; P, perennial.
[b] 16Sr group/subgroup affiliation determined by BlastN and iPhyClassifier analyses. Number of strains is given between parentheses.
[c] rp group/subgroup affiliation determined by BlastN analysis. Number of strains is given between parentheses.
[d] strain of 16SrIX phytoplasma group, identified in wild plants in Lebanon, which 16S rDNA and rp gene nucleotide sequences were deposited at NCBI GenBank.

Lebanon (four perennial and two annual) and six species of southern Lebanon (five perennial and one annual) have been found infected by phytoplasmas of taxonomic group 16SrIX.

Phytoplasma characterization by *16S rRNA* and ribosomal protein gene analyses. In order to determine the taxonomic subgroup of 16SrIX phytoplasmas detected in wild plants by real-time PCRs, further PCR analyses and sequencing were performed on larger 16S rDNA F2n/R2 fragments (1250 bp in size). PCR allowed to amplify the target fragment from the DNA of all the plants revealed as infected by real-time PCR assays. BlastN analysis of F2n/R2 fragments highlighted that phytoplasmas detected in the wild plants shared identical sequences among themselves and a sequence identity of >99.5% with the strains PEY (accession No. Y16389) and NaxY (accession No. HQ589191), reference strains of the subgroup 16SrIX-C. Moreover, PCR allowed to amplify the ribosomal protein gene target fragment (delimited by primer pair rpF1/rp(I)R1A) from the DNA of all the infected plants. As reported for 16S rDNA, BlastN analysis of rpF1/rp(I)R1A fragments showed that phytoplasmas detected in wild plants shared identical sequences among themselves and with the strain PEY (Acc. No. EF186802).

Phylogenetic analyses carried out on *16S rRNA* and *rplV-rpsC* genes evidenced that 16SrIX group phytoplasma strains, identified in wild plants in Lebanon, were positioned into a cluster with other phytoplasma strains of subgroup 16SrIX-C (Fig. 1, Fig. 2).

Based on the rules of phytoplasma taxonomy (Lee *et al.*, 1998; IRCPM, 2004; Wei *et al.*, 2007), group/subgroup affiliation is established by RFLP analysis of nucleotide sequence of a 16S rDNA fragment delimited by the annealing sites of the primer pair F2n/R2. iPhyClassifier analysis revealed one RFLP pattern among phytoplasma strains identified in wild plants in Lebanon (Table 3). This pattern was indistinguishable from that characteristic of strains classified in the subgroup 16SrIX-C (Fig. 3).

In addition to the iPhyClassifier analysis of 16S rDNA sequences of 16SrIX phytoplasmas identified in the present study, *in silico* digestions were carried out also from 16S rDNA nucleotide sequences (retrieved from GenBank) of other 16SrIX-C phytoplasmas or of 16SrIX phytoplasmas of unknown subgroup affiliation. The 16S rDNAs from phytoplasma strains JTBB (Accession No. JF508513), from tomato plant in Iran, and BN4 (Accession No. KJ825886), from *Argyranthemum frutescens* (L.) Webb & Berth plant in Italy, exhibited mutually distinct (similarity coefficient 92%) virtual RFLP patterns (Fig. 3) that also differed (similarity coefficient <97%) from those characteristic of previously described 16SrIX subgroups (Table 3). Therefore these strains may be tentatively considered as representative of two new subgroups 16SrIX-G (strain JTBB) and -H (strain GN4), respectively. The new tentative subgroups 16SrIX-G and -H can be distinguished from previously described subgroup 16SrIX-C (the most similar within group 16SrIX) by the enzymes *Hha*I and *Bfa*I, respectively (Fig. 3). Single nucleotide polymorphisms identified among strains of 16SrIX-C subgroup, including new tentative subgroups 16SrIX-G and -H, are shown in Table 4.

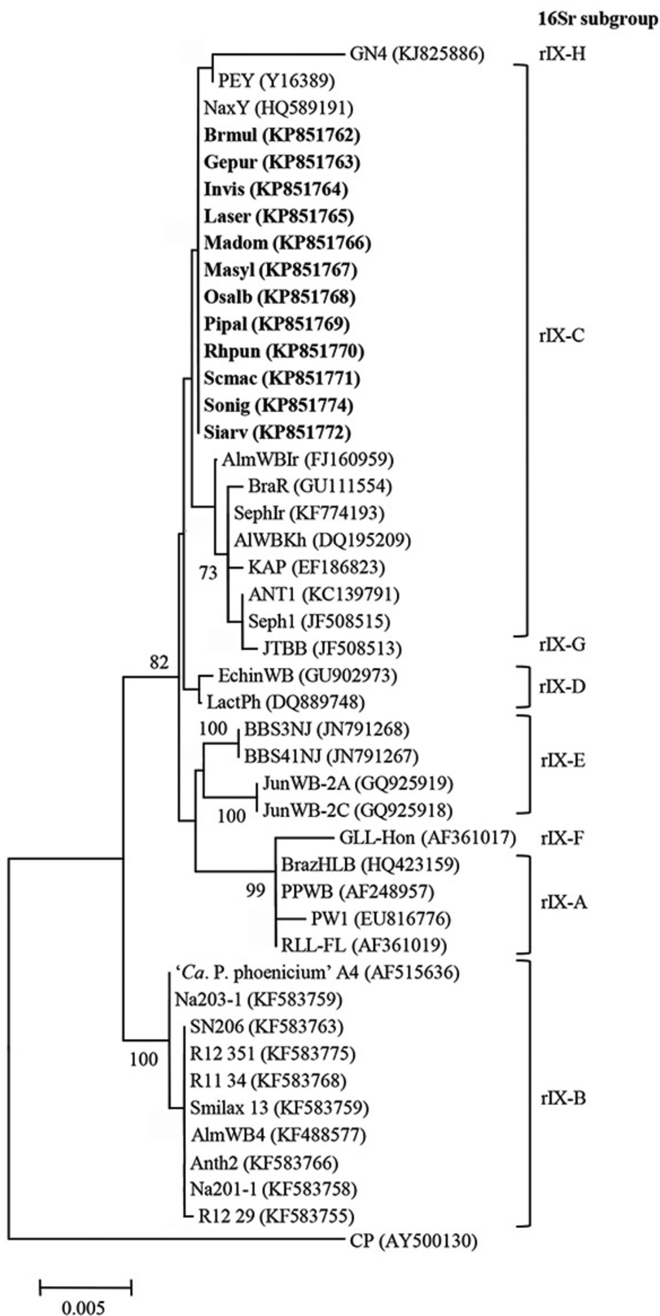

Fig. 1. Phylogenetic tree inferred from analyses of nucleotide sequences of *16S rRNA* gene. Phytoplasma strains and their nucleotide sequence accession numbers from GenBank are given in the trees. Nucleotide sequences from the present work are in bold characters. Bootstrap values lower than 70 are not shown.

DISCUSSION

Several plant diseases associated with phytoplasmas have a complex biological cycle, involving different host plants and/or insect vectors (Lee *et al*., 2000; Weintraub and Beanland, 2006). Normally, during their feeding activity, insect vectors transmit damaging phytoplasmas not only to crops, but also to wild plants that, even symptomless, can constitute phytoplasma reservoirs involved in the

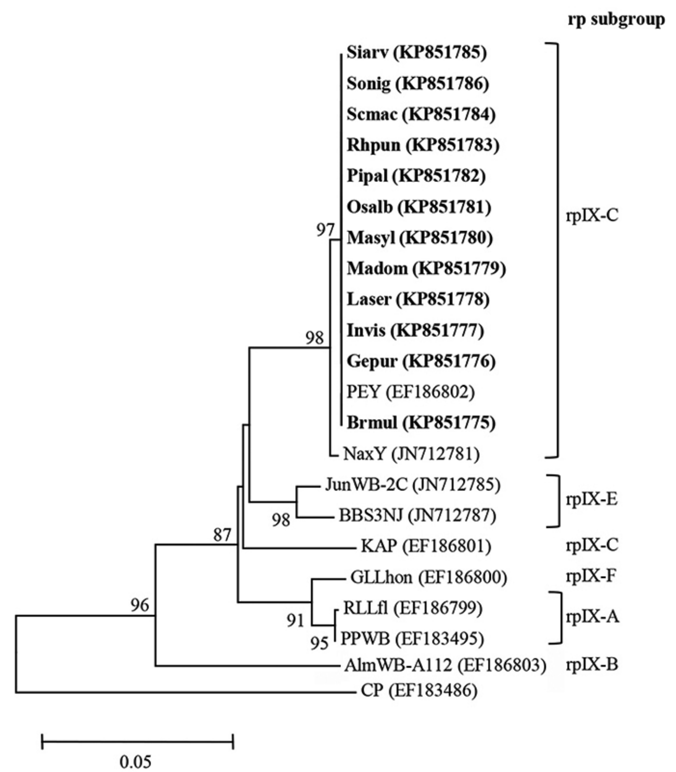

Fig. 2. Phylogenetic tree inferred from analyses of nucleotide sequences of *rplV-rpsC* gene. Phytoplasma strains and their nucleotide sequence accession numbers from GenBank are given in the trees. Nucleotide sequences from the present work are in bold characters. Bootstrap values lower than 70 are not shown.

pathogen diffusion (Langer and Maixner, 2004; Filippin *et al*., 2009; Tedeschi *et al*., 2009; Mori *et al*., 2015). In the present study, perennial and annual wild plants belonging to 12 species, recognized during field surveys in Lebanese orchards, were found infected by 16SrIX phytoplasmas. Molecular and sequence analyses of the genes *16S rRNA* and *rplV-rpsC* allowed to attribute such phytoplasmas to subgroup 16SrIX-C, associated with sesame phyllody and almond broomings in Middle East regions. Molecular characterization highlighted a great genetic homogeneity among phytoplasmas of subgroup 16SrIX-C, here identified in a wide range of wild plants. An accurate *in silico* digestion analysis, performed by iPhyClassifier online platform (Zhao *et al*., 2009), allowed the recognition of two new tentative subgroups among phytoplasma strains whose 16S rDNA nucleotide sequences were previously deposited in GenBank database.

Prior to the present study, six subgroups within group 16SrIX have been described: pigeon pea witches'-broom (PPWB) subgroup -A (Wei *et al*., 2007), '*Ca*. P. phoenicium' subgroup -B (Verdin *et al*., 2003), *Picris echioides* yellows (PEY) subgroup -C (Khan et al., 2007), *Lactuca sativa* phyllody subgroup -D (Lee *et al*., 2012), juniper witches'-broom subgroup -E (Davis *et al*., 2010), Honduran *Gliciridia* little leaf subgroup -F (Lee *et al*., 2012). Due to their common biological traits, subgroups 16SrIX-F and

Table 3. Similarity coefficients of *in silico* RFLP patterns of 16SrIX phytoplasma amplicon sequences obtained from wild plants in Lebanon.

Serial#	Strain / Acc. No.	rIX subgr	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	PPWB (AF248957)	A	1.00																						
2	GLL-Hon (AF361017)	F	0.97	1.00																					
3	BBS3NJ (JN791268)	E	0.91	0.94	1.00																				
4	PEY (Y16389)	C	0.89	0.92	0.98	1.00																			
5	EchinWB (GU902973)	D	0.84	0.85	0.91	0.93	1.00																		
6	CaPphoe A4 (AF515636)	B	0.75	0.76	0.82	0.84	0.86	1.00																	
7	ANT1 (KC139791)	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00																
8	Seph1 (JF508515)	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00															
9	JTBB (JF508513) *	G	0.86	0.89	0.93	0.95	0.88	0.81	0.95	0.95	1.00														
10	SephIr (KF774193)	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00													
11	GN4 (KJ825886) *	H	0.86	0.89	0.95	0.97	0.90	0.81	0.97	0.97	0.92	0.97	1.00												
12	Brmul (KP851762)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00											
13	Gepur (KP851763)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00										
14	Invis (KP851764)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00									
15	Laser (KP851765)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00	1.00								
16	Madom (KP851766)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00	1.00	1.00							
17	Masyl (KP851767)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00						
18	Osalb (KP851768)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00					
19	Pipal (KP851769)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00				
20	Rhpun (KP851770)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00			
21	Scmac (KP851771)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
22	Siarv (KP851772)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
23	Sonig (KP851774)[a]	C	0.89	0.92	0.98	1.00	0.93	0.84	1.00	1.00	0.95	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

* Strains JTBB and GN4 have been described as representative strains of two new tentative subgroups, -G and -H, of group 16SrIX.

[a] Phytoplasmas identified in *Bryonia multiflora* Boiss. & Heldr. (Brmul), *Geranium purpureum* Vill. (Gepur), *Inula viscosa* L. (Invis), *Lactuca serriola* L. (Laser), *Malus domestica* Borkh. (Madom), *Malva sylvestris* L. (Masyl), *Osyris alba* L. (Osalb), *Pistacia palaestina* Boiss. (Pipal), *Rhamnus punctata* Boiss. (Rhpun), *Scolymus maculatus* L. (Scmac), *Sinapis arvensis* L. (Sinap), *Solanum nigrum* L. (Sonig).

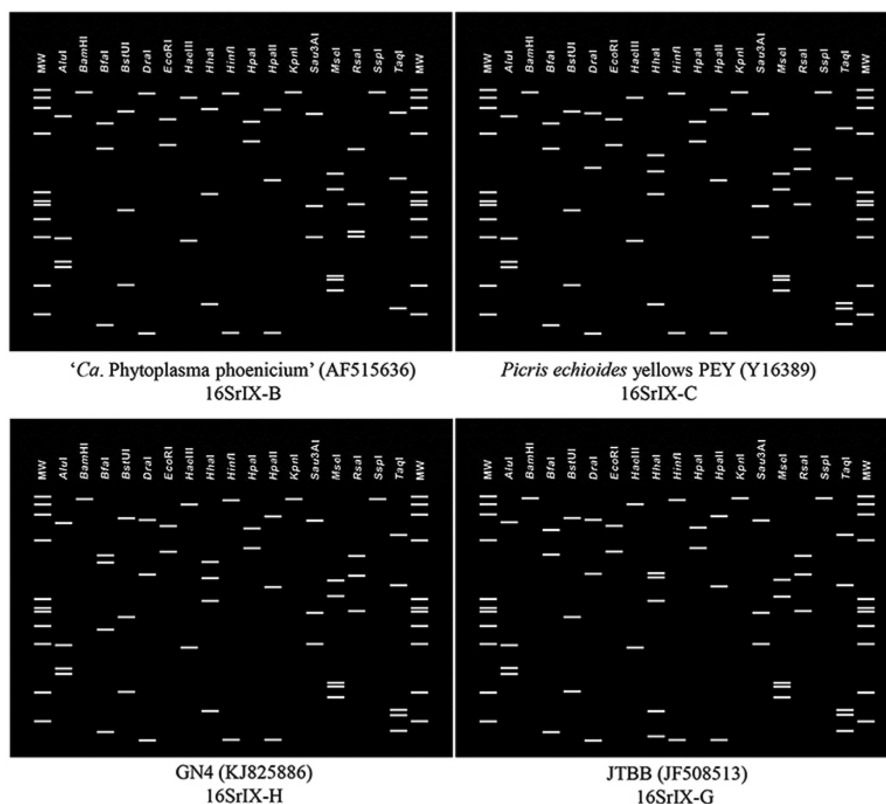

Fig. 3. Collective virtual-RFLP patterns (iPhyClassifier) of phytoplasma subgroups 16SrIX-B (A), 16SrIX-C (B), identified in wild plants in Lebanon, and of new tentative subgroups 16SrIX-G and -H.

Table 4. 16S rDNA single nucleotide polymorphisms (SNPs) discriminating phytoplasma strains within subgroup 16SrIX-C.

16SrIX-C strain	Plant host	Origin	SNP from annealing position of primer R16F2n																							
			6	7	8	18	75	255	263	275	284	305	409	478	487	541	568	658	689	690	940	964	1185	1212	1228	1229
BraR	*Brassica rapa* L.	India	A	G	T	A	A	T	A	T	T	–	–	A	T	G	A	C	–	–	–	T	–	T	C	T
KAP	*Knautia arvensis* (L.) Coulter	Italy	A	G	T	A	A	T	A	T	T	–	–	G	T	G	G	C	T	T	–	T	–	T	C	T
AlmWBIr	*Prunus dulcis* (Mill.) D.A.Webb	Iran	A	G	T	T	A	T	A	T	T	–	–	G	T	G	A	C	T	T	C	T	–	T	C	T
AlmWBKh	*Prunus dulcis* (Mill.) D.A.Webb	Iran	A	G	T	A	A	T	–	T	T	–	–	G	T	G	A	C	T	T	–	T	–	T	C	T
ANT1	*Sesamum indicum* L.	Turkey	A	G	T	A	A	T	A	T	T	–	–	G	T	G	A	C	T	T	–	T	–	C	C	T
Seph1	*Sesamum indicum* L.	Turkey	A	G	T	A	A	T	A	T	T	–	C	G	T	G	A	C	T	T	–	T	–	C	C	T
SephIr	*Sesamum indicum* L.	Iran	A	G	T	A	A	T	A	T	T	–	–	G	T	G	A	C	T	T	–	T	–	T	C	T
JTBB*	*Solanum lycopersicum* L.	Iran	A	G	T	T	A	T	A	T	T	A	C	G	T	C	A	C	T	T	–	T	C	C	C	T
GN4*	*Argyranthemum frutescens* (L.) Webb & Berth	Italy	G	A	C	T	G	C	A	C	C	–	–	G	C	G	A	T	T	T	–	C	–	T	G	A
NaxY	*Catharanthus roseus* (L.) G. Don	Italy	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	C	T	T	–	T	–	T	C	T
PEY	*Picris echioides* L.	Italy	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Brmul	*Bryonia multiflora* Boiss. & Heldr.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Gepur	*Geranium purpureum* Vill.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Invis	*Inula viscosa* L.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Laser	*Lactuca serriola* L.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Madom	*Malus domestica* Borkh.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Masyl	*Malva sylvestris* L.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Osalb	*Osyris alba* L.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Pipal	*Pistacia palaestina* Boiss.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Rhpun	*Rhamnus punctata* Boiss.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Scmac	*Scolymus maculatus* L.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Siarv	*Sinapis arvensis* L.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T
Sonig	*Solanum nigrum* L.	Lebanon	A	G	T	T	G	C	A	T	T	–	–	G	T	G	A	T	T	T	–	T	–	T	C	T

* Strains JTBB and GN4 have been described as representative strains of two new tentative subgroups, -G and -H, of group 16SrIX.

-G, originally proposed by Molino Lova *et al.* (2011), are now considered as genetic variants of subgroup 16SrIX-B ('*Ca*. P. phoenicium'). The results of the present study added two new tentative subgroups to group 16SrIX: (i) subgroup 16SrIX-G, represented by the strain JTBB identified in tomato plants showing big bud symptoms in Iran (deposited by Jamshidi *et al.* in GenBank at accession no. JF508513,); (ii) subgroup 16SrIX-H, represented by the strain GN4 identified in *A. frutescens* plant in Italy (Ferretti *et al.*, 2014).

No wild plants were found infected by '*Ca*. P. phoenicium' strain (16SrIX-B), indicating that only *Smilax aspera* and *Anthemis* spp. can be considered reservoir of such phytoplasma in Lebanon, as reported recently (Tedeschi *et al.*, 2015). The evidence that wild plant species were found infected by 16SrIX-C phytoplasma suggests that 16SrIX-C phytoplasma (i) can be transmitted plant-to-plant by an insect vector able to feed on a large number of plant species, or (ii) can be transmitted by diverse insect vectors involved in distinct ecological niches. To confirm the role of such wild plants in 16SrIX phytoplasma transmission, additional research should be carried out to investigate their association with the larval and adult stages of the known insect vector(s) (Abou-Jawdah *et al.*, 2014; Ikten *et al.*, 2014; Tedeschi *et al.*, 2015). Moreover, considering that perennial plants are the main phytoplasma reservoirs and hosts of the vectors (Weintraub and Beanland, 2006), it is interesting to report the presence of 16SrIX-C phytoplasma in three annual wild plants (*Geranium purpureum* Vill., *Scolymus maculatus* L., and *Sinapis arvensis* L.). The possible role of annual wild plants in phytoplasma diffusion could be explained by different hypotheses. Firstly, these plants could favour the phytoplasma diffusion over the years by means of seeds, as reported for other annual plants (Olivier *et al.*, 2010; Calari *et al.*, 2011). Secondly, some infections in the wild plants might result from alternative epidemiological cycles with or without relation to crops. Cixiids are known to be hypogeic organisms during nymphal stage feeding on roots of their host plants. They normally become infected during some of juvenile instars (Weintraub and Beanland, 2006). Some cixiid species, such as *Cixius* sp. and *Hyalesthes obsoletus* Signoret, were reported to be able to accomplish two generations per year in the Middle East. The same bivoltinism was observed in the genus *Tachycixius* as well (Tedeschi *et al.*, 2015). This behaviour highlights the possible role of the annual plants species in harbouring the vector(s) larval stage which might be able to acquire 16SrIX-C phytoplasma from these plants. Considering the activity period of adult planthoppers observed in previous study (Abou-Jawdah *et al.*, 2014; Tedeschi *et al.*, 2015), feeding of infective adult vectors on annual wild plants could explain the occurrence of infected plants. On the other hand, such plants could constitute the inoculation target and the acquisition source of bivoltine vector(s).

In the present study, eight wild plants (*Bryonia multiflora* Boiss & Heldr., *G. purpureum*, *I. viscosa*, *Osyris alba*

L., *P. palaestina*, *Rhamnus punctata* Boiss., *S. maculatus*, and *S. arvensis*) were found infected by phytoplasmas for the first time. Interestingly, the identification of 16SrIX-C phytoplasmas in wild plants of the genera *Pistacia* and *Sinapis* indicates the possibility that these phytoplasma strains could be transmitted also to the species *Pistacia vera* L. and *Sinapis alba* L., two important crops related to food production in the Mediterranean basin. Among the other plants, 16SrIX phytoplasmas have been detected in *Lactuca serriola* showing phyllodies in Iran (Salehi *et al*., 2007). On the other hand, *Malus domestica* Borch., *M. sylvestris* and *S. nigrum* were never reported before as host plants of 16SrIX phytoplasmas, but were reported in previous studies as potential reservoirs of phytoplasmas belonging to different taxonomic groups. *M. domestica* is the main crop plant infected by '*Ca*. P. mali' (subgroup 16SrX-A), the etiological agent of apple proliferation disease in Europe (Seemüller and Schneider, 2004). Recently, apple trees showing typical hyper-proliferation symptoms have been found infected by '*Ca*. P. asteris' (group 16SrI) and '*Ca*. P. aurantifolia' (group 16SrII) in Iran (Hashemi-Tameh *et al*., 2014). *M. sylvestris* was found infected by genetically distinct phytoplasmas in Europe, such as '*Ca*. P. solani' (subgroup 16SrXII-A), associated with bois noir disease of grapevine in vineyards (Mori *et al*., 2015), and '*Ca*. P. rubi' (subgroup 16SrV-E), associated with *Rubus* stunt disease (Jarausch *et al*., 2001). *S. nigrum* was recognized as host plant of '*Ca*. P. aurantifolia' in Iran (Samavi *et al*., 2012) and of '*Ca*. P. solani' in European countries (Weber and Maixner, 1998; Batlle *et al*., 2000; d'Aquilio *et al*., 2002; Credi *et al*., 2006; Sabaté *et al*., 2014; Mori *et al*., 2015). In previous work, '*Ca*. P. asteris', '*Ca*. P. mali' and '*Ca*. P. solani' were detected in cixiids from northern and southern Lebanon (Tedeschi *et al*., 2015). This evidence suggests the possibility that *M. domestica*, *M. sylvestris* and *S. nigrum* could act as plant hosts involved in the diffusion of such phytoplasmas in Lebanon and in the Euro-Mediterranean basin. Further studies should be carried out to investigate the presence of those phytoplasmas in a significant number of plants.

Results of the present study evidenced that wild plants, recognized in Lebanese orchards, harbour 16SrIX-C phytoplasma, similar but genetically distinct from the etiological agent of AlmWB disease (16SrIX-B). Intriguingly, 16SrIX-C phytoplasma is associated with AlmWB-like disease in Iran, *Sesamum indicum* L. disease in Iran and Turkey, and it is reported also in other continents (Bertaccini and Duduk, 2009; Azadvar and Baranwal, 2010; Martini *et al*., 2012; Ferretti *et al*., 2014). The wide range of 16SrIX-C phytoplasma host plants, detected in this study, highlights the elevated risk of its spread both within Middle East regions and throughout geographically distinct areas. Interestingly, the wide spread of 16SrIX-C phytoplasmas over large geographical areas suggests the presence of efficient insect vector(s). The knowledge of the insect vectors is one of the crucial keys for managing a disease and to avoid further spreading to other geographical areas. As nothing or very few is known about insect vectors of 16SrIX-C phytoplasma, further efforts will be required to identify these insects.

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