Association of seven '*Candidatus* Phytoplasma' species to an almond disease complex in Jordan, and preliminary information on their putative insect vectors

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Abstract

During field surveys carried out from June to October 2020 and in January 2021 in orchards of northern Jordan, phytoplasma-like symptoms (early flowering along with evergreen pattern; witches'-broom, yellowing, and dieback; slim leaf and leaf rolling; stem fasciation) were observed in almond trees. In 23 investigated orchards, symptomatic almond trees ranged from 20 to 85%. PCR-based amplification of 16S rRNA gene detected phytoplasmas in 21% of 140 collected symptomatic almond trees. Sequence analyses allowed attributing the detected phytoplasmas to '*Candidatus* Phytoplasma asteris' (taxonomic subgroups 16SrI-B and -R), '*Ca.* P. aurantifolia' (16SrII-B and -C), '*Ca.* P. omanense' (16SrXXIX-A and -B), '*Ca.* P. phoenicium'(16SrIX-B), '*Ca.* P. pyri' (16SrX-C), '*Ca.* P. solani' (16SrXII-A), and '*Ca.* P. ulmi'(16SrV-A). Such phytoplasmas were found associated with specific symptoms and differentially distributed in the considered locations. Moreover, further investigation identified '*Ca*. P. asteris' (subgroup 16SrI-R) in putative insect vectors such as *Empoasca* sp., *Reptalus* sp., and *Hyalesthes obsoletus*, '*Ca*. P. pyri' in *Cacopsylla bidens*, and '*Ca*. P. omanense' (subgroup 16SrXXIX-B) in the non-crop plant *Amaranthus* sp. In conclusion, this study described an almond disease complex associated with multiple phytoplasmas, including '*Ca*. P. pyri', '*Ca*. P. omanense', and '*Ca*. P. ulmi' that were never reported before in this crop. Further studies are needed to survey the diffusion of this almond disease complex in the region, demonstrate the transmission capability of the identified putative vectors, and in-depth investigate the ecologies of all phytoplasmas associated with the disease.

Keywords: '*Candidatus* Phytoplasma asteris'; '*Candidatus* Phytoplasma aurantifolia'; '*Candidatus* Phytoplasma omanense'; '*Candidatus* Phytoplasma phoenicium'; '*Candidatus* Phytoplasma pyri'; '*Candidatus* Phytoplasma solani'; '*Candidatus* Phytoplasma ulmi'; *Cacopsylla bidens*

Introduction

Almond (*Prunus dulcis* (Mill.) D.A. Webb, 1967) is one of the most important stone fruit crops in Middle East and North Africa (MENA) region, in which also wild almond is very common. In 2019, five out of the top ten almond producers over the world were from MENA including Iran, Morocco, Syria, Tunisia, and Algeria (https://www.fao.org/faostat/en/#rankings/countries_by_commodity). In Jordan, stone fruits are the most important exported fruit crops (59,425 ton in 2020) (MOA, 2021). Within stone fruits, almond cultivation is expanding in several rural rainfed areas as family farming instead of olive, the most common inherited economic tree. Recently, almond commercial orchards have been established in the irrigated areas of the Country, and the green fruits of almond are gaining huge popularity and recorded unprecedented prices. Many phytoplasmas were reported in association with almond diseases in MENA countries including Iran, Lebanon, and Tunisia (Hemmati et al., 2021). Phytoplasmas are a large group of phloem-restricted, cell wall-less bacteria that infect nearly a thousand of plant species worldwide (Gasparich, 2010). They are transmitted plant-to-plant by phloem-feeding insects, mainly leafhoppers (Cicadellidae) (Weintraub and Beanland, 2006; Alma et al., 2015), and their severe epidemic outbreaks can induce losses up to 70-100% (Bertaccini et al., 2014; Kumari et al., 2019). Almond witches'-broom (AlmWB) is the most destructive phytoplasma disease of almond, associated with '*Candidatus* Phytoplasma phoenicium' (taxonomic subgroup 16SrIX-B and its variants) in Middle East (Abou-Jawdah et al., 2002; Verdin et al., 2003; Salehi et al., 2006; Molino Lova et al., 2011; Mosayyebi et al., 2021). The most characteristic symptoms of AlmWB are shoot proliferation on the main trunk with the appearance of a witches'-broom, perpendicular development of many axillary buds with small and yellowish leaves, and general tree decline with final dieback. More than 100,000 of almond trees were eradicated in Lebanon due to AlmWB outbreak in 2002 (Abou-Jawdah et al., 2003). In Lebanon, AlmWB epidemiological cycle involves *Asymmetrasca decedens* (Paoli) (prevalent in almond), possibly responsible for the transmission of '*Ca*. P. phoenicium' from almond to almond, and cixiids of the genus *Tachycixius* (collected also on *Smilax aspera* L. and *Anthemis* sp., two wild plants harboring '*Ca*. P. phoenicium' as well), possibly responsible for the transmission from weeds to almond

(Abou-Jawdah et al., 2014; Tedeschi et al., 2015). In Iran, *Prunus scoparia*, a wild almond species harboring '*Ca*. P. phoenicium', could play a role in the phytoplasma transmission pathways to fruit trees (Salehi et al., 2015). Based on detection of '*Ca*. P. phoenicium' in insect body and saliva and the presence of consistent populations, the leafhopper *Frutioidea bisignata* Mulsant and Rey can be considered as potential vector of this phytoplasma in Iran (Taghizadeh and Salehi, 2002; Siampour et al., 2004). In the last years, '*Ca*. Phytoplasma phoenicium' was associated with diseases of stone fruits including peach, nectarine, apricot, and cherry (Abou-Jawdah et al., 2009; Salehi et al., 2018, 2020). '*Ca.* Phytoplasma phoenicium' is a quarantine pathogen in the European Union, being included in the List A1 of the European Plant Protection Organization (EPPO) by September 2018. In 2019, AlmWB was firstly reported in south Italy (Nigro et al., 2020). In Iran, AlmWB was found associated also with phytoplasmas belonging to subgroup 16SrIX-C (Salehi et al., 2006) that, in Lebanon, were reported only in wild plant species (Casati et al., 2016). Symptoms including decline, early fall, rosette, witches'-broom, yellowing, little leaf, leaf rolling, leaf scorch, and leaf reddening were observed in almond trees in Iran in association with *'Ca.* Phytoplasma aurantifolia, *'Ca.* Phytoplasma solani, *'Ca.* Phytoplasma trifolii', and '*Ca*. Phytoplasma asteris' (Zirak et al., 2009, 2021). Moreover, '*Ca*. Phytoplasma prunorum', the causal agent of European stone fruit yellows (ESFY), was identified in almond showing early leaf reddening in autumn, off-season growth in winter followed by dieback, and bore small and tasteless fruits in Tunisia (Ben Khalifa et al., 2011). In Jordan, few studies focused on phytoplasma-associated diseases as well as their epidemiology. Therefore, based on the increased attention for both almond cultivation and phytoplasma-associated diseases in MENA region, the current study aimed to (i) survey the presence of phytoplasma-like diseases of almond in orchards localized in northern Jordan, (ii) detect and identify by molecular analyses the phytoplasmas associated with such diseases, (iii) preliminarily investigate the presence of putative vectors and reservoir plants of the identified phytoplasmas.

Materials and Methods

Phytoplasma-like symptom observation, plant sampling, and insect collection

Phytoplasma-like symptoms were surveyed, from June to October 2020 and in January 2021, in 23 almond orchards localized in seven rainfed locations in two governorates in northern Jordan. In detail, the surveys were conducted in Kharja (32°39'48"N/35°54'32"E), Ezrit (32°39'5"N/35°50'30"E), Hofa (32°34'26.3"N/35°42'27.4"E) and Sydoor (32°39'7"N/35°41'47"E) in Irbid governorate, and in Ain Jana (32°20'59"N/35°47'40"E), Zatarah (32°35'17"N/

35°73’9”E), and Sikhrah (32°38’33.4”N/35°86’46.3”) in Ajloun governorate (Figure 1). In the surveyed orchards (19.7 ha), representative of the considered almond cultivation area in the northern regions of the Country (150 ha), 7060 almond trees were monitored. In each location, incidence of phytoplasma-like diseases was estimated as the percentage of symptomatic trees out of the observed ones. Most of the surveyed orchards were surrounded by olive trees, stone fruits, grapevine, and scattered pears. A lack of associated green cover weeds was observed in the orchards during summer due to the dry season and the agronomic soil management.

Leaves were collected from 140 almond trees showing phytoplasma-like symptoms and 16 symptomless almond trees (Table 1). Moreover, leaves were sampled from 20 symptomatic plants of four weed species (*Convolvulus arvensis* L., *Chenopodium* sp., *Amaranthus* sp., *Capparis* sp.) observed within and around investigated almond orchards (Table 1). Collected samples were labeled, signed by the areas coordinates, transported to the laboratories of National Agricultural Research Center (NARC), Baqaà, Jordan, and maintained at 4°C until total nucleic acids extraction. Additionally, during the field survey carried out in both governorates, insects within almond orchards were collected by entomological sweeping net and transferred to the NARC laboratories. Stereomicroscope observation was conducted for a preliminary selection of Hemiptera taxa with a particular attention to phloem-sap feeding ones. The selected insects were kept in 99% ethanol until their identification carried out at the Department of Agriculture, Forest, and Food Science (DISAFA), University of Turin, Italy. The insect identification was based on stereomicroscope observation of phenotypic characters and male genitalia after their dissection and clarification in a 10% potassium hydroxide solution (Ribaut, 1952; Ossiannilsson, 1981; Dmitriev, 2003; Holzinger et al., 2003; Biedermann and Niedringhaus, 2004). Insects recognized at genus/species level were maintained in 99% ethanol at -20°C until total nucleic acids extraction.

DNA extraction

DNAs were extracted from 0.5 g of petiole and midrib tissues of all the collected plant samples using a CTAB-based extraction protocol previously described by Angelini et al. (2001). DNAs were extracted from single insect specimens or from insect pools (2-5 specimens) based on their size and number of collected specimens using a CTAB-based extraction method previously described (Marzachì et al., 1998). The obtained DNAs were solved in 50 (insects) to 100 µl (plants) distilled sterile water and stored at -20 °C until further use. DNA quality and concentration were measured by Nanodrop system.

Phytoplasma detection and identification

DNAs extracted from plants and insects were used as templates in nested PCR reactions conducted to detect the presence of phytoplasmas. Each DNA was amplified in three independent nested PCRs (technical replicates). Nested PCRs were carried out to amplify the phytoplasma 16S rRNA gene using the primer pair P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) followed by the primer pair R16F1/R16R0 (Lee et al., 1995). PCRs were performed in 25 µl reaction volume containing 30 to 100 ng of template, 0.4 µM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.625 units of GoTaq® G2 DNA Polymerase in the buffer supplied by the manufacturer (Promega, Italy). PCRs were conducted in a thermal cycler (Life Touch, Bioer Technology, China**)** for 36 cycles as follows: 1 min denaturation at 94°C (3 min for the first cycle), 2 min annealing at 50°C (55°C in nested PCR), and 3 min of extension at 72°C (Lee et al., 1998; Quaglino et al., 2009). DNAs extracted from periwinkle [*Catharanthus roseus* L. (G. Don)] plants, infected by phytoplasma strains STOL ('*Ca*. Phytoplasma solani', subgroup 16SrXII-A) and AY1 ('*Ca*. P. asteris', subgroup 16SrI-B) and maintained in greenhouse at Department of Agricultural and Environmental Sciences, University of Milan (Italy), were employed as positive controls. DNA extracted from healthy periwinkle and reaction mixtures devoid of DNA were used in as negative controls. PCR products (6 µl) were analyzed by electrophoresis on 1% (w/v) agarose gels in 1X TBE buffer, stained with Midori Green, and visualized 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.

Three nested PCR products (F1/R0 fragment), amplified from each phytoplasma-infected plants and insects, 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 primers F1/R0 and aligned to obtain a consensus sequence in the software BioEdit, version 7.1.3.0 (Hall, 1999). Trimmed nucleotide sequences were aligned using the ClustalW Multiple Alignment program and analyzed by Sequence Identity Matrix in the sofware BioEdit to estimate their genetic diversity. For attribution to '*Ca*. Phytoplasma' species, 16S rDNA nucleotide sequences, representative of the phytoplasma strains detected in this study and deposited on NCBI GenBank (Accession Number OL873123-OL873133), were aligned with those of representative strains of the 49 '*Ca*. Phytoplasma' species described in literature

(Bertaccini et al., 2022) and checked for their sequence identity in the software Bioedit. Species attribution was confirmed searching the species-specific signature sequences, and by analysis on *i*PhyClassifier online tool (Wei et al., 2007). For ribosomal group/subgroup attribution, 16S rDNA sequences were analyzed by virtual 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 1424 positions in the final dataset. *Acholeplasma palmae* (GenBank Acc. No. L33734) was used for rooting the tree.

Results

Description of phytoplasma-like symptoms in almond and weeds

During the survey conducted in almond orchards in seven locations in North Jordan, five main categories of phytoplasma-like symptoms were observed in almond trees. (i) Witches'-broom, yellowing, and dieback (Figure 2A, B) were found in orchards localized in Ezrit, Hofa, and Sydoor (Irbid governorate), and Ain Jana (Ajloun governorate) with an incidence (percentage of symptomatic trees) of 40%, 25%, 30%, and 80%, respectively. (ii) Early flowering (started 30 days before the expected blossom period) along with evergreen pattern (leaf canopy was maintained during the winter season) (Figure 2C) was observed in orchards localized in Sikhrah (Ajloun), with an incidence around 85%. (iii) Witches'-broom, yellowing and leaf rolling (Figure 2D) were found in orchards localized in Kharja with an incidence around 55%. (iv) Slim leaves (Figure 2E) were observed in orchards localized in Zatarah (Ajloun) with an incidence around 20%. (V) Flat stem (Figure 2F) was exhibited by almond trees in Kharja (Irbid), with an incidence around 40%. Additionally, symptoms of yellowing on *Capparis* sp., reddening on *Chenopodium* sp., and little leaves and colour alteration on *Amaranthus* sp. (Figure 2G) were observed within and around almond orchards in Kharja and Ezrit.

Molecular detection and identification of phytoplasmas in plants

Nested PCR reactions allowed amplifying phytoplasma 16S rRNA gene in leaf samples of 31 out of 176 plants. In detail, F1/R0 amplicons of the expected size (around 1370 bp) were obtained in 30 out of 140 symptomatic almond trees (21.4%), and in one (*Amaranthus* sp.) out of 20 weeds (5%) (Table 1). No amplificons were obtained in leaf samples collected from symptomless almond trees and from *C. arvensis*, *Chenopodium* sp., and *Capparis* sp. Goodness of PCR reactions was supported by the amplification of F1/R0 fragment from periwinkles infected by phytoplasma strains STOL and AY1 (positive controls), while no amplification was obtained from healthy periwinkle and reaction mixture devoid of DNA (negative controls). Infection rates (percentage of phytoplasma-infected trees) varied among the surveyed localities as it follows: 67% in Sikhrah, 36.7% in Kharja, 32% in Hofa and Ain Jana, 13% in Zatarah 13%, and 12% in Sydoor and Ezrit.

Based on 16S rDNA sequence identity versus the reference strains of '*Ca*. Phytoplasma' species and on the presence of species-specific signature sequences, the phytoplasma strains detected in the present study in 30 symptomatic almond trees were attributed to the species '*Ca.* P. solani' (40%; 12 strains out of 30), '*Ca.* P. ulmi' (16.7%; 5 out of 30), '*Ca.* P. omanense' (16.7%; 5 out of 30), '*Ca*. P. asteris' (10%; 3 out of 30), '*Ca.* P. pyri'(10%; 3 out of 30), '*Ca.* P. aurantifolia' (3.3%; 1 out of 30), and '*Ca.* P. phoenicium' (3.3%; 1 out of 30) (Table 2). Phytoplasma clustering in phylogenetic tree confirmed the attribution to '*Ca*. Phytoplasma' species (Figure 3).

Based on similarity coefficient obtained by comparison of virtual RFLP patterns (Figure 4A), '*Ca*. P. solani' strains were attributed to taxonomic subgroup 16SrXII-A, '*Ca*. P. ulmi' strains to subgroup 16SrV-A (strain AL1) and its variant (strains AL2, AL3, AL5, and AL10), '*Ca*. P. omanense' strain AL408 to subgroup 16SrXXIX-A, '*Ca*. P. asteris' strains to subgroups 16SrI-B (strains AL7, AL2C) and a variant of the subgroup 16SrI-R (strain AL831), '*Ca*. P. pyri' strains AL198, AL222, and AL225 to subgroup 16SrX-C, '*Ca*. P. aurantifolia' strain AL214 to a variant of the subgroup 16SrII-C, and '*Ca*. P. phoenicium' strain AL1067 to subgroup 16SrIX-B. Moreover, '*Ca*. P. omanense' strains AL163, AL1052, AL1054, AL1056, and AL1058 were characterized by a common collective restriction profile sharing the higher similarity coefficient (0.97) with the profile of the reference strain of subgroup 16SrXXIX-A; such digestion patterns, distinguished by the enzyme *Alu*I, were confirmed by actual RFLP analysis (Figure 4B). Due to the similarity coefficient value, these five '*Ca*. P. omanense' strains were inserted in the new taxonomic subgroup 16SrXXIX-B.

'*Ca*. P. solani' strains have identical 16S rDNA nucleotide sequence (GenBank Acc. No. OL873130), distinct from the reference strain STOL 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. Within '*Ca*. P. ulmi', '*Ca.* P. omanense', and '*Ca*. P. asteris' the identified strains of each species have diverse 16S rDNA nucleotide sequences. In '*Ca*. P. ulmi', sequences of strains AL2, AL3, AL5, and AL10, identical between them (GenBank Acc. No. OL873125), and AL1 (GenBank Acc. No. OL873124) are distinct from the reference strain EY1 by three [positions 95 (C/T), 117 /A/C), 346 (A/C)] and two [positions 95 (C/T), 346 (A/C)] SNPs, respectively. In '*Ca.* P. omanense', sequences of strains AL163, AL1052, AL1056, and AL1058, identical between them (GenBank Acc. No. OL873126) are distinct from the reference strain IM-1 by SNPs at positions 152 (G/A), 274 (T/C), 331 (C/T), 344 (G/A), and 712 (G/A), while the sequence of strain AL408 (GenBank Acc. No. OL873127) is identical to the reference strain IM-1. In '*Ca*. P. asteris', sequences of strains AL7 and AL2C, identical between them (GenBank Acc. No. OL873123), and AL831 (GenBank Acc. No. OL873129) are distinct from the reference strain OAY by three [323 (G/-), 346 (G/-), 539 (C/T)] and seven [323 (G/-), 346 (G/-), 488 (C/T), 539 (C/T), 698 (C/T), 984 (A/G), 1122 (G/A)] SNPs, respectively. In '*Ca*. P. pyri', the sequences of the strains AL198, AL222, and AL225 (GenBank Acc. No. OL873132) were identical to the reference strain PD1. In '*Ca*. P. aurantifolia', the sequence of the strain AL214 (GenBank Acc. No. OL873131) is distinct from the reference strain WBDL by six SNPs at positions 62 (T/A), 83 (G/A), 285 (C/T), 559 (-/T), 793 (-/C), and 1032 (T/C). In '*Ca*. P. phoenicium', the sequence of the strain AL1067 (GenBank Acc. No. OL873128) is identical to the reference strain A4.

Phytoplasmas identified in symptomatic almond trees were found differentially distributed in the examined locations and associated with different symptoms. '*Ca*. P. solani' (16SrXII-A) was found in Hofa (7 strains out of 8 detected phytoplasmas), Sydoor (2 out of 3), Kharja (2 out of 5), and Ezrit (1 out of 3) in association with witches'-broom, yellowing, leaf rolling, and dieback (Figure 2A, B, D). '*Ca*. P. ulmi' was found only in Sikhrah (5 strains out of 7) in association with early flowering and evergreen pattern (Figure 2C). '*Ca*. P. omanense' was found in Ain Jana (2 strains out of 3), Ezrit (1 out of 3), and Sydoor (1 out of 3) in association with witches'-broom, yellowing, and dieback (Figure 2A, B), and was the sole phytoplasma identified in Zatarah (1 out of 1) in association with slim leaves (Figure 2E). '*Ca*. P. asteris' was identified in Sikhrah (2 strains out of 7) in association with early flowering and evergreen pattern (Figure 2C), and in Ezrit (1 out of 3) in association with witches'-broom, yellowing, and dieback (Figure 2A, B). '*Ca*. P. pyri' (3 strains out of 5) was identified in Kharja in association with flat stem. Finally, '*Ca*. P. aurantifolia' and '*Ca*. P. phoenicium' were identified

in Hofa (1 strain out of 8) and Ain Jana (1 out of 3), respectively, in association with witches'-broom, yellowing, and dieback (Figure 2A, B) (Table 2).

Concerning non-crop weeds, a '*Ca*. P. omanense strain, sharing identical 16S rDNA sequence with almond-infecting strains AL163, AL1052, AL1056, and AL1058 (newly reported subgroup 16SrXXIX-B), was identified in *Amaranthus* sp. exhibiting little leaf and yellowing in Ezrit (Figure 2G).

Molecular detection and identification of phytoplasmas in insects

During the field survey carried out in Ezrit, Kharja, and Sydoor localities in July and August 2020, 122 Auchenorrhyncha and 4 Sternorrhyncha adult insects were collected and distinguished, based on stereomicroscope analyses, in 12 taxonomic groups defined at genus (5) and species (7) level. Most of Auchenorrhyncha insects belong to the family Cicadellidae (106 specimens), while the remnant 16 collected specimens belong to the families Cixiidae, Issidae, Delphacidae, and Tettigometridae. Within Cicadellidae, the more abundant insect taxa were *Zygina flammigera* (Fourcrov) (25 specimens) (firstly reported in Jordan), *Anaceratagallia frisia* (Wagner) (20 specimens) (firstly reported in Jordan), *Cicadulina bipunctata* (Melichar), (18 specimens), *Balclutha incisa* (Matsumura) (15 specimens), and *Empoasca* sp. (15 specimens). All Sternorrhyncha insects (4) belong to the family Psyllidae. In Kharja, 10 out of 12 insect taxa were captured; in Ezrit and Sydoor only two and one insect taxa were captured, respectively. In these last two locations, the following three insect taxa were captured, namely *Z. flammigera*, *Hyalesthes obsoletus* (Signoret), and *B. incisa* (Table 3). Molecular analyses for phytoplasma detection and identification were conducted on 48 insect pools (35 from Kharja, 7 from Ezrit, and 6 from Sydoor) representative of the observed diversity. Nested PCR allowed detecting phytoplasmas in 13 insect pools (infection rate 27.1%), belonging to 8 different insect taxa, collected in Kharja (infection rate 34.3%) and Ezrit (infection rate 14.3%). No positive insect pools were found in Sydoor. Among Cicadellidae, two out of 34 insect pools (5.9%), belonging to the taxa *C. bipunctata* and *Empoasca* sp., were found phytoplasma-infected. Among the other families, 11 out of 14 insect pools (78.6%), belonging to six taxa, were found phytoplasma-infected. An infection rate of 66.7% (2 pools out of 3) was recorded in *Reptalus* sp., 50% (1 pool out of 2) in *H. obsoletus*; and 100% (2 pools out of 2) in *Cacopsylla bidens* (Šulc) (Psyllidae) (Table 3). Moreover, phytoplasmas were detected in *Agalmatium* sp. (Issidae) (3 pools out of 3), *Laodelphax striatellus* (Fallén) (Delphacidae) (2 pools out of 2) and in *Tettigometra* sp. (Tettigometridae) (1 pool out of 2) (Supplementary Figure 1).

Analyses of 16S rDNA nucleotide sequences allowed attributing the phytoplasma strains detected in insects to the species '*Ca*. P. asteris' (7 pools out of 13) and '*Ca*. P. pyri' (6 pools out of 13) (Table 4). Phytoplasma clustering in phylogenetic tree confirmed the attribution to '*Ca*. Phytoplasma' species (Figure 3). In detail, '*Ca*. P. asteris' strains found in *Empoasca* sp., *Reptalus* sp., *H. obsoletus*, and *Agalmatium* sp. share identical 16S rDNA nucleotide sequence with the almond-infecting strain AL831, attributed to a variant of taxonomic subgroup 16SrI-R. '*Ca*. P. pyri' strains found in insects *L. striatellus*, *Tettigometra* sp., *C. bidens*, and *C. bipunctata* shares identical 16S rDNA nucleotide sequence between them (GenBank Acc. No. OL873133), distinct from almond-infecting strains by SNPs at positions 251 (A/C), 470 (C/A), and 723 (G/A). Such strains were attributed to taxonomic subgroup 16SrX-C (Table 4).

Discussion

Recently, almond became a very important stone fruit crop in Jordan, gaining huge popularity among family farming, investors, and consumers. In this work, field surveys allowed observing almond trees exhibiting five different symptom categories in North Jordan, and molecular analyses identified in symptomatic almond trees the presence of seven genetically distinct '*Ca*. Phytoplasma' species, including '*Ca*. P. solani', '*Ca*. P. aurantifolia', '*Ca*. P. asteris', '*Ca*. P. ulmi', '*Ca* P. pyri', '*Ca*. P. phoenicium', and '*Ca*. P. omanense', belonging to nine taxonomic subgroups (16SrI-B, I-R, II-C, V-A, IX-B, X-C, XII-A, XXIX-A and -B). Remarkably, '*Ca*. P. solani', '*Ca*. P. aurantifolia', '*Ca*. P. asteris', and '*Ca*. P. ulmi', identified in almond in the present study, were previously reported in Jordan in association with diseases affecting grapevine, plum, peach, tomato, date palm, and pomegranate (Anfoka et al., 2003; Anfoka & Fattash, 2004; Alhudaib et al., 2019; Salem et al., 2013, 2019, 2020; Abu Alloush et al., unpublished). On the other hand, '*Ca.* P. pyri', '*Ca*. P. phoenicium', and '*Ca*. P. omanense' are firstly reported in Jordan. Furthermore, '*Ca*. P. phoenicium', '*Ca.* P. solani', '*Ca*. P. aurantifolia', and '*Ca*. P. asteris' were already found in association with almond diseases in MENA region (Abou-Jawdah et al., 2003; Salehi et al., 2006; Ghayeb Zamharir, 2014; Zirak et al., 2021). Based on the findings of this study, '*Ca*. P. pyri', '*Ca*. P. omanense', and '*Ca*. P. ulmi' are reported for the first time in almond around the world.

Most of the symptoms observed in almond trees in Jordan (early flowering along with evergreen pattern, leaf rolling, witches'-broom, yellowing, and dieback) were already reported in MENA countries (Abou-Jawdah et al., 2003; Salehi et al., 2006), except for flat stem (stem fasciation) and slim leaves, firstly reported here in association with infection by '*Ca*. P. pyri' and '*Ca*. P. omanense' (newly described subgroup 16SrXXIX-B), respectively. Such results

suggested that the symptoms observed in almond could be related to differences in phytoplasma-plant interactions and/or in specific environmental features of the examined locations, as reported for other crops (Bisognin et al., 2008; Hren et al., 2009; Murolo and Romanazzi, 2015; Quaglino et al., 2016). Even if the incidence of phytoplasma-like symptoms was high in examined orchards, only 21% of collected symptomatic almond trees were found phytoplasma infected. This can be due to: (i) the uneven distribution of phytoplasmas in phloem tissues of infected plants (Constable et al., 2003); (ii) the possible absence or low concentration of phytoplasma cells in leaf tissues in the different sampling periods (from June to January) (Martini et al., 2011); (iii) the possibility that the highest titer of phytoplasmas is detectable in the root tissues of infected trees (Baric et al., 2011; Jawhari et al., 2015); (iv) the possibility that observed symptoms are caused by other etiological agents or to abiotic stresses; (v) the presence of PCR inhibitors in extracted DNAs.

Among the seven phytoplasma species identified in almond in this study, '*Ca*. P. solani was the most prevalent and detected in all Irbid locations. Such finding confirms previous reports about the large dispersal of this phytoplasma species throughout the Country in association with grapevine 'bois noir' disease, plum yellowing and witches'-broom (Salem et al., 2013, 2020), and pomegranate yellowing and little leaf (Abu Alloush et al., unpublished). In Europe and in MENA countries, diffusion of '*Ca*. P. solani' is strictly related to the vectoring activity of the polyphagous planthopper *H. obsoletus* and the presence of its preferred host plants (mainly nettle and bindweed) in agroecosystems (Maixner, 1994; Choueiri et al., 2019; Kosovac et al., 2019; Jamshidi et al., 2019; Quaglino et al., 2021). In this study, '*Ca*. P. solani' was not detected in either *H. obsoletus* or bindweed, but the very low number of tested samples cannot allow us any inference on the epidemiology of the pathogen in the study area. Similarly, '*Ca.* P. solani' was not detected in *Reptalus* sp. a genus already reported as alternative vector of '*Ca.* P. solani' to periwinkle (Jović et al., 2009; Cvrković et al., 2014; Chuche et al., 2016). The limited number of samples collected in a narrow time window during the growing season did not allow to collect useful data for the study of the epidemiology of '*Ca.* P. solani' in Jordan. Further studies are thus necessary to investigate accurately the ecological cycle of '*Ca.* P. solani' in this Country, with a particular focus on its transmission pathways to almond, thus on the putative insect vectors and reservoir plants.

Four phytoplasma-infected insect taxa, including *Agalmatium* sp., *Empoasca* sp., *H. obsoletus* and *Reptalus* sp., were found in Kharja carrying '*Ca*. P. asteris' strains belonging to a variant of taxonomic subgroup 16SrI-R, undistinguishable from a strain identified only in one almond tree exhibiting witches'-broom, yellowing, and dieback in Ezrit. On the other hand, '*Ca.* P.

asteris' strains belonging to subgroup 16SrI-B were identified in almond trees showing early flowering along with evergreen pattern in Sikhrah, but no weeds and insects were found infected by this phytoplasma. Phytoplasma strains of subgroup 16SrI-R were already reported in association with diseases of cherry and pomegranate in Lituania and Jordan, respectively (Jomantiene et al., 2011; Abu Alloush et al., unpublished) while *H. obsoletus* is known as putative vector of '*Ca*. P. asteris' (Maixner, 1994; Chuche et al., 2016; Zambon et al., 2018; Pierro et al., 2020). On the contrary, *Empoasca* spp. are mainly mesophyll feeders, but ingestion from phloem elements has been demonstrated for some species such as the potato leafhopper *E. fabae* (Harris) (Backus et al. 2005), *E. flavescens* Fabricius and *E. vitis* Göethe (Tavella and Arzone, 1992). So not surprisingly some *Empoasca* species have been founded being positive for the presence of phytoplasmas such as '*Candidatus* Phytoplasma aurantifolia' in *Empoasca papayae* Oman (Arocha et al. 2005, 2007) and in *Empoasca decipiens* Paoli (Alhudaib et al. 2019), '*Ca.* P. asteris' and '*Ca.* P. aurantifolia' in *E. decipiens* (Parrella et al. 2008). Moreover, field collected *E. papayae* were able to transmit 'Ca. Phytoplasma aurantifolia' to healthy papaya (Pérez et al., 2010), while *E. decipiens* proved to be an experimental vector of '*Ca.* P. asteris' in controlled conditions. (Galetto et al., 2011; Kumar et al., 2015; Perilla-Henao et al., 2016)

Agalmatium is a genus of planthoppers belonging to the family Issidae, subfamily Issinae, a taxon that in the Western Palearctic mostly inhabit arid or semiarid biotypes and Mediterranean and steppe communities, but the ecological and biological characters are still poorly known (Gnezdilov et al., 2014) as well as its possible ability in acquiring and transmitting phytoplasmas.

Based on these findings, it is reasonable to suggest that diffusion of at least '*Ca.* P. asteris' subgroup 16SrI-R to almond in Jordan can involve the insects *H. obsoletus*, *Reptalus* sp., and *Empoasca* sp. Upscaling the surveyed orchards and surroundings could provide better insights on the '*Ca*. P asteris' diffusion in almond and its epidemiology.

Interestingly, '*Ca*. P. pyri' (16SrX-C), associated with pear decline (PD) and other diseases of stone fruits (Paltrinieri et al., 2001; Bohunická et al., 2018) and included in the EPPO A2 List (OEPP/EPPO, 2007), was identified in almond trees showing stem fasciation in Kharja location. Molecular analyses allowed identifying '*Ca*. P. pyri′ in insect taxa *C. bidens*, *L. striatellus*, *Tettigometra* sp., and *C. bipunctata* in the same location. It is well known that *Cacopsylla pyri* is one of the main vectors of '*Ca*. P. pyri' in the Mediterranean area (Garcia-Chapa et al., 2005), where it transmits this phytoplasma also to stone fruits (Sabaté et al., 2018).

Consequently, based on evidence from this and previous studies (Etropolska et al., 2015), it is reasonable to hypothesize that *C. bidens* can be a vector of '*Ca*. P. pyri' to almond in Jordan. Previous studies reported that *L. striatellus* is a vector of '*Ca*. P. solani' to grapevine (Quaglino et al., 2019) and a potential vector of 16SrI, 16SrIII and 16SrXII-A phytoplasmas to *Myrthus communis* L. (Prota et al., 2007) and of Toria phyllody phytoplasma (16SrIX) (Azadvar et al., 2011). On the contrary *C. bipunctata* is a potential vector of '*Ca*. P. asteris'-related strain to date palm (Alhudaib et al., 2007), while Tettigometridae were reported as potential vectors of Peanut witches´-broom group (16SrII) in Ethiopia (Tessema et al., 2010).

Anyhow, no one of these taxa have been previously associated with '*Ca.* P. pyri'. Further studies should be planned to investigate the vector activity of these insects by transmission trials, and to describe their ecology, including cultivated and wild host plants.

'*Ca*. P. omanense', previously reported in MENA Countries in association with grapevine 'bois noir' and yellowing, reddening, dwarfing, die-back and decline diseases in *Prunus persica*, *Prunus domestica*, *Diospyros kaki*, and *Sophora alopecuroides* (Foissac et al., 2019; Esmaeilzadeh-Hosseini et al., 2019, 2020), was identified in this study for the first time in almond trees exhibiting three symptom categories (witches'-broom, yellowing, dieback; little leaf, yellowing; slim leaves) in three Jordanian localities. Interestingly, the majority of '*Ca*. P. omanense' strains have been classified in a new taxonomic subgroup, named 16SrXXIX-B, and are undistinguishable from strains recently identified in table grapes in Jordan (Abu Alloush et al., unpublished). Molecular analyses of weeds revealed the presence of '*Ca*. P. omanense', subgroup 16SrXXIX-B, in *Amaranthus* sp., suggesting a potential role of this plant as reservoir for phytoplasma diffusion. Recent studies reported *H. obsoletus* and *Reptalus* sp. as putative vectors of '*Ca.* P. omanense' in Lebanon (Foissac et al., 2019), but in the present work these and the other examined insects were not found infected by this phytoplasma. Due to the association of the new subgroup 16SrXXIX-B to almond and grapevine diseases in Jordan, it will be useful to focus further studies on improving the knowledge on its epidemiology throughout the Country, in different agroecosystems.

Early flowering is one of the most common symptoms of almond witches'-broom disease, associated with *'Ca*. P. phoenicium' in Lebanon and Iran (Salehi et al., 2006; Molino Lova et al., 2011). In this study, early flowering along with evergreen pattern was associated with '*Ca.* P. asteris' (16SrI-B) and '*Ca.* P. ulmi' (16SrV-A), not detected in examined weeds and insects. To the best of our knowledge, '*Ca.* P. ulmi', previously reported in Jordan in association with date palm stunting and yellowing (Alhudaib et al., 2019), is firstly associated in this study with an almond disease worldwide.

Interestingly, '*Ca*. P. phoenicium' (16SrIX-B), the etiological agent of AlmWB in Middle East (Fiore et al., 2018), was reported in one almond tree in Ain Jana. Studies investigating '*Ca*. P. phoenicium' epidemiology reported that insect vectors *A. decedens* and *Tachycixius* sp. and reservoir plants *Slimax aspera* and *Anthemis* sp. are involved in its diffusion to almond (Abou-Jawdah et al., 2014; Tedeschi et al., 2015; Casati et al., 2016). Moreover, several insect species were reported as putative vectors in Lebanon and Iran (Dakhil et al., 2011; Zirak et al., 2021). Except for *H. obsoletus*, none of these putative and confirmed vectors were collected during field surveys in Jordan, and '*Ca.* P. phoenicium' was not detected in collected insects and putative host plants. However, considering the capability of this pathogen to adapt to diverse environmental conditions and novel agroecosystems (Quaglino et al., 2015; Salehi et al., 2018, 2020), and to quickly spread in large areas (Abou-Jawdah *et al*., 2009), its report in Jordan sounds like an alarm and needs to be accurately monitored in the next years. In particular, the movement of propagation materials between MENA Countries and within the local areas suggests giving special attention to stone fruits nurseries with regularly sampling to investigate the phytoplasma presence.

In conclusion, the present study evidenced that seven '*Candidatus* Phytoplasma' species are associated with an almond disease complex characterized by five symptom categories, including witches'-broom, yellowing, leaf rolling, and stem fasciation. Most phytoplasma species, identified in almond in this study, are associated with destructive plant diseases in different parts of the world. Moreover, obtained results provided new insights on almond phytoplasmas diffusion, identifying putative vectors and non-crop plants at least for '*Ca*. P. asteris', '*Ca*. P. pyri', and '*Ca*. P. omanense'. Further studies have to focus on (i) verify the capability of putative insect vectors, identified in the present work, to transmit '*Ca*. P. asteris' and '*Ca*. P. pyri' to almond by transmission trials; (ii) survey the insect population diversity and dynamics throughout the whole season in the almond cultivation areas; (iii) upscale the survey of almond disease complex in the whole Country, focusing on the canopy and the roots of symptomatic trees and exploring differences among the available cultivars; (iv) controlling the sanitary status of the propagation materials by molecular analyses of regularly collected samples, with special attention for the presence of '*Ca*. P. phoenicium' and '*Ca*. P. pyri'.

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760 **Table 1**. Plant samples collected from surveyed locations in northern Jordan.

761

Governorate	Location	Plant	No. of collected samples	No. of phytoplasma-infected samples
Irbid	Kharja	Symptomatic almond	30	5
		Asymptomatic almond	3	0
		Convolvulus arvensis L.	6	0
		Chenopodium sp.	2	0
		Amaranthus sp.	4	0
	Ezrit	Symptomatic almond	25	3
		Asymptomatic almond	2	0
		Amaranthus sp.	4	1
		Capparis sp.	2	0
		Chenopodim sp.	2	0
	Hofa	Symptomatic almond	25	8
		Asymptomatic almond	3	0
	Sydoor	Symptomatic almond	25	3
		Asymptomatic almond	2	0
Ajloun	Sikhrah	Symptomatic almond	12	7
		Asymptomatic almond	2	0
	Ain Jana	Symptomatic almond	8	3
		Asymptomatic almond	2	0
	Zatarah	Symptomatic almond	15	1
		Asymptomatic almond	2	0
		Overall total	176	31

762

763 **Table 2**. Attribution to species and taxonomic subgroups of phytoplasmas detected in plants

Strain	Plant host	Location	Symptoms	Phtoplasma species	Identity % versus reference strain	16Sr subgroup	Acc. No.
AL7	almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. asteris'	99.9	I-B (1.00)	OL873123 (a)
AL2C	almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. asteris'	99.9	I-B (1.00)	a
AL1	almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.9	V-A (1.00)	OL873124
AL2	almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.8	V-A* (0.98)	OL873125 (b)
AL3	almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.8	V-A* (0.98)	b
AL5	almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.8	V-A* (0.98)	b
AL10	almond	Sikhrah	early flowering, evergreen pattern	'*Ca*. P. ulmi'	99.8	V-A* (0.98)	b
AL163	almond	Zatarah	slim leaves	'*Ca*. P. omanense'	99.6	XXIX-B (new)	OL873126 (c)
AL1056	almond	Ain Jana	witches'-broom, yellowing, dieback	'*Ca*. P. omanense'	99.6	XXIX-B (new)	c
AL1058	almond	Ain Jana	witches'-broom, yellowing, dieback	'*Ca*. P. omanense'	99.6	XXIX-B (new)	c
AL1067	almond	Ain Jana	witches'-broom, yellowing, dieback	'*Ca*. P. phoenicium'	100	IX-B (1.00)	OL873128
AL831	almond	Ezrit	witches'-broom, yellowing, dieback	'*Ca*. P. asteris'	99.6	I-R* (0.98)	OL873129 (d)
AL1042	almond	Ezrit	witches'-broom, yellowing, dieback	'*Ca*. P. solani'	99.7	XII-A (1.00)	OL873130 (e)
AL1052	almond	Ezrit	witches'-broom, yellowing, dieback	'*Ca*. P. omanense'	99.6	XXIX-B (new)	c
AL1054	*Amaranthus* sp.	Ezrit	little leaf, yellowing	'*Ca*. P. omanense'	99.6	XXIX-B (new)	c
AL169	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL170	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL171	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL172	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL173	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL174	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL175	almond	Hofa	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL214	almond	Hofa	witches'-broom, yellowing, dieback	'*Ca*. P. aurantifolia'	99.7	II-C* (0.99)	OL873131
AL408	almond	Sydoor	witches'-broom, yellowing, dieback	'*Ca*. P. omanense'	100	XXIX-A (1.00)	OL873127
AL167	almond	Sydoor	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL168	almond	Sydoor	witches'-broom, yellowing, dieback	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL11	almond	Kharja	witches'-broom, yellowing, leaf rolling	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL1299	almond	Kharja	witches'-broom, yellowing, leaf rolling	*'Ca*. P. solani'	99.7	XII-A (1.00)	e
AL198	almond	Kharja	flat stem	'*Ca*. P. pyri'	100	X-C (1.00)	OL873132 (f)
AL222	almond	Kharja	flat stem	'*Ca*. P. pyri'	100	X-C (1.00)	f
AL225	almond	Kharja	flat stem	'*Ca*. P. pyri'	100	X-C (1.00)	f

764

765 **Table 3**. Phytoplasma infection of Auchenorrhyncha and Sternorrhyncha insects collected in northern Jordan.

766

Governorate	Location	Family	Species	Collection date	No. of collected insects	No. of pools	No. of positive pools	Phytoplasma positive insect (%)
Ajloun	Sydoor	Cicadellidae	*Zygina flammigera*[a]	Jul	25	6	0	0
Irbid	Ezrit	Cixiidae	*Hyalesthes obsoletus*	Jul	2	2	1	50
Irbid	Ezrit	Cicadellidae	*Balclutha incisa*	Jul	15	5	0	0
Irbid	Kharja	Cicadellidae	*Anaceratagallia frisia*[a]	Aug	20	6	0	0
Irbid	Kharja	Cicadellidae	*Cicadulina bipunctata*	Aug	18	6	1	16.7
Irbid	Kharja	Cicadellidae	*Zygina flammigera*	Aug	12	5	0	0
Irbid	Kharja	Cicadellidae	*Empoasca* sp.	Aug	15	5	1	20
Irbid	Kharja	Cicadellidae	*Eupelix* sp.	Aug	1	1	0	0
Irbid	Kharja	Cixiidae	*Reptalus* sp.	Aug	3	3	2	66.7
Irbid	Kharja	Delphacidae	*Laodelphax striatellus*	Aug	4	2	2	100
Irbid	Kharja	Issidae	*Agalmatium* sp.	Aug	3	3	3	100
Irbid	Kharja	Tettigometridae	*Tettigometra* sp.	Aug	4	2	1	50
Irbid	Kharja	Psyllidae	*Cacopsylla bidens*	Aug	4	2	2	100
				Overall	126	48	13	27.1

767 [a] species firstly reported in Jordan

768 **Table 4**. Attribution to species and taxonomic subgroups of phytoplasmas detected in insects.
769

Strain	Species	Location	Phytoplasma species	Identity % versus reference strain	16Sr subgroup	Acc. No.
K7-15	*Laodelphax striatellus*	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	g
K7-16	*Laodelphax striatellus*	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	g
K11-17	*Tettigometra* sp.	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	g
K17-18	*Cicadulina bipunctata*	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	g
K28-19	*Empoasca* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	d
KPS-20	*Cacopsylla bidens*	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	OL873133 (g)
KPS-21	*Cacopsylla bidens*	Kharja	'*Ca*. P. pyri'	99.8	X-C (1.00)	g
K24-7	*Agalmatium* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	d
K24-8	*Agalmatium* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	d
K24-9	*Agalmatium* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	d
K25-10	*Reptalus* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	d
K25-11	*Reptalus* sp.	Kharja	'*Ca*. P. asteris'	99.6	I-R* (0.98)	d
EA-12	*Hyalesthes obsoletus*	Ezrit	'*Ca*. P. asteris'	99.6	I-R* (0.98)	d

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771

772 **Figure Legends**

773 **Figure 1.** Maps of governorates and locations in North Jordan where the surveys on
774 phytoplasma-like diseases in almond orchards were conducted.

775 **Figure 2.** Phytoplasma-like symptoms observed in almond trees and weeds in northern Jordan.
776 (i) Witches'-broom, yellowing, and dieback in Ezrit, Hofa, Sydoor, and Ain Jana (A, B); (ii)
777 early flowering along with evergreen pattern in Sikhrah (C); witches'-broom, yellowing and
778 leaf rolling in Kharja (D); (iv) slim leaves in Zatarah (E); (v) flat stem in Kharja (F); little
779 leaves and colour alteration on *Amaranthus* sp. in Kharja and Ezrit (G).

780 **Figure 3**. Phylogenetic tree based on the alignment of 16S rDNA nucleotide sequences of
781 representative phytoplasma strains identified in almond, putative insect vectors, and reservoir
782 plants in Jordan (bold characters), and reference strains of previously described '*Candidatus*
783 Phytoplasma' species. The optimal tree with the sum of branch length = 0.91431819 is shown.
784 The percentage of replicate trees in which the associated taxa clustered together in the bootstrap
785 test (1000 replicates) are shown next to the branches.

786 **Figure 4.** Virtual (A) and actual (B) RFLP profiles of 16S rDNA nucleotide sequences of
787 phytoplasma strains identified in almond, weeds, and insects in northern Jordan. (A) One strain
788 among those sharing identical 16S rDNA sequence (Tables 2 and 4) was selected as
789 representative strain for *i*PhyClassifier analyses; (B) actual *Alu*I-RFLP patterns distinguishing
790 strains of subgroups 16SrXXIX-A (strain AL408) and -B (strains AL163, AL1052, AL1054,
791 AL1056, AL1058), firstly reported in this study.

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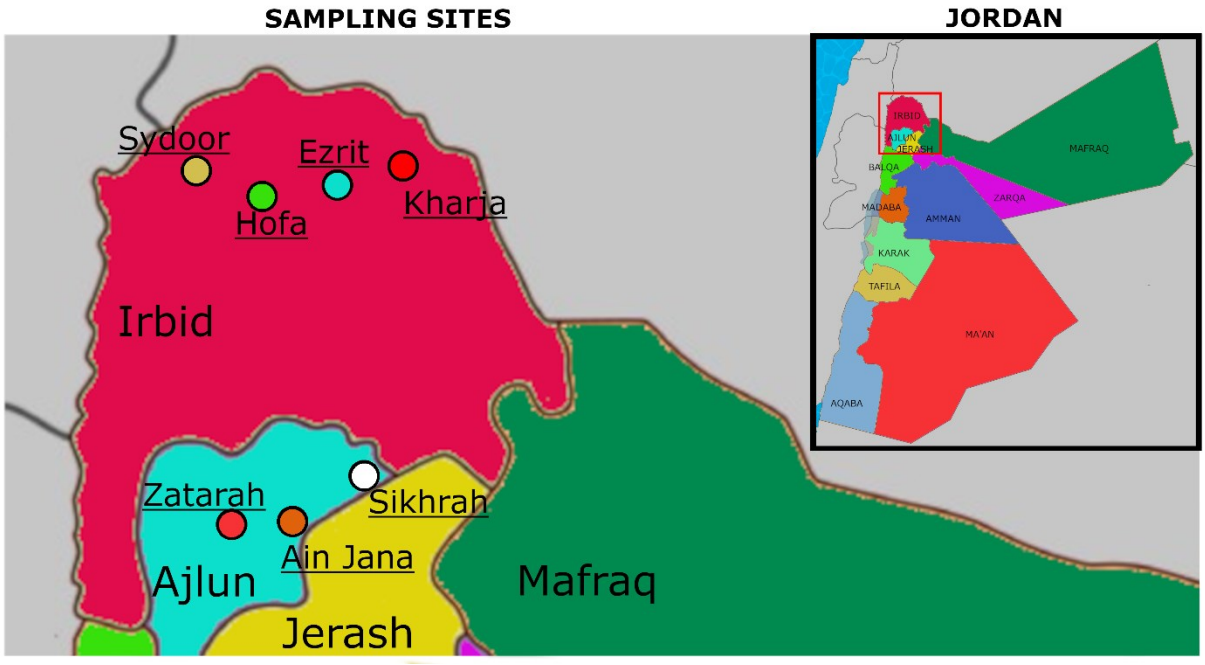

796
797
798 **Figure 1**
799
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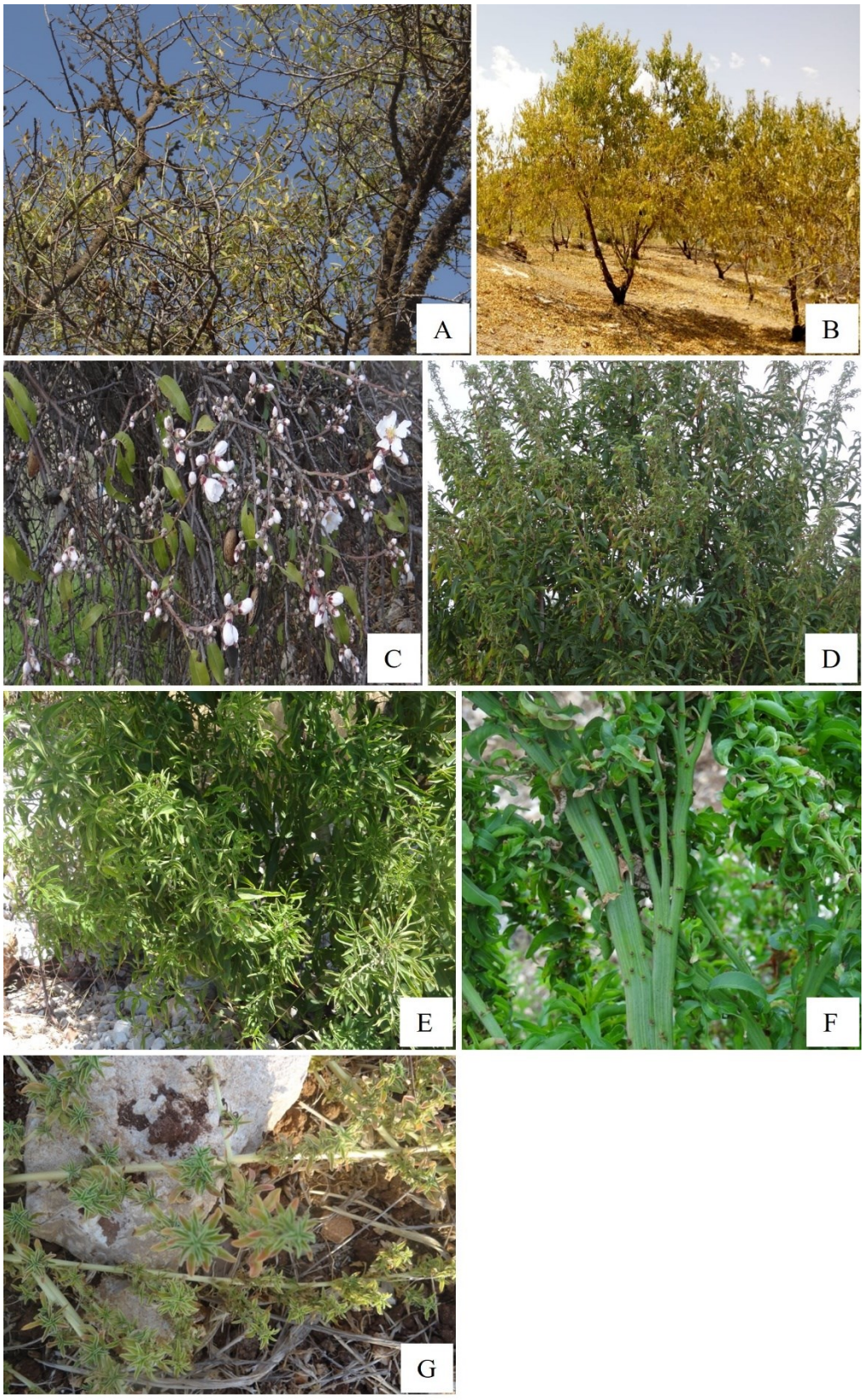
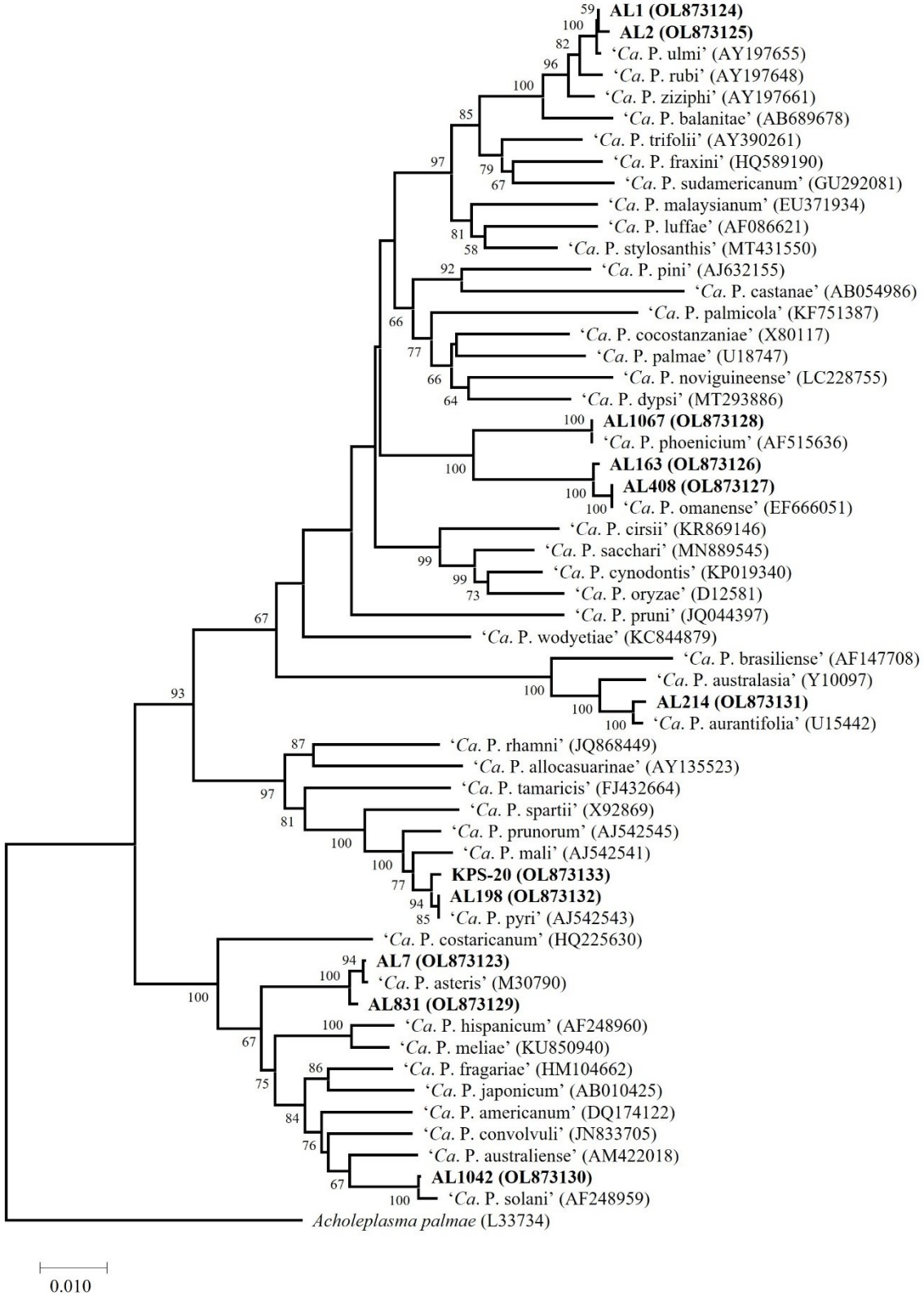


803

804 **Figure 2**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'Ca. P. balanitae' (AB689678)
'Ca. P. trifolii' (AY390261)
'Ca. P. fraxini' (HQ589190)
'Ca. P. sudamericanum' (GU292081)
'Ca. P. malaysianum' (EU371934)
'Ca. P. luffae' (AF086621)
'Ca. P. stylosanthis' (MT431550)
'Ca. P. pini' (AJ632155)
'Ca. P. castanae' (AB054986)
'Ca. P. palmicola' (KF751387)
'Ca. P. cocostanzaniae' (X80117)
'Ca. P. palmae' (U18747)
'Ca. P. noviguineense' (LC228755)
'Ca. P. dypsi' (MT293886)
AL1067 (OL873128)
'Ca. P. phoenicium' (AF515636)
AL163 (OL873126)
AL408 (OL873127)
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'Ca. P. cirsii' (KR869146)
'Ca. P. sacchari' (MN889545)
'Ca. P. cynodontis' (KP019340)
'Ca. P. oryzae' (D12581)
'Ca. P. pruni' (JQ044397)
'Ca. P. wodyetiae' (KC844879)
'Ca. P. brasiliense' (AF147708)
'Ca. P. australasia' (Y10097)
AL214 (OL873131)
'Ca. P. aurantifolia' (U15442)
'Ca. P. rhamni' (JQ868449)
'Ca. P. allocasuarinae' (AY135523)
'Ca. P. tamaricis' (FJ432664)
'Ca. P. spartii' (X92869)
'Ca. P. prunorum' (AJ542545)
'Ca. P. mali' (AJ542541)
KPS-20 (OL873133)
AL198 (OL873132)
'Ca. P. pyri' (AJ542543)
'Ca. P. costaricanum' (HQ225630)
AL7 (OL873123)
'Ca. P. asteris' (M30790)
AL831 (OL873129)
'Ca. P. hispanicum' (AF248960)
'Ca. P. meliae' (KU850940)
'Ca. P. fragariae' (HM104662)
'Ca. P. japonicum' (AB010425)
'Ca. P. americanum' (DQ174122)
'Ca. P. convolvuli' (JN833705)
'Ca. P. australiense' (AM422018)
AL1042 (OL873130)
'Ca. P. solani' (AF248959)
Acholeplasma palmae (L33734)
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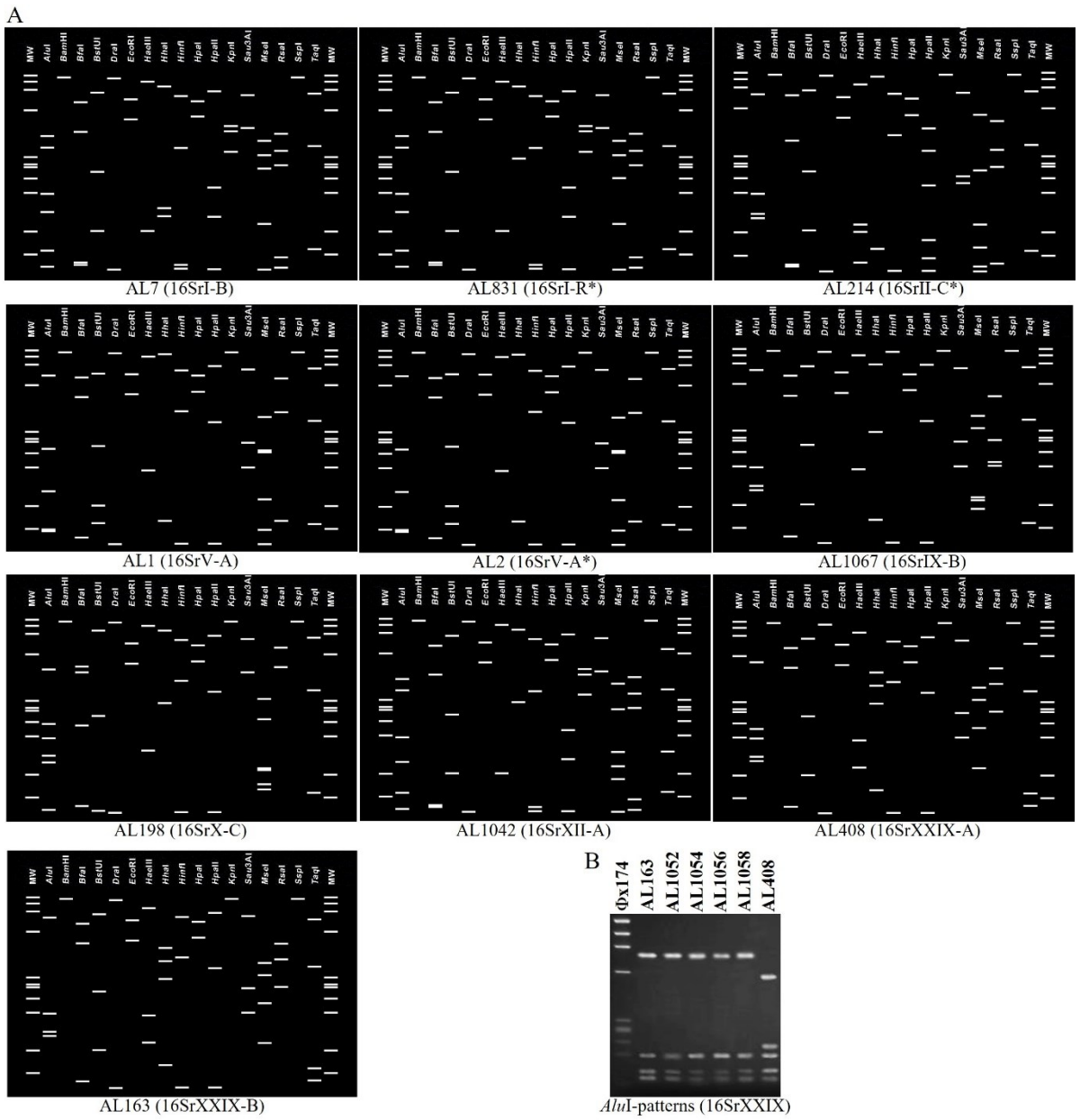
Figure 3


A
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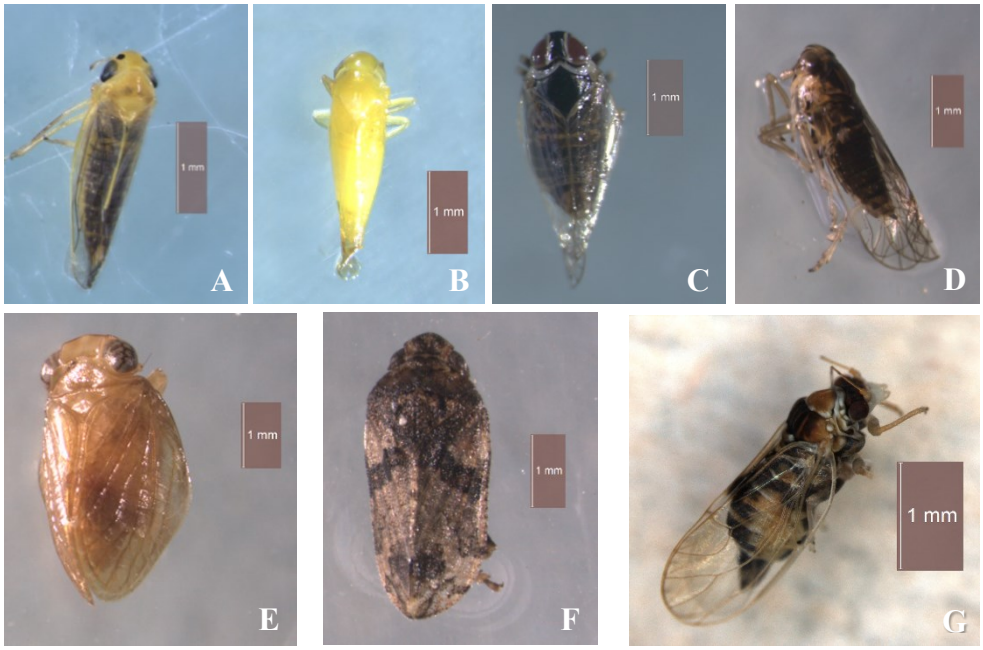
AL7 (16SrI-B)
AL831 (16SrI-R*)
AL214 (16SrII-C*)
AL1 (16SrV-A)
AL2 (16SrV-A*)
AL1067 (16SrIX-B)
AL198 (16SrX-C)
AL1042 (16SrXII-A)
AL408 (16SrXXIX-A)
AL163 (16SrXXIX-B)
B
Φx174
AL163
AL1052
AL1054
AL1056
AL1058
AL408
AluI-patterns (16SrXXIX)

Figure 4

Highlights

- Seven '*Candidatus* Phytoplasma' species were reported in Jordan in association with an almond disease complex.
- '*Ca*. Phytoplasma omanense', '*Ca*. Pytoplasma ulmi', and '*Ca*. Phytoplasma pyri' were firstly reported in almond around the world.
- New subgroup XXIX-B was described.
- Eight insect taxa were found positive to '*Ca*. Phytoplasma pyri' and '*Ca*. P. asteris'.



Supplementary Fig. 1 – Insects collected in Jordan almond orchards and tested positive to phytoplasmas. Cicadellidae: (A) *Cicadulina bipunctata* (positive to 16SrX-C), (B) *Emposasca* sp. (positive to 16SrI-R); Cixiidae: (C) *Hyalesthes obsoletus* (positive to 16SrI-R); Delphacidae: (D) *Laodelphax striatellus* (positive to 16SrX-C), Issidae: (E) *Agalmatium* sp. (positive to 16SrI-R); Tettigometridae: (F) *Tettigometra* sp. (16SrX-C); Psyllidae: (G) *Cacopsylla bidens* (positive to 16SrX-C).