Identification of four distinct ‘Candidatus Phytoplasma’ species in pomegranate trees showing witches' broom, little leaf and yellowing in Jordan, and preliminary insights on their putative insect vectors and reservoir plants

Identification of four distinct '*Candidatus* Phytoplasma' species in pomegranate trees showing witches' broom, little leaf and yellowing in Jordan, and preliminary insights on their putative insect vectors and reservoir plants

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Abstract

During field surveys conducted in northern Jordan from June to November 2020, phytoplasma-like symptoms, including leaf yellowing/reddening and rolling, little leaf and witches' broom were observed in pomegranate. Disease incidence in 22 surveyed orchards ranged from 30% to 65%. Nested PCR-based amplification of 16S rRNA gene detected phytoplasmas in 17% of collected symptomatic pomegranate trees. Amplicon nucleotide sequence analyses allowed attributing the detected phytoplasmas to '*Candidatus* Phytoplasma solani', '*Ca*. P. aurantifolia', '*Ca*. P. asteris' and '*Ca*. P. ulmi'. These phytoplasmas were found in plants showing specific symptoms and differentially distributed in the considered locations. Additionally, three cicadellids (*Macrosteles sexnotatus*, *Cicadulina bipunctata* and *Psammotettix striatus*) and two non-crop plants (*Plantago major* and *Capsicum annuum*) resulted hosting '*Ca*. P. asteris' strains, and one cicadellid (*Balclutha incisa*) was carrying a '*Ca*. P. solani' strain. A new pomegranate disease complex associated with multiple phytoplasmas, including '*Ca*. P. aurantifolia' and '*Ca*. P. ulmi', never reported before in this host plant, is described here. Moreover, preliminary indications are provided on its possible epidemiology in Jordan, involving two putative insect vectors (*M. sexnotatus*, *B. incisa*) first reported in the Country.

1 INTRODUCTION

Pomegranate (*Punica granatum* L.) is a high value horticultural crop of tropical and subtropical regions of the world native to Central Asia (Still, 2006). It is a woody deciduous shrub, or a small tree adapted to a wide range of environments from mild temperate to sub-tropical, and relatively drought tolerant (Kahramanoglu & Usanmaz, 2016). Pomegranate fruits have nutritional benefits and are used to obtain many pharmaceutical products (Calani et al., 2013; Fahmy et al., 2020; Sun et al., 2021). In Jordan, pomegranate is one of the oldest cultivated fruit trees and a major source of income for the farmers.

Recently, pomegranate diseases associated with phytoplasmas presence were reported in Turkey and Iran (Gazel et al., 2016; Salehi et al., 2016). Phytoplasmas are obligate bacteria restricted to the phloem tissue of host plants and transmitted by phloem-feeding insects (Weintraub & Beanland, 2006). Based on 16S rRNA gene nucleotide sequence identities, 47 '*Candidatus* Phytoplasma' species have been described (Bertaccini & Lee, 2018; Jardim et al., 2021; Jones et al., 2021; Kirdat et al., 2021; Zhao et al., 2021). Phytoplasma-infected plants exhibit various symptoms including stunting, phyllody, shortened internodes, yellows, little leaf, witches' broom and vascular discoloration. Such symptoms are related to diseases in over a 1000 plant species worldwide and may cause losses up to 70%–100% in the case of severe epidemic outbreaks (Bertaccini et al., 2014; Ermacora & Osler, 2019; Hemmati et al., 2021; Kumari et al., 2019).

In Jordan, phytoplasma-associated diseases as well as their epidemiology are still poorly studied. '*Ca*. P. solani' was associated with 'bois noir' of grapevine (Salem et al., 2013) and plum yellowing and witches' broom (Salem et al., 2020), '*Ca*. P. trifolii' with tomato big bud (Anfoka et al., 2003), '*Ca*. P. asteris' with peach yellowing and reddening (Anfoka & Fattash, 2004), '*Ca*. P. aurantifolia' with potato reddening and tuber deformation (Salem et al., 2019) and '*Ca*. P. ulmi' with date palm stunting and yellowing (Alhudaib et al., 2019). Considering the increasing importance in Jordan of both pomegranate and phytoplasma diseases, the present study aimed to (i) survey the presence of phytoplasma-associated diseases of pomegranate in family and commercial orchards, (ii) detect and identify the phytoplasmas associated with such diseases and (iii) explore the presence of putative insect vectors and reservoir plants of the identified phytoplasmas.

2 MATERIALS AND METHODS

2.1 Field surveys, plant sampling and insect collection

From June to November 2020, field surveys were conducted in 20 family and two commercial pomegranate orchards, including local (Khdary, Mawardy, Erqaby) and imported (Wonderful) cultivars, in five locations in the governorates of Irbid (Kufr soum and Jdita), Ajloun (Arjan) and Al-Mafraq (Sabha and Umm jamal) in northern Jordan (Figure 1). In the surveyed orchards (8.6 ha), representative of the considered pomegranate cultivation area in the northern regions of the Country (90 ha), 5160 pomegranate trees were monitored. In each location, incidence of phytoplasma symptoms was estimated as the percentage of symptomatic trees out of the observed ones. Leaf samples were collected from 112 pomegranate trees exhibiting different symptoms (Table 1). Moreover, leaf samples were collected from 13 symptomless pomegranate trees and 30 plants of 11

non-crop weed species (*Amaranthus* sp., *Bidens* sp., *Chenopodium album*, *Capsicum annuum*, *Convolvulus arvensis*, *Inula* sp., *Lactuca* sp., *Malva sylvestris*, *Origanum vulgare*, *Plantago major*, *Rubus* sp.) showing suspicious symptoms, observed within and around the surveyed orchards. Collected samples were transferred to the laboratories of National Agricultural Research Center, Baqaà, Jordan, and maintained at 4°C until total nucleic acids (TNAs) extraction. In parallel, during the field survey carried out in Irbid and Ajloun governorates, insects within pomegranate orchards were collected by entomological sweeping net and transferred to the laboratories. Stereomicroscope observation was conducted for preliminary selection of phloem-feeding insects. The selected insects were kept in 99% ethanol until their identification based on stereomicroscope observation of phenotypic characters and male genitalia after their dissection and soaking into a 10% potassium hydroxide solution to dissolve soft tissues and clear the cuticle. Insects recognized at genus/species level were maintained in 99% ethanol at −20°C until nucleic acids extraction.

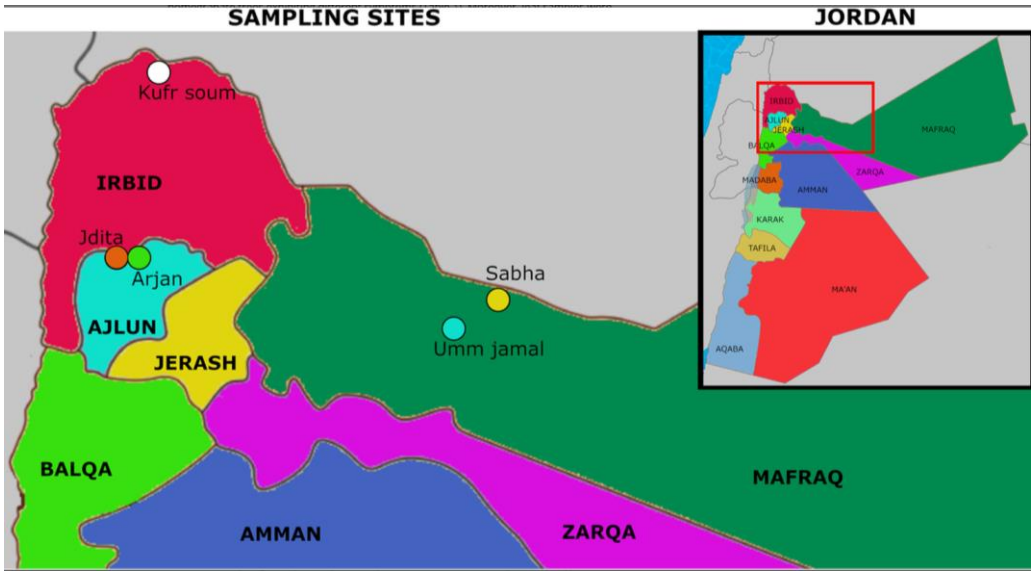

FIGURE 1 Maps of governorates and locations in North Jordan where the surveys on phytoplasma-like diseases in pomegranate orchards were conducted.

TABLE 1. Results of sampling and testing the pomegranate trees in the surveyed locations in northern Jordan

Governorate	**Location**	**Pomegranate cultivar**	**No. orchards**	**Average orchard dimension (ha)**	**No. surveyed trees**	**No. symptomatic trees**	**Disease incidence (%)**	**No. collected trees**	**No. positive trees**	**Infection rate (%)**
Irbid	Kufr soum	Khdary	12	0.5	3600	1440	40	50	11	22

Governorate	Location	Pomegranate cultivar	No. orchards	Average orchard dimension (ha)	No. surveyed trees	No. symptomatic trees	Disease incidence (%)	No. collected trees	No. positive trees	Infection rate (%)
	Jdita	Mawardy	7	0.3	1260	630	50	20	7	35
Ajloun	Arjan	Unknown	1	0.2	120	78	65	12	0	0
Al-Mafraq	Sabha	Wonderful	1	0.1	60	18	30	10	1	10
	Umm jamal	Erqaby	1	0.2	120	36	30	20	0	0
	Overall		22	8.6	5160	2202	42.7	112	19	17

2.2 Phytoplasma detection

TNAs were extracted from the collected plants and insects as described respectively by Angelini et al. (2001) and Marzachì et al. (2008), with some modifications. Concerning plant samples, for each pomegranate tree, leaf midribs and petioles from eight to 11 leaves were mixed, weigthed (1 g) and ground in 4 ml of prewarmed 2.5% CTAB-based buffer in sterile mortars. Regarding insects, for each species, TNA extraction was done from single, or pooled (2–5) individuals based on their size and/or number of captured specimens. Individual or pooled insects were crushed by sterile pestles in a 1.5 ml tubes containing sand and 0.5 ml of prewarmed 2% CTAB-based buffer. Extracted TNA was washed by 0.3 ml of 70% ethanol, dissolved in 50 (insects) or 100 (plants) µl of TE buffer, measured for quantity and quality by Nanodrop system, and stored at −20°C.

Nested PCRs were carried out on 25–50 ng of extracted TNAs (three replicates per sample) using the primer pair P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995) followed by the primer pair R16F1/R16R0 (Lee et al., 1995). Reaction mixtures and conditions were as previously described (Quaglino et al., 2009). TNAs extracted from periwinkle (*Catharanthus roseus* L. [G. Don]) plants, infected by phytoplasma strains STOL (‘*Ca*. P. solani’, subgroup 16SrXII-A, GenBank Acc. No. AF248959) and AY1 (‘*Ca*. P. asteris’, subgroup 16SrI-B, GenBank Acc. No. AY265210) and maintained in greenhouse at Department of Agricultural and Environmental Sciences, University of Milan (Italy), were employed as positive controls. TNAs extracted from healthy periwinkle and reaction mixtures devoid of TNAs were used as negative controls. PCR products (6 µl) were analysed by electrophoresis on 1% (w/v) agarose gels in 1× TBE buffer, stained with Midori Green, and visualised on UV transilluminator.

Based on the obtained results, phytoplasma infection rate was estimated (i) in plants, as the percentage of infected plants out of the examined ones in each location; (ii) in insects, as the percentage of infected pools out of the examined ones for each species.

2.3 Phytoplasma identification

Three F1/R0 PCR products (technical replicates), amplified independently from each phytoplasma-infected plant and insect, were sequenced in both strands by a commercial service (Eurofins Genomics, Germany). For each sample, nucleotide sequences of the three F1/R0 fragments were assembled by the Contig Assembling Program, trimmed to the annealing sites of the F1/R0 primer pairs, and aligned to obtain a consensus sequence in the software BioEdit, version 7.1.3.0 (Hall, 1999). Trimmed nucleotide consensus sequences were aligned using the ClustalW Multiple Alignment program and analysed by Sequence Identity Matrix in the software BioEdit to estimate their genetic diversity. For attribution to '*Ca*. Phytoplasma' species, one 16S rDNA nucleotide sequence randomly selected among each group of identical sequences obtained in this study was aligned with those of the 49 '*Ca*. Phytoplasma' species described in literature (Bertaccini et al., 2022) and compared for their sequence identity in the software Bioedit. '*Ca*. Phytoplasma' species attribution was confirmed searching the species-specific signature sequences within the analysed F1/R0 nucleotide sequences, and by analysis on *i*PhyClassifier online tool (Wei et al., 2008). For ribosomal group/subgroup attribution, 16S rDNA sequences were analysed by virtual Restriction Fragment Length Polymorphism (RFLP) using the online tool *i*PhyClassifier (Zhao et al., 2009).

Nucleotide sequences of 16S rRNA gene of phytoplasmas identified in the present study (one strain among those sharing identical 16S rDNA sequence) and reference strains of '*Ca*. Phytoplasma' species were employed for phylogenetic analyses with the software MEGA X (Kumar et al., 2018). The evolutionary distances were computed using the Maximum Composite Likelihood method. The Minimum-Evolution tree was searched using the Close-Neighbor-Interchange algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree and bootstrap replicated 1000 times. All ambiguous positions were removed for each sequence pair. There were a total of 1286 positions in the final dataset. *Acholeplasma palmae* (GenBank Acc. No. L33734) was used for rooting the tree.

3 RESULTS

3.1 Phytoplasma-like symptoms observed in pomegranate trees and weeds

In Irbid governorate, Khdary pomegranate cultivar trees in Kufr soum orchards were found exhibiting leaf reddening and rolling, leaf yellowing and little leaf and leaf discoloration (Figure 2a–c) with an incidence of around 40%. In the same governorate in Jdita orchards, Mawardy pomegranate cultivar trees showed witches'-broom, little leaf and yellowing (Figure 2d,e) with an incidence of around 50%. In Ajloun governorate, pomegranate trees showing leaf reddening (Figure 2f) were observed with an incidence of around 65%. In Al-Mafraq governorate, leaf reddening and/or yellowing symptoms (Figure 2g) were observed in Wonderful (Sabha) and Erqaby (Umm jamal) pomegranate cultivars with an incidence of around 30%. Within and around pomegranate orchards, leaf yellowing and/or reddening and little leaf were observed in *Convolvulus arvensis* L., *Capsicum annuum* L., *Rubus* sp., *Malva sylvestris* L., *Chenopodium album* L., *Plantago major* L., *Origanum vulgare* L., *Bidens* sp., *Inula* sp., *Amaranthus* sp. and *Lactuca* sp. (Figure 2h).

FIGURE 2. Phytoplasma-like symptoms observed in pomegranate trees and weeds in northern Jordan. Leaf reddening and rolling (a), leaf yellowing and little leaf (b) and leaf discoloration (c) exhibited by Khdary pomegranate cultivar in Kufr soum, Irbid governorate; witches'-broom, little leaf and yellowing (d,e) exhibited by Mawardy pomegranate cultivar in Jdita, Irbid governorate; leaf reddening (f) in pomegranate in Arjan, Ajloun governorate; leaf reddening and/or yellowing (g) in Wonderful and Erqaby pomegranate cultivars respectively in Sabha and Umm jamal, Al-Mafraq governorate; leaf yellowing and little leaf (h) in *Convolvulus arvensis* in Kufr soum, Irbid governorate.

3.2 Molecular detection and identification of phytoplasmas in plants

Nested PCRs allowed detecting the presence of phytoplasmas in 26 out of 155 analysed plant samples. In detail, F1/R0 amplicons of the expected size (around 1310 bp) were obtained in 19 out of 112

symptomatic pomegranate trees (17%). Infection rate in pomegranate changed in relation to the orchard locations. Higher infection rate was found in Jdita (35%), followed by Kufr soum (22%) and Sabha (10%). No phytoplasmas were identified in symptomatic trees in Arjan and Umm jamal locations (Table 1). No amplification was obtained in symptomless pomegranate plants. Regarding weeds, three out of 11 analysed weed species were found positive to phytoplasma presence: *C. arvensis* (5 out of 8), *C. annuum* (1 out of 2) and *P. major* (1 out of 1).

The phytoplasma strains detected in the present study in symptomatic pomegranate trees were attributed to '*Ca*. P. solani' (10 strains out of 19), '*Ca*. P. aurantifolia' (4 out of 19), '*Ca*. P. ulmi' (3 out of 19) and '*Ca*. P. asteris' (2 out of 19) (Table 2). Phytoplasma clustering in phylogenetic tree confirmed the attribution to these '*Ca*. Phytoplasma' species (Figure 3). Based on similarity coefficient obtained by comparison of virtual RFLP patterns, '*Ca*. P. solani' strains were attributed to ribosomal subgroup 16SrXII-A, '*Ca*. P. aurantifolia' strains to subgroup 16SrII-B, '*Ca*. P. ulmi' strains to subgroup 16SrV-A (strains PG1 and PG17) and its variant (strain PG7) and '*Ca*. P. asteris' strains to subgroups 16SrI-B (strain PG18) and a variant of subgroup 16SrI-R (strain PG784) (Table 2; Figure 4).

TABLE 2. '*Candidatus* Phytoplasma' species and ribosomal subgroups of phytoplasmas detected in pomegranate trees surveyed

Location	**Plant host**	**Symptoms**	**Phytoplasma species**	**16Sr subgroup (similarity coefficient)**	**No. of strains**	**Representative strain**	**Acc. No.**
Kufr soum	*Punica granatum* L.	Leaf discoloration	'*Ca*. P. asteris'	I-R* (0.98)	1	PG784	OL873108
Kufr soum	*Punica granatum* L.	Leaf reddening and rolling	'*Ca*. P. aurantifolia'	II-B (1.00)	4	PG795	OL873109
Kufr soum	*Punica granatum* L.	Yellowing, little leaf	'*Ca*. P. solani'	XII-A (1.00)	4	PG797	OL873110
Kufr soum	*Punica granatum* L.	Leaf reddening and rolling	'*Ca*. P. ulmi'	V-A (1.00)	1	PG1	OL873111
Kufr soum	*Punica granatum* L.	Leaf reddening and rolling	'*Ca*. P. ulmi'	V-A* (0.98)	1	PG7	OL873112
Kufr soum	*Plantago major* L.	Symptomless	'*Ca*. P. asteris'	I-B (1.00)	1	PM14	OL873113
Kufr soum	*Capsicum annuum* L.	Yellowing	'*Ca*. P. asteris'	I-B (1.00)	1	CAn22	[a]

Location	**Plant host**	**Symptoms**	**Phytoplasma species**	**16Sr subgroup (similarity coefficient)**	**No. of strains**	**Representative strain**	**Acc. No.**
Kufr soum, Umm jamal	*Convolvulus arvensis* L.	Yellowing, little leaf	'*Ca*. P. aurantifolia'	II-C* (0.99)	5	CAr403	OL873114
Jdita	*Punica granatum* L.	Witches'-broom, little leaf	'*Ca*. P. asteris'	I-B (1.00)	1	PG18	[a]
Jdita	*Punica granatum* L.	Witches'-broom, little leaf	'*Ca*. P. solani'	XII-A (1.00)	5	PG624	[b]
Jdita	*Punica granatum* L.	Witches'-broom, little leaf	'*Ca*. P. ulmi'	V-A (1.00)	1	PG17	[c]
Sabha	*Punica granatum* L.	Yellowing	'*Ca*. P. solani'	XII-A (1.00)	1	PG404	[b]

- [a] Nucleotide sequences identical to OL873113.
- [b] Nucleotide sequences identical to OL873110.
- [c] Nucleotide sequences identical to OL873111. *subgroup variant.

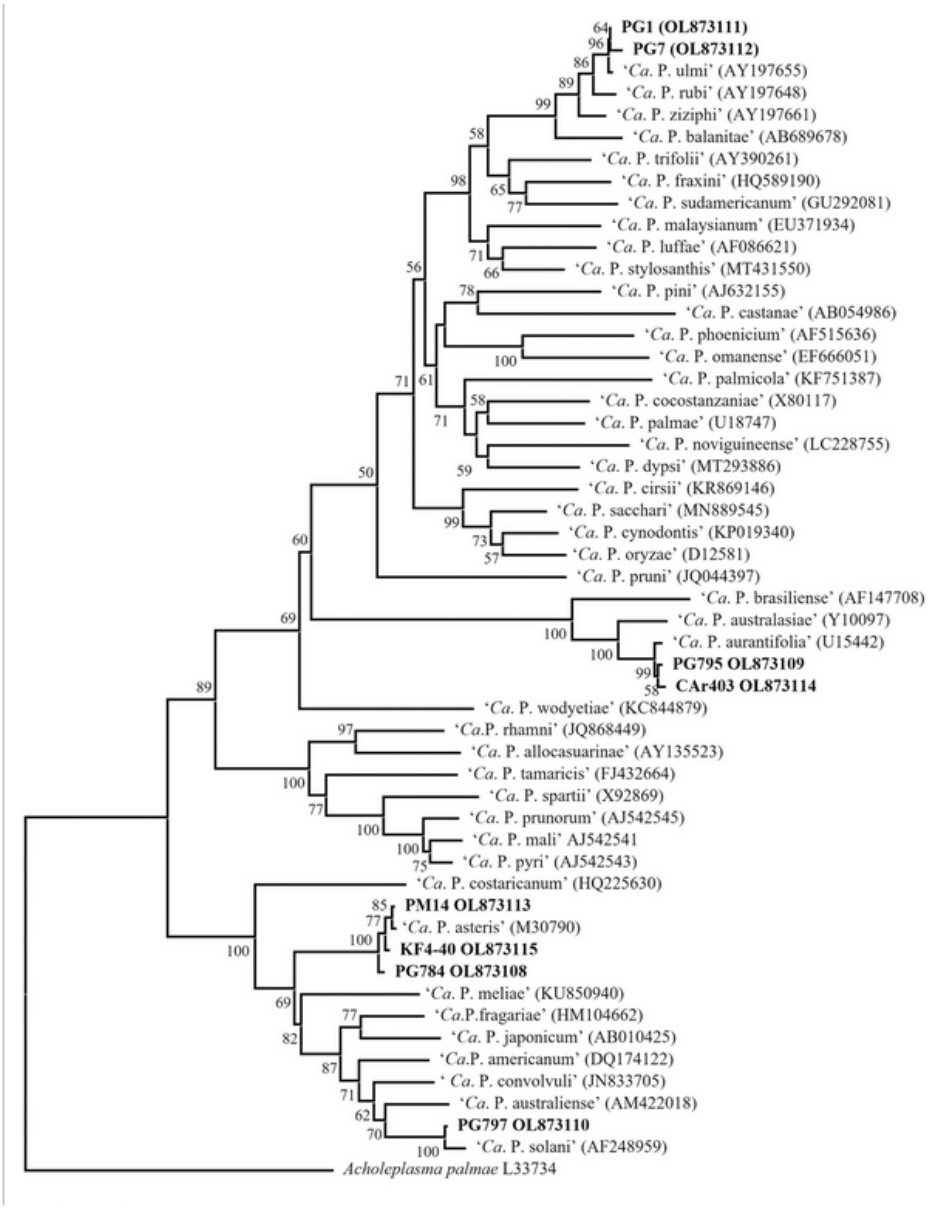

FIGURE 3. Phylogenetic tree based on the alignment of 16S rDNA nucleotide sequences of representative phytoplasma strains identified in pomegranate, putative insect vectors and reservoir plants in Jordan (bold characters), and described '*Candidatus* Phytoplasma' species. The optimal tree with the sum of branch length = 0.91360565 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

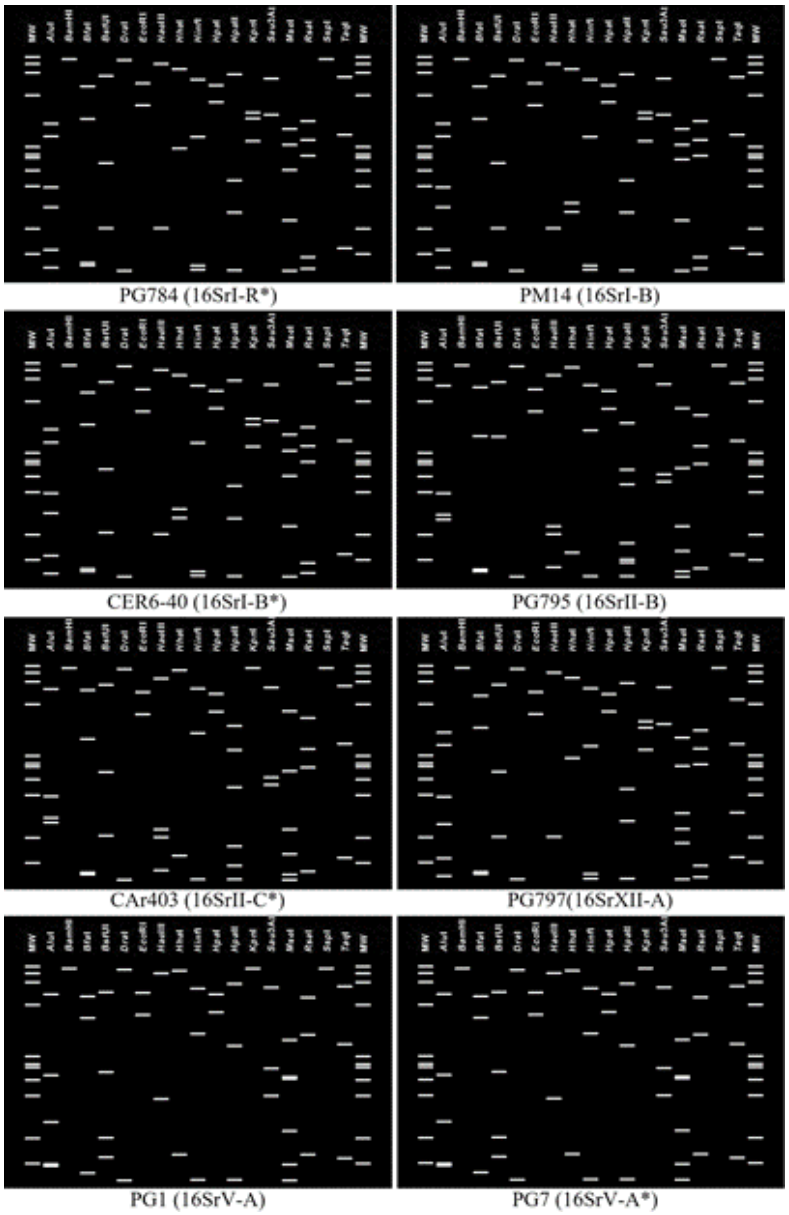

FIGURE 4. Virtual RFLP profiles of 16S rDNA nucleotide sequences of phytoplasma strains identified in pomegranate, weeds and insects in northern Jordan. One strain among those sharing identical 16S rDNA sequence (Tables 2 and 3) was selected as representative strain for *i*PhyClassifier analyses shown in the diverse pictures.

'*Ca*. P. solani' strains have identical 16S rDNA nucleotide sequence (GenBank Accession Number [Acc. No.] OL873110), distinct from the reference strain STOL (GenBank Acc. No. AF248959) by four single nucleotide polymorphisms (SNPs) at positions 504 (T/A), 595 (A/G), 888 (C/T) and 1084 (T/C) from the annealing site of the primer R16F1. '*Ca*. P. aurantifolia' strains have identical 16S rDNA nucleotide sequence (GenBank Acc. No. OL873109), distinct from the reference strain WBDL (GenBank Acc. No. U15442) by four SNPs at positions 285 (C/T), 559 (-/G), 793 (-/C) and 1032

(T/C) from the annealing site of the primer R16F1. Within '*Ca*. P. asteris' and '*Ca*. P. ulmi', the identified strains of each species have diverse 16S rDNA nucleotide sequences. In '*Ca*. P. ulmi', sequences of strains PG1 and PG17, identical between them (GenBank Acc. No. OL873111), and PG7 (GenBank Acc. No. OL873112) are distinct from the reference strain EY1 (GenBank Acc. No. AY197655) by two (positions 95 [C/T], 346 [A/C]) and three (positions 95 [C/T], 117 [A/C], 346 [A/C]) SNPs, respectively. In '*Ca*. P. asteris', sequences of strains PG18 (GenBank Acc. No. OL873113) and PG784 (GenBank Acc. No. OL873108) are distinct from the reference strain OAY (GenBank Acc. No. M30790) by three [323 (G/-), 346 (G/-) and 539 (C/T)] and seven [323 (G/-), 346 (G/-), 488 (C/T), 539 (C/T), 698 (C/T), 984 (A/G) and 1122 (G/A)] SNPs, respectively.

Phytoplasmas identified in symptomatic pomegranate trees were found differentially distributed in the examined locations. Moreover, different symptoms exhibited by pomegranate were found associated with infection by distinct phytoplasmas: witches' broom and little leaf with '*Ca*. P. asteris', '*Ca*. P. solani' and '*Ca*. P. ulmi'; leaf reddening and rolling with '*Ca*. P. aurantifolia' and '*Ca*. P. ulmi'; leaf discoloration with '*Ca*. P. asteris'; yellowing and little leaf with '*Ca*. P. solani' (Table 2; Figure 2).

Concerning non-crop weeds, '*Ca*. P. asteris' (16SrI-B) strains sharing identical 16S rDNA sequence between them and with pomegranate-infecting strain PG18 were identified in *P. major* and *C. annum* in Kufr soum. Moreover, '*Ca*. P. aurantifolia' strains sharing identical 16S rDNA sequence (GenBank Acc. No. OL873114) were identified in *C. arvensis* from Kufr soum (one plant) and Umm jamal (four plants). Such strains, all attributed to a variant of ribosomal subgroup 16SrII-C by *i*PhyClassifier analysis, are distinct from '*Ca*. P. aurantifolia' pomegranate-infecting strains identified in Kufr soum (subgroup 16SrII-B) (Table 2; Figure 2).

3.3 Molecular detection and identification of phytoplasmas in insects

During the field survey, 1918 Cicadomorpha and Fulgoromorpha adult insects were collected and identified in 10 taxonomic groups defined at species (5) and genus (5) level (Table 3). Molecular analyses for phytoplasma detection and identification were conducted on 187 insect pools (146 from Kufr soum, 27 from Jdita and 14 from Arjan) representative of the observed species diversity. Nested PCR allowed detecting phytoplasmas in eight insect pools (infection rate 4.3%), belonging to five taxa, collected in Kufr soum (infection rate 4.8%) and Jdita (infection rate 3.7%). No positive insect pools were found in Ajloun governorate. Infection rate of the phytoplasma-infected insect taxa was 100% in *P. striatus* (Linnaeus) (2 pools out of 2), 14.3% in *Z. sohrab* (2 pools out of 14) and *B. incisa*

(1 pool out of 7), 6.7% in *M. sexnotatus* (2 pools out of 30) and 2.5% in *C. bipunctata* (1 pool out of 40) (Table 3). Analyses of 16S rDNA nucleotide sequences allowed attributing the phytoplasma strains detected in insects to '*Ca*. P. asteris' (7 pools out of 8) and '*Ca*. P. solani' (1 pool out of 8) (Table 3). In detail, '*Ca*. P. asteris' strains KF1-27 and -28 (detected in *M. sexnotatus*), and KF2-33 (detected in *C. bipunctata*) share 16S rDNA nucleotide sequences undistinguishable from those of weed-infecting strains PM14 and CAn22 (from the same location) and pomegranate-infecting strain PG18 (from another location), all attributed to subgroup 16SrI-B. '*Ca*. P. asteris' strains RK3A-1 and -2 (detected in *P. striatus*) share 16S rDNA nucleotide sequences undistinguishable from those of pomegranate-infecting strain PG784 (from the same location), attributed to a variant of taxonomic subgroup 16SrI-R. '*Ca*. P. asteris' strains KF4-40 and -41 (detected in *Z. sohrab*) have identical 16S rDNA nucleotide sequences (GenBank Acc. No. OL873115) distinct from the reference strain OAY (GenBank Acc. No. M30790) by three SNPs at positions 488 (C/T), 539 (C/T) and 698 (C/T). Based on similarity coefficient obtained by comparison of virtual RFLP patterns, '*Ca*. P. asteris' strains KF4-40 and -41 were attributed to a variant of subgroup 16SrI-B (Table 3; Figure 4). '*Ca*. P. solani' strain RJ2-44 (detected in *B. incisa*) shares identical 16S rDNA nucleotide sequence with the pomegranate-infecting strain PG624 (from the same location), attributed to ribosomal subgroup 16SrXII-A (Table 3).

TABLE 3. Results of collecting and testing the insects in northern Jordan, and attribution of detected phytoplasmas to '*Candidatus* Phytoplasma' species and ribosomal subgroups

Governorate	**Location**	**Insect code**	**Family**	**Species**	**Collection date**	**No. of collected insects**	**No. of positive/analysed pools**	**Phytoplasma species (no. of strains)**	**16Sr subgroup (similarity coefficient)**	**Acc. No.**
Irbid	Kufr soum	KF1	Cicadellidae	*Macrosteles sexnotatus*[a]	November	700	2/30	'*Ca*. P. asteris' (2)	I-B (1.00)	[b]
Irbid	Kufr soum	KF2	Cicadellidae	*Cicadulina bipunctata*	August	440	1/40	'*Ca*. P. asteris' (1)	I-B (1.00)	[b]
Irbid	Kufr soum	KF3	Cicadellidae	*Anaceratagallia* sp.	November	40	0/20			
Irbid	Kufr soum	KF4	Cicadellidae	*Zyginidia sohrab*	August	135	2/14	'*Ca*. P. asteris' (2)	I-B* (0.98)	OL873115

Governorate	Location	Insect code	Family	Species	Collection date	No. of collected insects	No. of positive/analysed pools	Phytoplasma species (no. of strains)	16Sr subgroup (similarity coefficient)	Acc. No.
Irbid	Kufr soum	KF7	Cicadellidae	*Balclutha incisa*[a]	November	175	0/24			
Irbid	Kufr soum	KF5	Cicadellidae	*Eupteryx stachydearum*[a]	November	49	0/10			
Irbid	Kufr soum	RK2	Delphacidae	*Laodelphax striatellus*[a]	August	35	0/6			
Irbid	Kufr soum	RK3A	Cicadellidae	*Psammotettix striatus*	August	4	2/2	'*Ca*. P. asteris' (2)	I-R* (0.98)	[c]
Irbid	Jdita	RJ1	Cicadellidae	*Balclutha incisa*	October	120	1/7	'*Ca*. P. solani' (1)	XII-A (1.00)	[d]
Irbid	Jdita	RJ1A	Delphacidae	*Laodelphax striatellus*	October	25	0/9			
Irbid	Jdita	RJ1C	Delphacidae	*Laodelphax* sp.	October	10	0/4			
Irbid	Jdita	RJ3	Cicadellidae	*Cicadulina bipunctata*	October	55	0/7			
Ajloun	Arjan	AR1	Cicadellidae	*Balclutha incisa*	October	100	0/6			
Ajloun	Arjan	AR2	Delphacidae	*Laodelphax striatellus*	October	20	0/5			
Ajloun	Arjan	AR3	Cicadellidae	*Cicadulina bipunctata*	October	10	0/3			
				Total		1918	8/187			

- [a] Insect first reported in Jordan.
- [b] Nucleotide sequences identical to OL873113.
- [c] Nucleotide sequences identical to OL873108.
- [d] Nucleotide sequences identical to OL873110.

4 DISCUSSION

Results obtained from field surveys and molecular analyses conducted during this study revealed the presence of four '*Ca*. Phytoplasma' species ('*Ca*. P. solani', '*Ca*. P. aurantifolia', '*Ca*. P. asteris' and '*Ca*. P. ulmi'), including five 16Sr ribosomal subgroups (16SrXII-A, II-B, I-B, I-R and V-A), in pomegranate showing witches' broom, little leaf and yellowing/reddening in orchards located in northern Jordan. Interestingly, the majority of these phytoplasmas identified in symptomatic pomegranate trees were already reported in Jordan in association with diseases of other important crops (tomato, grapevine, plum, potato and date palm; Alhudaib et al., 2019; Anfoka et al., 2003; Anfoka & Fattash, 2004; Salem et al., 2013, 2019, 2020). Phytoplasmas detected in symptomatic pomegranate were enclosed in five '*Ca*. Phytoplasma' species and associated with different symptoms. Pomegranate yellows were found associated with '*Ca*. P. asteris' (16SrI-B) and '*Ca*. P. solani' (16SrXII-A) in Turkey (Gazel et al., 2016). Pomegranate decline and little leaf were found associated respectively with '*Ca*. P. pruni' (16SrIII) and '*Ca*. P. australasia' (16SrII-D) in Iran (Karimishahri et al., 2015; Salehi et al., 2016). Pomegranate fasciation in China was found associated with '*Ca*. P. asteris' (16SrI-B) (Gao et al., 2018). In India, pomegranate little leaf, yellows and malformation were associated with the presence of '*Ca*. P. australasia' (16SrII-D), while pomegranate leaf yellowing and reddening were associated with '*Ca*. P. oryzae' (16SrXI-B) presence (Rao et al., 2020). In Guadeloupe (Carribean area), pomegranate little leaf, yellows and dried branch were associated with '*Ca*. P. asteris', subgroups 16SrI-B and -F (Castañeda-Alvarez et al., 2018). Nevertheless, the presence of pomegranate symptoms associated with '*Ca*. P. aurantifolia' (16SrII-B), '*Ca*. P. ulmi' (16SrV-A) and '*Ca*. P. asteris' (variant of subgroup 16SrI-R) is reported for the first time in this study. The diversity of symptoms and phytoplasmas detected in this study can be related also to differences in phytoplasma–plant interactions in pomegranate cultivars and/or in specific environmental features of the examined locations, as reported for other crops (Bisognin et al., 2008; Hren et al., 2009; Murolo & Romanazzi, 2015; Quaglino et al., 2016).

Even if the incidence of symptoms was high in examined orchards, only 17% of collected symptomatic pomegranate trees were positive to phytoplasma presence. This can be because of: (i) the erratic distribution of phytoplasmas in phloem tissues (Constable et al., 2003); (ii) the low concentration of phytoplasmas in the sampling periods (Martini et al., 2011); (iii) the possibility that observed symptoms are associated with the presence of other agents or abiotic stresses; and (iv) the presence of PCR inhibitors in extracted TNAs. '*Ca*. P. solani' is the prevalent phytoplasma infecting pomegranate throughout the investigated areas. This evidence confirmed previous studies conducted in the Country revealing the large presence of this phytoplasma associated with "bois noir" of

grapevine and plum yellowing and witches' broom (Salem et al., 2013, 2020). Interestingly, '*Ca*. P. solani' strains, undistinguishable based on 16S rDNA nucleotide sequences, were found in three different pomegranate cultivars, in three distinct locations, showing distinct symptoms: yellowing and little leaf (cultivar Khdary), witches' broom and little leaf (Mawardy) and yellowing (Wonderful). To investigate this aspect, further analyses should be conducted to type more accurately the '*Ca*. P. solani' strains identified in different pomegranate cultivars using molecular markers on variable genes (i.e., *secY, stamp* and *vmp1*) and obtain information on possible convergence in their virulence. '*Ca*. P. solani' was identified in the insect *B. incisa* (with 14.3% infection rate) in Jdita location, while no weeds were positive to phytoplasma presence. *B. incisa*, first reported in this study in Jordan, is reported as putative vector of '*Ca*. P. australasia' (16SrII-D) associated with fenugreek phyllody in Pakistan (Malik et al., 2020), and 16SrII phytoplasmas associated with cactus witches'-broom in Indonesia (Wulandari et al., 2021). This insect is present worldwide and prefers feeding on grasses (Narhardiyati & Bailey, 2005). This overall evidence suggests that *B. incisa* can be a potential vector of '*Ca*. P. solani' also in Jordan. Remarkably, even if '*Ca*. P. asteris' (subgroups 16SrI-B and 16SrI-R variant) was found only in two pomegranate trees in Irbid governorate, most phytoplasma-infected insects (seven specimens belonging to four distinct taxa) collected in the same locations were carrying this '*Ca*. Phytoplasma'. Molecular analyses evidenced that *M. sexnotatus* (first reported in Jordan in this study) and *C. bipunctata* hosted phytoplasma strains identical to pomegranate-infecting strain PG18 ('*Ca*. P. asteris', 16SrI-B), while *P. striatus* hosted phytoplasma strains undistinguishable from pomegranate-infecting strain PG784 ('*Ca*. P. asteris', 16SrI-R variant). Previous studies reported that *M. sexnotatus* and *P. striatus* are vectors of 16SrI group phytoplasmas ('*Ca*. P. asteris' and '*Ca*. P. tritici', respectively), while *C. bipunctata* was reported as potential phytoplasma vector (Alhudaib et al., 2007; Alma et al., 2015; Weintraub & Beanland, 2006; Wu et al., 2010). Moreover, '*Ca*. P asteris' (16SrI-B) strains, identical to those identified in pomegranate and *M. sexnotatus*, were found also in non-crop plants *P. major* and *C. annuum* in Kufr soum.

Noteworthy, '*Ca*. P. aurantifolia' (16SrII-B) and '*Ca*. P. ulmi' (16SrV-A and its variant), identified in pomegranate, were not detected in both analysed insects and non-crop plants. In fact, all phytoplasma-infected bindweeds were found hosting '*Ca*. P. aurantifolia' strains attributed to a variant of subgroup 16SrII-C. Considering that insect survey was preliminary and conducted in short period and restricted area in each location, further samplings are required to extend the knowledge on entomofauna diversity in pomegranate orchards in Jordan and its role in the epidemiology of phytoplasma-associated pomegranate diseases.

Further studies are needed to verify the diffusion of pomegranate phytoplasma-associated diseases in the region, demonstrate the transmission capability of the identified phytoplasma positive insects, and appropriately manage these emerging diseases.

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